



NON-CONVENTIONAL YEAST IN THE WINE INDUSTRY

EDITED BY : Albert Mas, Jose Manuel Guillamón and Gemma Beltran
PUBLISHED IN: Frontiers in Microbiology



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ISSN 1664-8714

ISBN 978-2-88945-053-4

DOI 10.3389/978-2-88945-053-4

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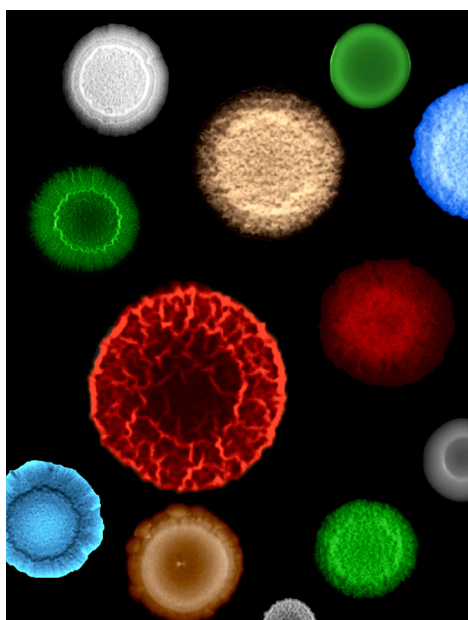
NON-CONVENTIONAL YEAST IN THE WINE INDUSTRY

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Differential colony growth of wine yeasts on culture plates

Image by Beatriz Gonzalez

Saccharomyces cerevisiae strains that exhibit high ethanol tolerance and excellent fermentative ability are extensively used in winemaking as selected starters. However, a side-effect of the widespread use of these commercial starter cultures is the elimination of native microbiota, which might result in wines with similar analytical and sensory properties, depriving them from the variability, complexity and personality that define the typicality of a wine. Nonetheless, a way of balancing control and yeast population diversity during wine fermentation is the selection of non-*Saccharomyces* yeasts with optimal oenological traits. Therefore, a current trend in enology is the implementation of mixed- or multi-starter cultures, combining *S. cerevisiae* that remains the yeast species required for the completion of fermentation and non-*Saccharomyces* yeasts isolated from the native flora of grape juices. This research topic mainly deals with possible applications of different non-*Saccharomyces* yeast to wine production such as aroma production, ethanol reduction or biocontrol.

Citation: Mas, A., Guillamón, J. M., Beltran, G., eds. (2016). Non-conventional Yeast in the Wine Industry. Lausanne: Frontiers Media. doi: 10.3389/978-2-88945-053-4

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Editorial: Non-conventional Yeast in the Wine Industry

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Keywords: non-saccharomyces, torulaspora, hanseniaspora, starmerella

The Editorial on the Research Topic

Non-conventional Yeast in the Wine Industry

The alcoholic fermentation of grape musts to wines is a rather complex process that involves the sequential development of microorganisms, mainly yeasts, but also filamentous fungi, lactic acid bacteria, etc. In the early stages of wine fermentation, several yeast species may be present but, as the alcohol concentration increases, *Saccharomyces* species progressively take over. The winemaking process cannot be understood without knowing how the different microorganisms leave their microbial footprint. The footprint depends on how long these microorganisms are present and their dominance during the winemaking process.

The first source of this microbial population diversity is grape, which is an ecological niche for freely proliferating microorganisms. The grapes have populations of native or indigenous yeasts that are between 10^4 and 10^6 cells/g of grapes, which are mainly Non-*Saccharomyces* yeasts. The populations of *Saccharomyces* are very low in grapes, although they are not completely absent. The Non-*Saccharomyces* strains have been regarded for many years as the responsible for wine spoilage and different preventive actions have been taken to avoid them. These populations change slightly when they enter in contact with the cellar environment (presses, pumps, tanks) where they join the resident microbiota. This microbiota is rare in new wineries, particularly if the equipment has not been used previously. The cellar is a good niche for *S. cerevisiae*, which becomes the main cellar-resident yeast (Beltran et al., 2002).

According with the distribution of yeasts in the grape surface, yeasts with low fermentation activity, such as *Candida* spp., *Hanseniaspora* spp., *Pichia* spp., *Rhodotorula* spp., *Kluyveromyces* spp. and *Schizosaccharomyces* spp. are predominant in grape musts and during the early stages of fermentation. *Saccharomyces cerevisiae* develops shortly afterwards, to become the dominating microorganism and completing the wine fermentation. *S. cerevisiae* strains have unique physiological properties that are not found in other yeasts. The most important is the high ability to ferment sugars vigorously to produce alcohol under both aerobic and anaerobic conditions. This aptitude allows them to colonize quickly substrates with high sugar concentrations and overgrow other yeasts (Fleet and Heard, 1993).

The role of *S. cerevisiae* yeasts is not only related with conducting the alcoholic fermentation, but is also heavily related to wine quality. The activities of the different yeast species and strains have an impact on the sensory profiles of wine by increasing its complexity and organoleptic richness (Fleet, 2003). Currently, winemakers use available commercial starters of *S. cerevisiae* to have a reproducible and predictable wine by controlling the fermentation. The selection of these commercially available starters has been based on different criteria, and many different presentations can be found. There are *S. cerevisiae* strains selected to increase aromatic expression, to ferment musts with high sugar concentration (osmophilic yeast), to resist high or low fermentation temperatures, or to survive in wines with high ethanol content, also able to perform

OPEN ACCESS

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Reviewed by:

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Specialty section:

This article was submitted to
Food Microbiology,
a section of the journal
Frontiers in Microbiology

Received: 05 August 2016

Accepted: 07 September 2016

Published: 27 September 2016

Citation:

Mas A, Guillamón JM and Beltran G
(2016) Editorial: Non-conventional
Yeast in the Wine Industry.
Front. Microbiol. 7:1494.
doi: 10.3389/fmicb.2016.01494

second fermentation (for instance for sparkling wine production), among other properties. Nonetheless, the main characteristic of all of them is that they are good fermenters and they are able to finish the alcoholic fermentation. However, the use of these commercial starter cultures produces the quick take over of the fermentation and the elimination of native microorganisms and their impact on the final wines. As consequence, the massive use of these selected yeasts derives in very uniform wines with small differences. These differences are due to similar analytical values and organoleptic profiles, and thus, limiting the personality (in terms of variability and complexity) that define the typicality of a wine. Wine typicality could be defined as the characteristics that allow the identification of a wine with the territory (terroir, AOC, for instance) where it has been produced. The defense of this typicality can be done by the use of native or indigenous yeasts, as certain microbial diversity is associated to a given area (Bokulich et al., 2013). This diversity has been analyzed in different agronomic conduction systems (Setati et al.).

Nonetheless, a way of balancing control and yeast population diversity during wine fermentation is the selection of Non-*Saccharomyces* yeasts with optimal oenological traits. Non-*Saccharomyces* species contribute increasing the concentration of volatile compounds and the chemical composition of wines due to higher production of secondary metabolites that contribute to the organoleptic properties of the wines (glycerol, aromas such esters, acetates,...). Some extracellular enzymes (esterases, pectinolytic, beta-glucosidase, etc.) produced by these yeasts may be responsible for the appearance in the wine of unique properties that allow its identification from the region where is produced (reviewed in Jolly et al., 2014). The use of Non-*Saccharomyces* yeast can reduce the ethanol content as reviewed in the present issue by Ciani et al. The ethanol reduction is a critical aspect in winemaking due to climate change, which produces an increased concentration of sugars. However, the common opinion of winemakers on Non-*Saccharomyces* yeasts is that they are mostly spoilage microorganisms and thus they restrict its use. However, there is an increasing interest on Non-*Saccharomyces* yeast species for the selection of starters and their use in mixed fermentations with *S. cerevisiae*,

which is probably changing the traditional bias of winemakers now.

The selection of Non-*Saccharomyces* yeast has focused on their direct positive effects on wine quality either by providing new aromas such as volatile fatty acids, esters, aldehydes, etc or by removing detrimental compounds that would affect wine quality. A couple of articles have approached this aspect in this special topic (Belda et al.; Padilla et al.). *Torulaspora delbrueckii* has been proposed to reduce volatile acidity produced by *Saccharomyces*. In this special topic, several articles are dealing with the use of this species in wine making (Ramírez et al.; Velázquez et al.; Renault et al.). Currently, there are various commercial preparations of this yeast. Another Non-*Saccharomyces* yeast that is commercially available is *Metschnikowia pulcherrima*, recommended for the release of some volatile thiols and terpenes in white wines, thus increasing their aromatic intensity. Finally, *Lachancea thermotolerans* is also commercially available to increase glycerol and lactic acid (Gobbi et al., 2013). Although there are still few commercial preparations of Non-*Saccharomyces* yeasts, they will probably increase in the near future. These include *Starmerella bacillaris*, with production of large amounts of glycerol, and its fructophilic character, which favors the end of fermentation. However, in this topic, antimicrobial activity has been described by several strains of this species (Fernandes Lemos et al.). Some species from the *Hanseniaspora* genus are also considered for future applications. In fact, in this topic a couple of articles on *H. uvarum* (Albertin et al.; Masneuf-Pomerade et al.) and another on *H. vineae* (Lleixà et al.) have been proposed as increasing the wine quality. However, some attention should be paid to some non-conventional *Saccharomyces*, which could also be relevant in terms of new activities of interest in winemaking (Pérez-Torrado et al.). Finally, the interaction between *Saccharomyces* and Non-*Saccharomyces* yeast during winemaking should be clearly known and controlled when mixed inocula are going to be used (Wang et al.).

AUTHOR CONTRIBUTIONS

All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

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- Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Sequence-based Analysis of the *Vitis vinifera* L. cv Cabernet Sauvignon Grape Must Mycobiome in Three South African Vineyards Employing Distinct Agronomic Systems

OPEN ACCESS

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Specialty section:

This article was submitted to
Food Microbiology,
a section of the journal
Frontiers in Microbiology

Received: 02 July 2015

Accepted: 16 November 2015

Published: 30 November 2015

Citation:

Setati ME, Jacobson D and Bauer FF
(2015) Sequence-based Analysis
of the *Vitis vinifera* L. cv Cabernet
Sauvignon Grape Must Mycobiome
in Three South African Vineyards
Employing Distinct Agronomic
Systems. *Front. Microbiol.* 6:1358.
doi: 10.3389/fmicb.2015.01358

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Recent microbiomic research of agricultural habitats has highlighted tremendous microbial biodiversity associated with such ecosystems. Data generated in vineyards have furthermore highlighted significant regional differences in vineyard biodiversity, hinting at the possibility that such differences might be responsible for regional differences in wine style and character, a hypothesis referred to as “microbial *terroir*.” The current study further contributes to this body of work by comparing the mycobiome associated with South African (SA) Cabernet Sauvignon grapes in three neighboring vineyards that employ different agronomic approaches, and comparing the outcome with similar data sets from Californian vineyards. The aim of this study was to fully characterize the mycobiomes associated with the grapes from these vineyards. The data revealed approximately 10 times more fungal diversity than what is typically retrieved from culture-based studies. The Biodynamic vineyard was found to harbor a more diverse fungal community ($H = 2.6$) than the conventional ($H = 2.1$) and integrated ($H = 1.8$) vineyards. The data show that ascomycota are the most abundant phylum in the three vineyards, with *Aureobasidium pullulans* and its close relative *Kabatella microsticta* being the most dominant fungi. This is the first report to reveal a high incidence of *K. microsticta* in the grape/wine ecosystem. Different common wine yeast species, such as *Metschnikowia pulcherrima* and *Starmerella bacillaris* dominated the mycobiome in the three vineyards. The data show that the filamentous fungi are the most abundant community in grape must although they are not regarded as relevant during wine fermentation. Comparison of metagenomic datasets from the three SA vineyards and previously published data from Californian vineyards revealed only 25% of the fungi in the SA dataset was also present in the Californian dataset, with greater variation evident amongst ubiquitous epiphytic fungi.

Keywords: wine yeasts, next-generation sequencing, grapevine mycobiome, microbial diversity, microbial *terroir*

INTRODUCTION

Vitis vinifera L. is an economically important crop plant that has been cultivated since ancient times. Throughout growth and development, the grapevines interact with a wide range of filamentous fungi and yeasts that colonize vegetative tissues and reproductive organs (Pancher et al., 2012). The fungal population comprises endophytic and epiphytic communities that may be pathogenic, neutral, or beneficial to the host (Pancher et al., 2012; Martins et al., 2014). Many studies employing culture-dependent and culture-independent approaches have shown that the grape berry endosphere is mainly colonized by ascomycetous filamentous fungi of the genera *Alternaria*, *Botryotinia*, *Epicoccum*, *Davidiella*, *Neofusicoccum*, and *Cladosporium* (Martini et al., 2009; Gonzalez and Tello, 2011). The endophytic fungi play a crucial role in plant health as they can retard the growth of detrimental phytopathogens (Martini et al., 2009). In contrast, the epiphytic fungal community comprises saprophytic filamentous fungi of the genera *Aspergillus*, *Penicillium*, *Rhizopus*, and obligate parasites including *Erysiphe necator* and *Plasmopara viticola*, as well as oxidative and fermentative yeasts that influence wine fermentation processes and contribute to the aroma and flavor of wine (Diguta et al., 2011; Rousseaux et al., 2014). The yeast population on grape surfaces is mainly dominated by basidiomycetous yeasts of the genera *Cryptococcus*, *Rhodsporidium*, and *Rhodotorula* pre-véraison, while the ascomycetous yeasts, particularly species of the genera *Hanseniaspora*, *Metschnikowia*, and *Candida*, increase in numbers as the fruit ripens. The yeast-like fungus *Aureobasidium pullulans* is dominant throughout the berry development and has been shown to exist as both an endophyte and epiphyte (Martini et al., 2009). The presence of other yeast genera depends upon various factors including vineyard practices (Setati et al., 2012; Martins et al., 2014), disease pressure and the level of damage of the grapes (Barata et al., 2012).

Although many studies have been performed to describe both the endophytic and epiphytic fungal communities associated with grape berries, most are based on culture-dependent methods and either target the two groups separately, or are mainly focused on the yeast population and not the entire fungal population. Recently, metagenomic approaches have become an important tool for assessment of the grape microbiome. Bokulich et al. (2014) comprehensively examined the communities of both bacteria and fungi in crushed Chardonnay and Cabernet Sauvignon fruit in California using Illumina amplicon sequencing approaches and showed that the microbiomes not only differed by region, but were also conditioned by climate, year, and cultivar. Similarly, Taylor et al. (2014) demonstrated regional distinction in fungal communities in vineyards across New Zealand. The diversity of fungi associated with grapes and present in grape must were shown to resemble that present on leaves (Bokulich et al., 2014; Pinto et al., 2014), and the community composition is influenced by chemical treatments, agronomic practices, and climatic conditions (Bokulich et al., 2014; David et al., 2014; Pinto et al., 2014).

Metagenomic surveillances were shown to reveal greater diversity than other community fingerprinting methods and

culture-based methods (David et al., 2014; Taylor et al., 2014). In fact, Taylor et al. (2014) suggested that culture-based methods might miss up to approximately 95% of the community in some samples. Consequently, these methods are increasingly becoming the preferred tool to evaluate the grape microbial community structures. The aim of the current study was therefore to employ a sequence-based metagenomic approach to better characterize fungal community structures associated with Cabernet Sauvignon grapes from three neighboring vineyards that employ different agronomic strategies and were shown through community fingerprinting and culture-based methods to harbor distinct communities. In addition, the fungal community structures associated with grape berries in South Africa and California (USA) were compared to determine continental distribution and prevalence of fungal species.

MATERIALS AND METHODS

Grape Sampling and DNA Extraction

Cabernet Sauvignon grapes were collected from 3 vineyards located in the Polkadraai area of Stellenbosch, South Africa. The viticultural practices applied in these vineyards [referred to as biodynamic (BD), conventional (CONV) and integrated production of wine (IPW)], their lay-out and relevant characteristics are described in detail in Setati et al. (2012). The three vineyards are located next to each other; BD (33°57'39.33" S 18°45'13.46" E elev 183 m), CONV (33°57'41.50" S, 18°45'11.87" E elev 179 m) and IPW (33°57'40.65" S 18°45'08.23" E elev 184 m). The CONV and BD vineyard had the same Cabernet Sauvignon rootstock (R101-14) while the integrated vineyard has rootstock R110-CS23A. Briefly, the BD vineyard applies sulfur, copper oxide as well as organic fungicide for control of powdery mildew and downy mildew while the integrated vineyard applies biofertilizers, mycorrhizae, as well as a combination of systemic and surface protectants for pest control. In contrast, the CONV vineyard mainly applies chemical fungicides and biofertilizers. The grapes were collected from the vineyards based on a sampling design described previously (Setati et al., 2012). From each vineyard 5 kg of grapes were collected from the selected sampling sites and pooled into a composite sample, hand de-stemmed and crushed under aseptic conditions in the laboratory. Only healthy undamaged grapes were used for the analysis. The chemical composition of the must was analyzed by Fourier Transform Infrared (FT-IR) spectroscopy using the GrapeScan 2000 instrument (FOSS Electric, Denmark). Fifty milliliters of grape must were collected immediately after crushing and used for DNA extraction. The grape must was centrifuged at 5000 rpm for 5 min and the pellet washed three times with a buffer comprising 0.15 M NaCl, 0.1 M EDTA, and 2% (w/v) Polyvinylpyrrolidone (Jara et al., 2008), followed by three washes with TE buffer (pH 7.6). DNA extraction was carried out according to Wilson (2003) with minor modifications. Briefly, the pellet was re-suspended in 2.3 ml TE buffer, followed by the addition of proteinase K, SDS, and 500 µl of fine glass beads. The mixture was vortexed for 3 min. A volume of 20 µl of a 10 mg/ml lysozyme solution

was added and the mixture incubated at 37°C for 50 min. Then 400 µl of 5 M NaCl and 240 µl CTAB/NaCl (CTAB: Cetyl-methyl ammonium bromide) was added and the mixture was incubated for 10 min at 65°C, followed by phenol/chloroform/isoamyl extractions and precipitation with isopropanol.

Sequencing Library Construction

Amplification of the ITS1-5.8S rDNA-ITS2 was performed using fusion primers consisting of the ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') primers and Illumina MiSeq platform specific adaptor sequences. In a study comparing primers targeting the ITS1, ITS2, and whole ITS, Bokulich and Mills (2013) showed that no primer pair could accurately reconstruct the known taxonomic distribution of a mock community. Consequently, for the current study we chose to target the whole ITS region for better taxonomic assignment of reads. The PCR was performed in 25 µl reactions containing 1 × Ex-Taq buffer, 0.2 mM dTNPs, 0.25 µM of each primer and 100 ng DNA template. Triplicate reactions were performed for each DNA sample. Cycling conditions consisted of an initial denaturation at 94°C for 3 min, followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 45 s; and a final extension of 10 min at 72°C. The PCR products were purified using the Zymoclean™ Gel DNA recovery kit (The Epigenetics Company™, Zymo Research, Inqaba Biotechnical Industries (Pty Ltd., South Africa) and quantified using the NanoDrop™ 1000 spectrophotometer (Thermo Scientific). The amplicons from triplicate PCR reactions were combined at equal concentrations and used for Illumina library preparation and sequencing. Samples were subjected to standard quality control measures (fluorometric quantification and normalization). One nanogram of each amplicon pool was used in a standard indexing PCR protocol for a paired-end sequencing library (Nextera) and samples were sequenced using MiSeqV3 chemistry (2 × 300 reads).

Data Analysis

Raw Illumina fastq files were uploaded onto the MG-RAST server (Meyer et al., 2008) and de-replicated (Gomez-Alvarez et al., 2009). The sequences were screened for plant (host-specific) DNA (Langmead et al., 2009) and low quality sequences with a Phred score below 30 were identified using the dynamic trimming (Cox et al., 2010) and removed. The Fastq join script was used to join overlapping paired-end reads. Since the ITS-5.8S region of some fungi is larger than 600 and would therefore not overlap, both joined reads and those that did not overlap were retained (i.e., no sequences were discarded) for further analysis. All sequences were processed for quality analysis. The resulting data sets were pre-screened using qiime-ucclust (Edgar, 2010) clustered at 97% identity by picking the longest sequence within each cluster as a representative of that cluster. Taxonomic assignment was performed in MG-RAST using the Blast Like-Alignment Tool (BLAT) search against the M5RNA database with an *E*-value and similarity cut-off of $1e^{-10}$ and 99%, respectively, and a minimum alignment length of 150 bp. Pearson correlation was used to compare the taxa

derived from forward reads (mainly representing partial ITS1-5.8S rDNA), reverse reads (mainly representing partial ITS2-5.8S rDNA) as well as the mix containing joined reads (representing both the partial and full ITS1-5.8S rDNA-ITS2). The MG-RAST accession codes for the libraries are: 4561567.3, 4561568.3, and 4561569.3. Classical ecology indices such as Shannon Wiener diversity index (*H'*) and Simpson dominance and diversity (*D*, *1-D*) were calculated using the free software package, PAST Version 3.0 (Hammer et al., 2001). The estimated richness was computed on a subsample of 20000 reads. Following taxonomic assignment the data was transformed into a presence/absence matrix and analysis of variance (ANOVA) was performed. A Perl program was written to create a weighted co-occurrence network depicting the species present in and across vineyards. The resulting network was visualized with Cytoscape (Shannon et al., 2003). In addition, the data generated in the current study was compared to yeast isolates that we obtained in a parallel study from the three grape musts by culture-based methods (Bagheri et al., 2015) and also to the metagenomic data generated from grape musts obtained from vineyards in different regions of California (Bokulich et al., 2014). Composite lists of the of the fungal species in the SA and California amplicon sequencing data were compiled and compared with the yeast isolates using Venn's diagrams, constructed on <http://bioinformatics.psb.ugent.be/webtools/Venn/>.

RESULTS

Sequence Analysis and Taxonomic Assignment

We previously assessed the grape berry associated diversity in the three vineyards and demonstrated using Automated Ribosomal Intergenic Spacer analysis (ARISA) that the fungal community structure was distinct (Setati et al., 2012). In addition, data derived from culture dependent microbiological analysis suggested that the BD vineyard had a more diverse fungal community than the CONV and integrated (IPW) vineyard (Setati et al., 2012). In the current study, Illumina paired end sequencing was used to explore the fungal biota (mycobiome) of the different vineyard samples. ITS1-5.8S rDNA-ITS2 libraries were generated from genomic DNA extracted from freshly crushed grape must samples prepared from composite samples. Chemical analysis of the musts shows differences in the ripeness level of the grapes (Supplementary Table S1). For the sequence data, quality filtering removed 29% of the reads from the BD and CONV libraries while only 24% was removed from the IPW library (Supplementary Table S2). The Streptophyta (data not included in further analysis) only accounted for less than 1% of the total sequence data in the three libraries. Unassigned sequences accounted for 295, 777, and 153 reads of the total reads in the BD, CONV, and IPW libraries, respectively. Our data revealed good correlation between taxonomic assignments from the forward reads (mainly containing partial ITS1-5.8S sequences) and the data sets containing all reads (i.e., joined ITS1-5.8S-ITS2) and single reads (partial ITS1-5.8S and ITS2-5.8S), while the reverse reads (containing partial ITS2-5.8S

sequences) from the BD and IPW showed poor correlation with the forward and joined reads (Supplementary Table S3). Based on this, we chose to use the dataset containing both joined and single reads. Therefore, for yeast species with short ITS-5.8S rRNA regions the taxonomic assignment was based on the full ITS1-5.8S rRNA-ITS2 gene while for other yeasts only the partial gene would have been used. Rarefaction curves showed that the sampling depth and sequencing coverage were good for all three samples, especially for the CONV sample which had clearly reached a plateau (Figure 1). Diversity analysis revealed that the BD library comprised a more diverse mycobiome with low dominance ($H' = 2.6$; $D = 0.11$) followed by the CONV ($H' = 2.1$; $D = 0.21$), while the IPW had the lowest diversity and highest dominance ($H' = 1.77$; $D = 0.3$). ANOVA analysis performed on the presence/absence transformed data showed that the community in the three vineyards was significantly different ($p = 0.025$).

Taxonomic assignment was performed using the MG-RAST pipeline. The data indicated some overall similarities in the species composition, but also significant differences. The Ascomycota was found to be the predominant phylum represented in all three grape mycobiomes, but their total contribution varied significantly between 79 and 98% of the total fungal population. In contrast, the Basidiomycota which is commonly the dominant phylum on unripe berries only accounted for 0.4% of the population in the BD vineyard, while in the CONV and IPW vineyard it represented 3.4 and 2%, respectively. In contrast, the BD grape must displayed a high incidence of fungi from the phylum Zygomycota (20%) while in

the CONV and IPW vineyard this phylum represented less than 0.1% of the fungal population. Further analysis shows that fungi of the order Dothidiales were dominant across the three libraries. The Saccharomycetales were also present in high levels in the BD and CONV libraries, while the Botryosphaeriales were the second most dominant in the IPW library (Figure 2). In addition, in the BD must sample the Mucorales were present at the same level as the Saccharomycetales accounting for 20% of the taxa. Dominant ascomycetous filamentous fungi included members of the genera *Alternaria*, *Botryotinia*, *Cladosporium*, *Davidiella*, *Kabatiella*, *Neofussicoccum*, *Pleospora*, and the yeast-like fungus *A. pullulans*, while *Rhodospiridium* sp., *Sporobolomyces* sp. and *Rhodotorula* sp. where the predominant basidiomycetous fungi. Twenty nine fungal species were common across the three vineyards (Figure 3). There were evidently more species shared between the BD and IPW vineyard, than between the BD and CONV, or CONV and IPW.

Distribution of the Filamentous Fungal Taxa

Our data revealed two fungi as the most abundant taxa in the must samples from the three vineyards. The yeast-like fungus *A. pullulans*, which has been reported as both an endophyte and an epiphyte of grapevine, accounted for 13, 25, and 38% of the total population in the BD, CONV, and IPW vineyard, respectively. Similarly, *Kabatiella microsticta* which is closely related to *A. pullulans*, accounted for 11, 25, and 38% of the population in the BD, CONV, and IPW vineyard must sample, respectively. Amongst common grapevine endophytes,

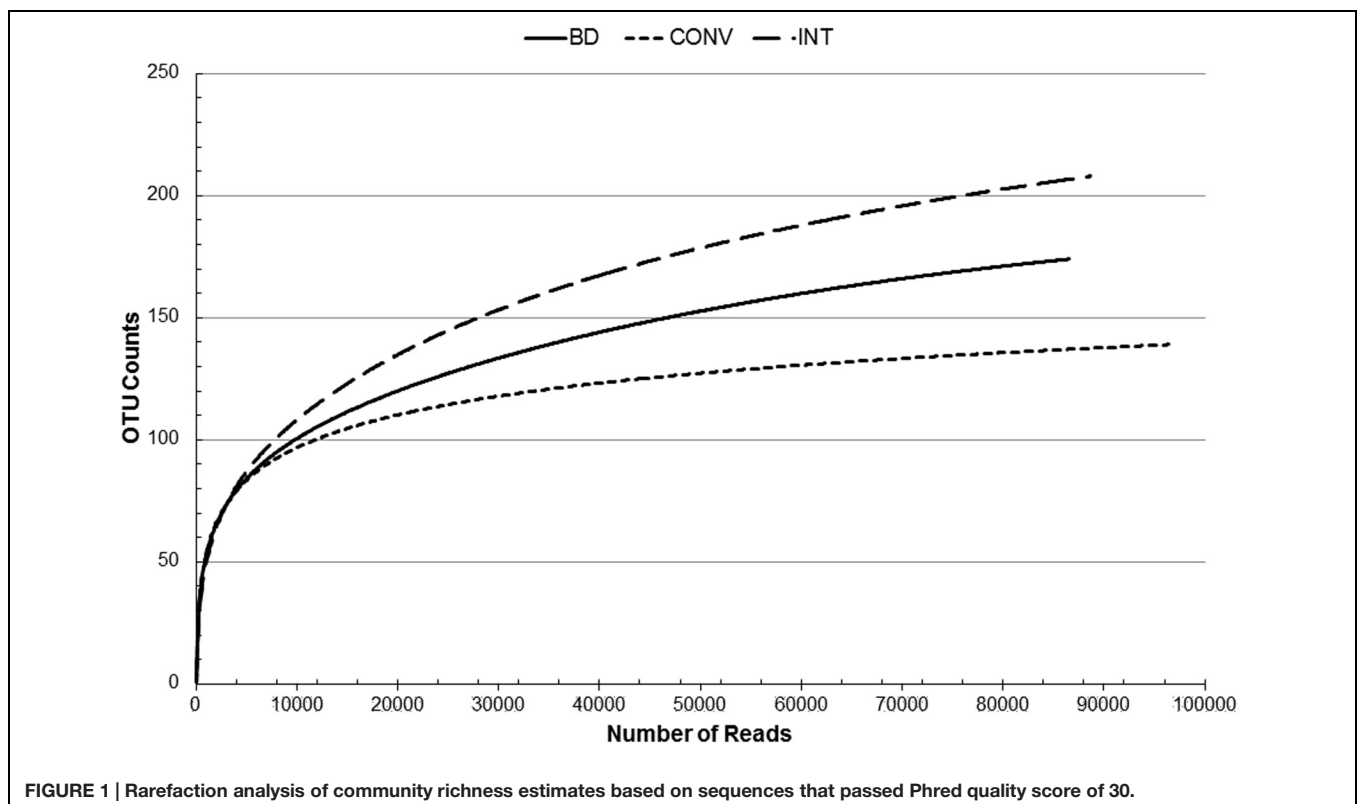


FIGURE 1 | Rarefaction analysis of community richness estimates based on sequences that passed Phred quality score of 30.

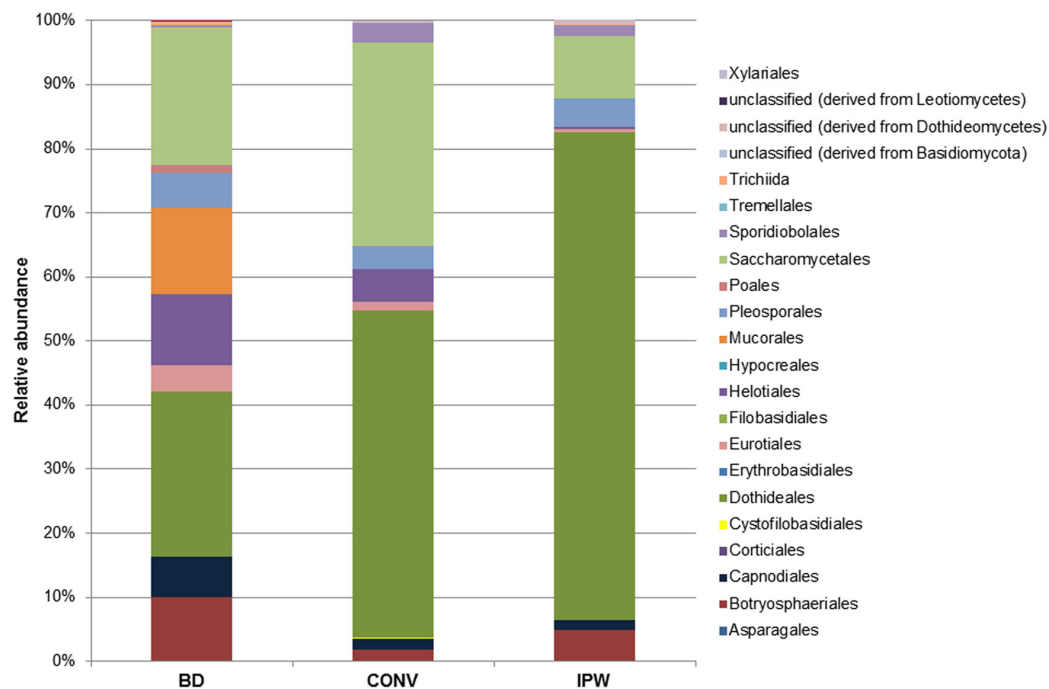


FIGURE 2 | Distribution of fungal species recovered across the orders of Ascomycetes, Basidiomycetes, and Zygomycetes.

Botryotinia fuckeliana, *Neofusicoccum australe*, *Cladosporium cladosporioides*, *Davidiella tassiana*, *Lewia infectoria*, and *Mucor* sp., were abundant in the BD vineyard must, while the IPW must displayed a more diverse *Neofusicoccum* community, with *N. parvum* being the dominant species of this genus. *Phoma herbarum* and *Diplodia seriata* were more dominant in the CONV vineyard (Figure 4). Fungi that were abundant amongst typical epiphytic taxa in the three vineyards were *Penicillium brevicompactum*, *P. corylophilum*, *P. glabrum* and *Pleospora herbarum*. In contrast, *Aspergillus tubingensis* was only present in BD and IPW, while *Botrytis elliptica* was present only in BD and CONV. The CONV exhibited a lower diversity of grapevine phytopathogens compared to the BD and IPW. Some of the fungi detected in the mycobiome were not previously known to associate with grapevine such as *Ascochyta rabiei*, *Ascochyta fabae*, *P. sojicola* (synonym, *A. sojicola*), *Lophodermium pinastri*, and *Sphaeropsis sapinea* (synonym, *D. pinea*). These fungi were, however, present at levels below 1%. Overall, fungi that are potential grapevine pathogens accounted for 50% of the total population in the must from the BD vineyard, while in the CONV and IPW, they accounted for 10 and 8%, respectively.

Analysis of the Yeast Community

Yeasts that constitute the wine microbial consortium have been grouped into previously described categories: (i) oligotrophic oxidative yeasts, e.g., (*Cryptococcus* sp., *A. pullulans*, *Rhodospiridium* sp., *Sporobolomyces* sp.), (ii) copiotrophic oxidative and weakly fermentative yeasts, e.g., (*Candida* sp., *Pichia* sp., *Hanseniaspora* sp., *Metschnikowia pulcherrima*, *Rhodotorula glutinis*, *Lachancea thermotolerans*), and (iii)

copiotrophic strongly fermentative yeasts, e.g., (*Torulaspora delbrueckii*, *Saccharomyces* sp., *Zygosaccharomyces* sp.), (Ocón et al., 2010; Barata et al., 2012). These groups of yeasts accounted for 22, 35, and 12% of the total fungal diversity in the BD, CONV, and IPW, grape must samples, respectively. The oxidative yeasts mainly comprised *Sporobolomyces* sp., *Rhodospiridium* sp., and *Rhodotorula* sp., which were only present at low levels (Figure 5). *M. pulcherrima* was the most dominant weakly fermentative yeast in the BD and CONV mycobiome, while *Starmerella bacillaris* (synonym, *Candida zemplinina*) was the most dominant in the IPW mycobiome. *Hanseniaspora uvarum* was present in similar amounts in the three mycobiomes. The strongly fermentative yeasts were generally present at very low levels. Amongst them, *L. thermotolerans* was detected in higher levels in the BD and CONV mycobiome, *T. delbrueckii* was only detected in the IPW mycobiome while *Kazachstania unispora* was only detected in the BD mycobiome and *Saccharomyces cerevisiae* only in the CONV mycobiome (Figure 5). Overall, 11 fermentative yeast species were detected in the BD mycobiome while 8 were detected in the CONV and 9 in the IPW mycobiomes. A comparison of the sequence data with the yeasts isolated from the same must samples shows the most commonly isolated yeasts could be detected by both methods with 11 species shared between them (Figure 6).

Comparative Analysis of SA and California Data

Composite lists of the fungal species detected in the grape musts from the three SA vineyards and those found in Californian

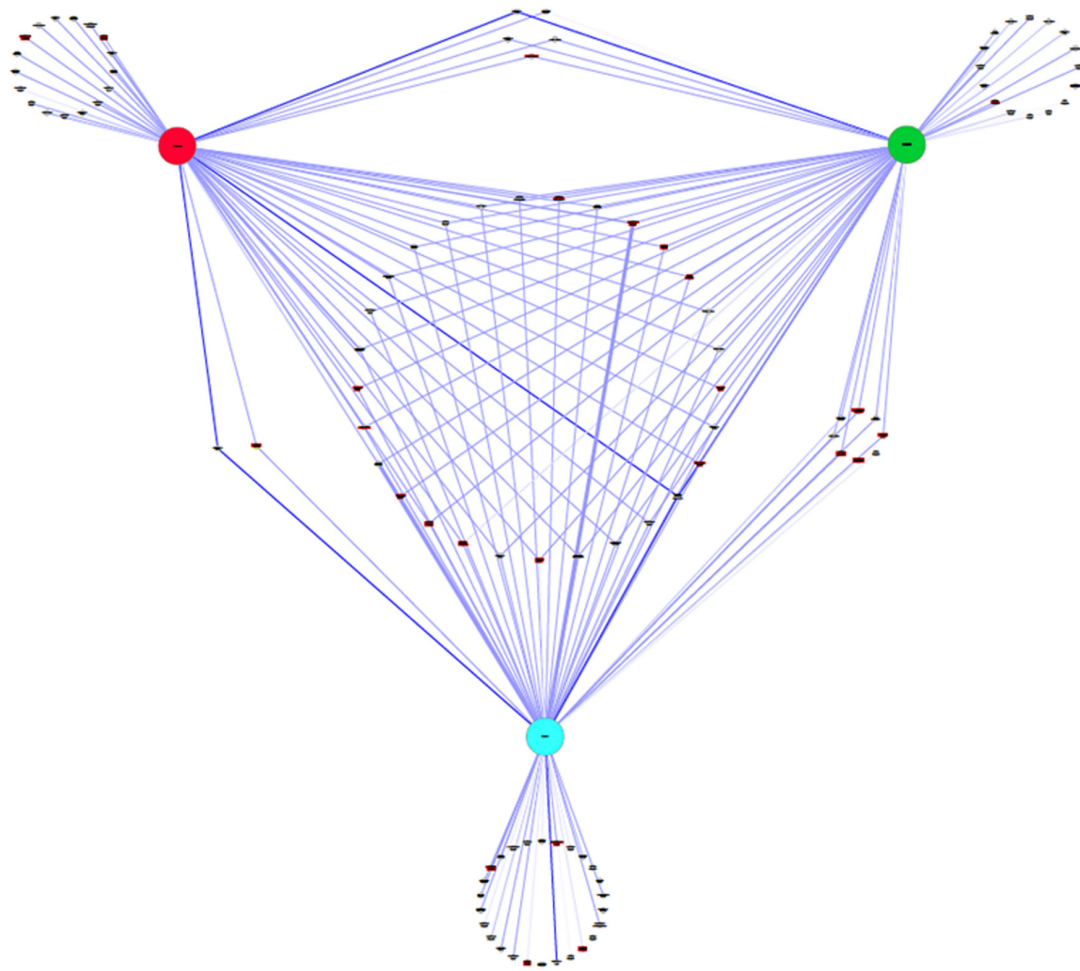


FIGURE 3 | A weighted co-occurrence network of the fungal communities in the grape must prepared from grapes obtained from the biodynamic (BD; green), conventional (CONV; red) and integrated (blue) vineyard.

vineyards through Illumina amplicon sequencing were generated and matched against the list of yeast isolates from the SA vineyards. The data revealed vast differences in fungal diversity detected through amplicon sequencing from the two countries with only 29 fungal species shared between the two data sets (**Figure 7**). Fifteen species were common between the SA and California mycobiomes, while 10 species were common across SA yeast isolates, SA mycobiomes and California mycobiomes. An additional four species were common between the SA isolates and California mycobiomes. The common fungi can be broadly grouped into (i) yeasts typically found in the wine microbial consortium such as *L. thermotolerans*, *T. delbrueckii*, *S. bacillaris*, *S. cerevisiae*, *I. ssatchenka terricola*, *H. uvarum*, and *Hanseniaspora guilliermondii*, (ii) genera that are frequent components of plant endophyte surveys such as *Alternaria*, *Davidiella*, *Lewia*, *Phoma*, *Aureobasidium*, and *Epicoccum* and (iii) ubiquitous epiphytes such as *Penicillium* and *Aspergillus* species. Our data also revealed one yeast species isolate (*M. pulcherrima*) that was detected only in the musts from SA mycobiomes and not the California mycobiomes (**Figure 7**).

DISCUSSION

The diversity of yeast and fungi associated with the grape berry and grape must have been the focus of many studies in the past. However, most of these studies have mainly relied on culture-dependent methods to define the diversity. Recently, culture-independent methods including ARISA, DGGE, and CE-SSCP have been employed especially in comparative studies as they provide a better overview of microbial community structures in different samples. However, confident identification of taxa represented in the community fingerprints is not always easy or reliable. Consequently, metagenomic approaches are the methods of choice for unraveling the microbiome associated with different ecosystems. In the current study, Illumina sequencing of the ITS1-5.8S rDNA-ITS2 gene sequences directly amplified from grape must samples derived from a CONV, integrated and BD vineyard. Our data show that the Ascomycota are the most dominant phylum constituting the grape must mycobiome. This is in agreement with data reported by Bokulich et al. (2014) and also with cultivation based studies that have shown that

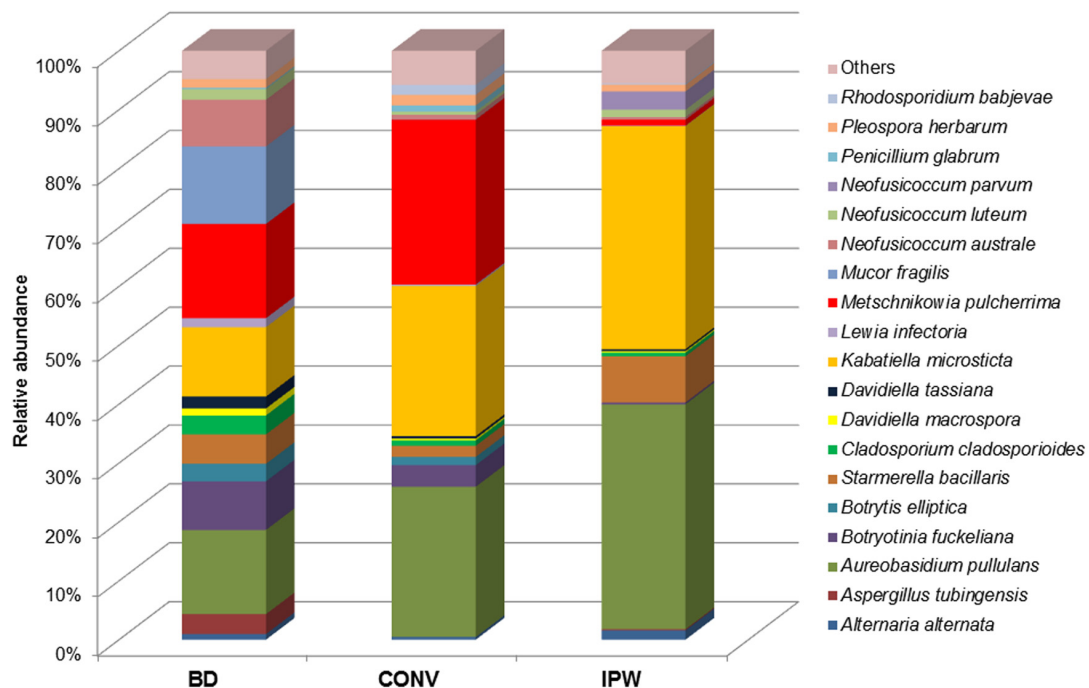


FIGURE 4 | The frequency of occurrence of the abundant fungal taxa as well as major grapevine associated taxa.

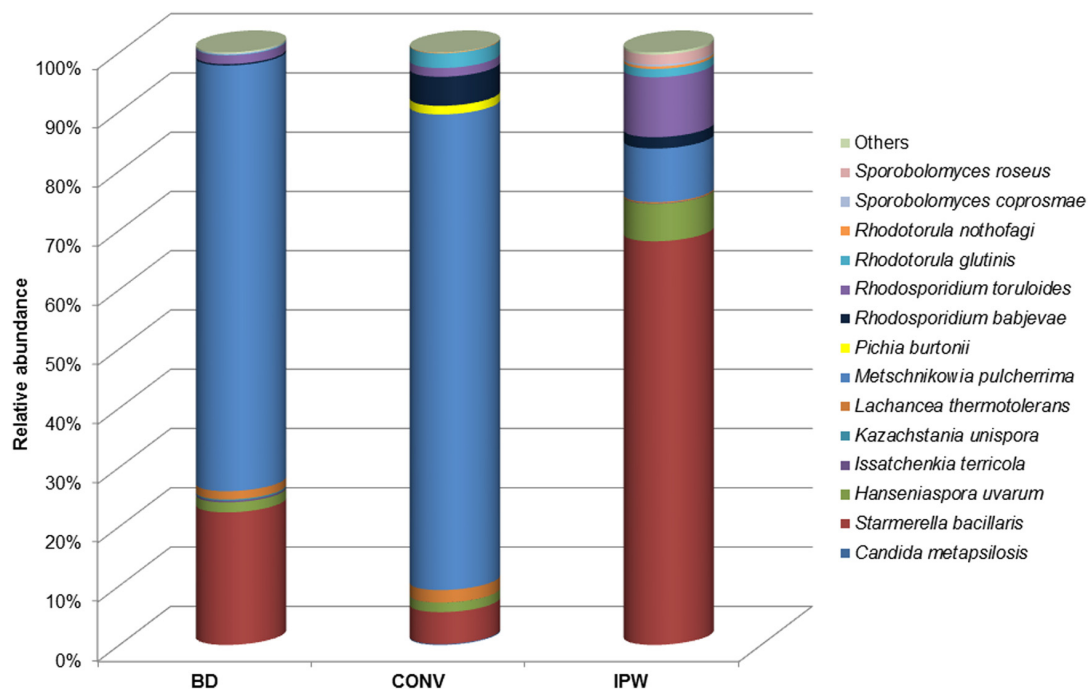
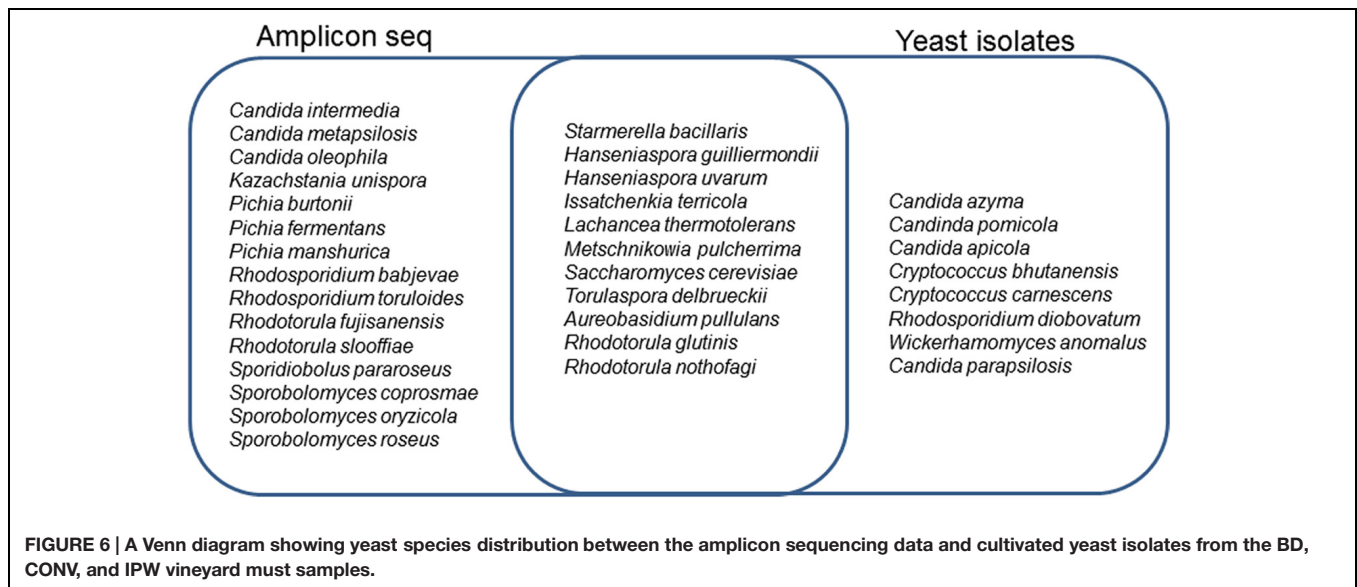


FIGURE 5 | Relative abundance of yeast species frequently encountered in the wine microbial consortium.

the grapevine fungal endophytes mainly comprise ascomycetous fungi while the epiphytic community has also been shown to shift from a basidiomycetes dominated community at berry-set to an ascomycetes dominated community at full ripeness

(Prakitchaiwattana et al., 2004; Renouf et al., 2005, 2007). The BD vineyard displayed a higher incidence of Zygomycetes mainly represented by *Mucor* and *Rhizopus* species. Pinto et al. (2014) recently reported such fungi belonging to early diverging fungi

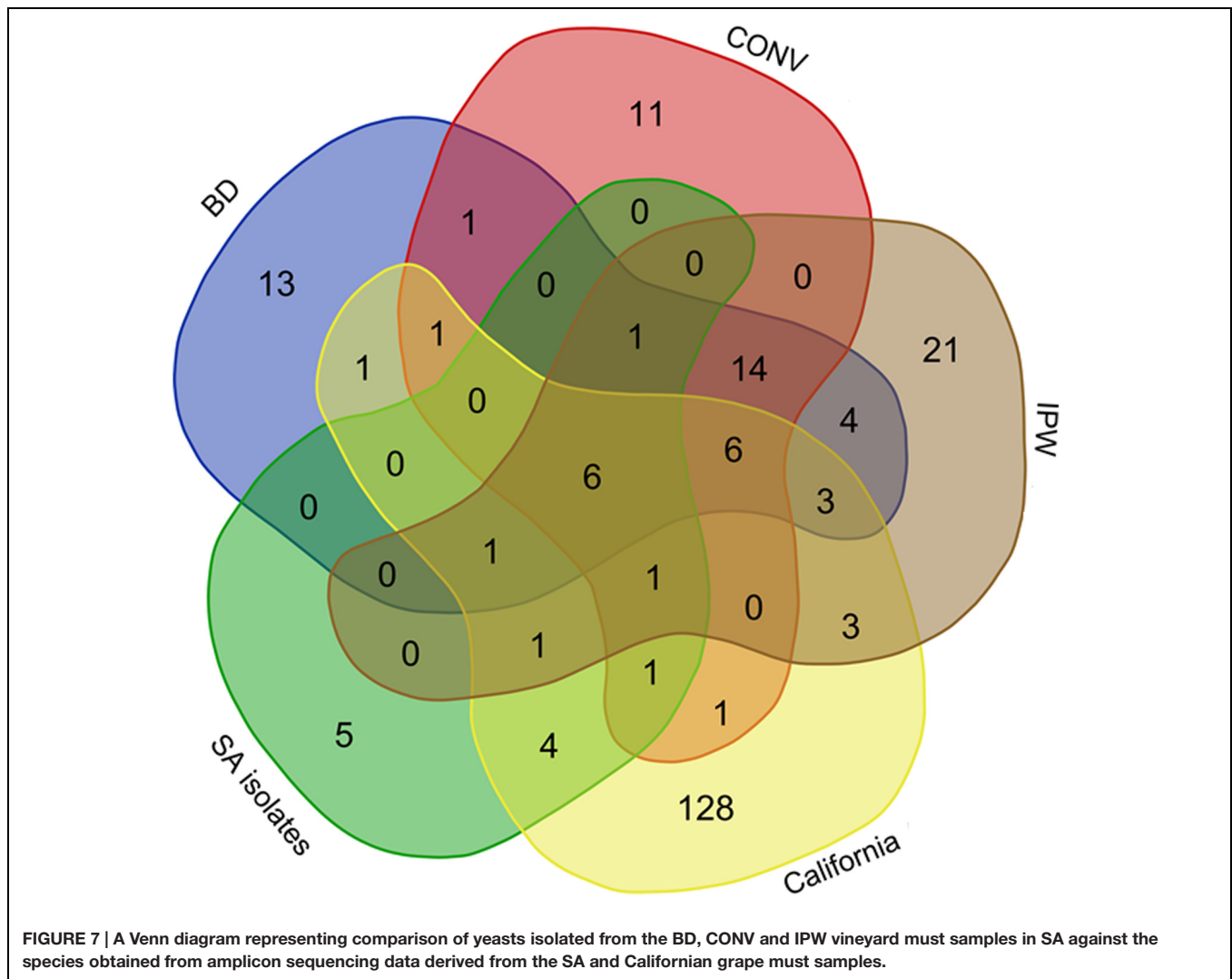


to account for close to 28% of the total mycobiota of grapevine leaves. *Mucor* sp. are most known to cause post-harvest rot in table grapes but rarely in wine grapes (Kassemeyer and Berkelmann-Lohnertz, 2009). Our previous data using ARISA analysis demonstrated that the epiphytic fungal community associated with the three vineyards was distinct from each other (Setati et al., 2012).

The current data revealed that members of the order Dothidiales were the most abundant in the three vineyards, albeit with very significant differences, since the two most prominent species, *A. pullulans* and *K. microsticta* accounted for 24, 50, and 76% in the BD, CONV, and IPW vineyard, respectively. *A. pullulans* is a common inhabitant of the grapevine ecosystem and has previously been shown to be present amongst both the endophytic and epiphytic fungal communities. In our previous study, we also found this yeast-like fungus to be the most abundant yeast isolated from the grape surface where it accounted for more than 50% of the yeast isolates (Setati et al., 2012). *K. microsticta* on the other hand, has never been isolated from grape vine before. In fact, members of this genus have not been successfully cultured and are only known from their sporodochial stages (Zalar et al., 2008). Importantly, the genus *Kabatiella* is a plant pathogen known to cause leaf spot on specific plant species. Its presence in grape must might be due to the transfer of spores from the epiphytic microbiota of the leaves to the grape berries. This fungus has not been shown to be a pathogen of grapevine. It would therefore be highly relevant to investigate whether this pathogen can impact on grapevine, and whether its presence is of wider relevance for the SA and global wine industry, or whether its occurrence is locally restricted. Interestingly, other *Kabatiella* sp. have been found associated with the Proteaceae family, characteristic of the fynbos biome endemic to the Western Cape province of South Africa (Taylor and Crous, 2000), suggesting that they might indeed be common members of the regional plant microbiota.

The BD grape must exhibited a higher incidence of phytopathogenic molds with potential to cause post-harvest rot. Some of these fungi, e.g., *Alternaria* sp. and *Cladosporium* sp., have previously been isolated from the grape endosphere (Pancher et al., 2012). Importantly, only healthy grapes have been used for our analysis, and the overall health status of all three vineyards at harvest appeared visually similar (i.e., no apparent diseased state). This not suggests that these fungi find it easier to colonize vineyards that are not treated with fungicides, but that their increased presence in the vineyard may itself not be problematic. Indeed, Dugan et al. (2002) demonstrated that grape berries were progressively infected with quiescent fungi, mainly members of the genera *Alternaria*, *Aureobasidium*, *Cladosporium*, and *Ulocladium*. Invasion by the fungus may occur via the stigma and style, resulting in latent infection of the berry. By harvest time, as much as 25–78% of the grape clusters may be colonized with various fungi including *B. cinerea*/*B. fuckeliana* (Dugan et al., 2002). These fungi, therefore may reside in the berry without causing any disease. The overall higher biodiversity within the BD vineyard may indeed act as a protective element.

Only the musts from the IPW vineyard contained a diverse group of *Neofussicoccum* species. These fungi, especially *N. parvum* are opportunistic pathogens of grapevine, proven to cause *Botryosphaeria* dieback. *N. parvum* which was only detected in the IPW vineyard must and *N. australe* which was most dominant in the BD vineyard must, are some of the most virulent species in South Africa (van Niekerk et al., 2004). Another *Botryosphaeriaceae* fungus detected in the IPW mycobiome was *Lasiodiplodia theobromae*, which has been reported as the most virulent of this group of fungi (Úrbez-Torres and Gubler, 2009). Although these fungi are often isolated from the woody grapevine, they have also been isolated from grapes (Yan et al., 2013). *L. theobromae* typically infects grape berries during véraison. However, the germination of *Botryosphaeriaceae* fungi is highly dependent on temperature



and humidity (Yan et al., 2013). Interestingly, *Ampelomyces quisqualis* was detected in the mycobiome of the IPW vineyard. This fungus is a naturally occurring mycoparasite of several powdery mildew species and is used as a biocontrol agent against *E. necator* and other powdery mildew species (Falk et al., 1995; Angeli et al., 2009). Some of the fungi detected in the mycobiome of the different vineyards are known as pathogens of other plants. These include *A. fabae* and *A. rabiei* which are known to cause blight disease in chick pea, wheat, barley, oats, rye, triticale, and turf grasses and may have been transferred from neighboring plants or plants such as oats which are commonly used as cover crops in the vineyard.

Common wine yeasts were found in the mycobiome from the three vineyards. *M. pulcherrima* and *S. bacillaris* were the most abundant weakly fermentative yeasts, followed by *H. uvarum*. Most of the other fermentative yeasts could be detected albeit at low levels. These included various *Candida* sp., *Pichia* sp., *L. thermotolerans*, *T. delbrueckii*, and *S. cerevisiae*. *K. unisporea* was only detected in the must from the BD vineyard. Members of this genus were previously isolated amongst the epiphytic

community in the same vineyard (Setati et al., 2012). Our data show that *S. bacillaris* is most abundant in the must with the highest sugar level, which is consistent with previous studies that have shown this yeast to be dominant in high sugar musts (Tofalo et al., 2009). This suggests that some of the differences in the three must samples can in part be attributed to differences in ripeness levels. Fermentations performed on the must from the three vineyards showed that the non-*Saccharomyces* yeast species that were already well represented in the must, persisted longer in fermentation (Bagheri et al., 2015). However, our data show disparity between culture-based method and high throughput amplicon sequencing with regard to the yeasts retrieved. For instance, different basidiomycetous yeasts of the genera *Rhodosporeidium* and *Rhodotorula* were detected using culture-based method compared to those detected in the metagenome. For instance, in the metagenomic datasets *Rhodosporeidium babjevae* and *R. toruloides* were detected, while the culture-based approach found *R. diobovatum*. In addition, yeasts such as *C. parapsilosis* and *Wickerhamomyces anomalus* previously shown to dominate fermentations in the BD and

IPW must, respectively (Bagheri et al., 2015), could not be detected in the metagenomic data even though they were found to account for at least 10% of the initial population in the must. Similar disparities between culture-based methods and direct sequencing were reported by David et al. (2014) where for instance, *Sporobolomyces roseus* and *Bulleromyces albus* were found to account for 18–21% of the population in the middle of fermentation by culture-based method but could not be detected in the sequence data, while *T. delbrueckii* was found to be 15.9% of the population in one fermentation through direct sequencing but was not retrieved by culture-based methods. The reasons for such disparities could differ from species to species and might include DNA extraction biases in complex communities, PCR amplification bias, better cultivability as well as rapid growth for some species. The ratio of weakly fermentative to strongly fermentative yeasts was shown to influence fermentation rate. Pinto et al. (2014) demonstrated that some of the fermentative species of the genera *Saccharomyces*, *Hanseniaspora*, and *Metschnikowia* were present in the microbiome of leaves proving these organisms to be natural colonizers of the vine even before the appearance of the grape berries.

The data generated in the current study revealed huge differences in fungal assemblages between SA and Californian vineyards with approximately 25% of fungal species present in SA mycobiomes detected in the Californian mycobiomes. This was surprising especially since the California dataset covers an extensive number of vineyards which potentially should increase the probability of finding similar species when matching the SA dataset to the California data. However, given this difference in the community composition is probably acceptable given that these are cross-continental comparisons. The common fungal species mostly represented plant endophytes with antifungal properties useful against several plant diseases, as well as common constituents of the wine microbial consortium that drive fermentation processes. This suggests that there are only minor variations in resident mutualistic endophytes of grapevine, and the existence of a core group of species defining vineyard microbial ecosystem. The data show that there are two groups of fungal endophytes associated with *V. vinifera*. The first group comprises *Alternaria tenuissima*, *D. tassiana*, *Epicoccum nigrum*, *L. infectoria*, *Massarina corticola*, *P. herbarum*, and *Stemphylium* sp., which are intimately associated with *V. vinifera* globally, while the second group is characterized by fungi that are “host neutral” (i.e., generalist fungal pathogens) e.g., *D. seriata*, *N. parvum*, *N. australe*, and *L. theobromae*, that maybe horizontally transmitted between plant species and whose host affinity is strongly influenced by the environment (Slippers and Wingfield, 2007). This second group largely comprises botryosphaeriaceous fungi that have an endophytic phase and a pathogenic phase which can lead to rapid development of disease following the onset of stress due to factors such as extreme weather conditions (Slippers and Wingfield, 2007). Surprisingly, 9 species of this group

of fungi were detected in SA vineyards while only 1 was detected in the Californian data sets. In contrast, a higher incidence of leaf spot inducing saprophytic fungi of the genera *Leptosphaeria*, *Phaeosphaeria*, and *Leptosphaerulina* was apparent in Californian vineyards. These findings highlight critical differences in plant pathogenic fungal clusters. Regarding yeasts of oenological relevance, similar yeast species could be detected across SA and California samples. Most of the species were also retrievable by cultivation, suggesting that strain variation as well as the combination and concentrations of individual species and strains are pivotal in determining stylistic distinction.

Overall, the current study shows the highly significant differences ($p = 0.025$) in fungal species assemblages between neighboring vineyards. Also, the data reveal interesting groupings of fungi and major distinctions in the *V. vinifera* mycobiomes across continents but also delineates a group of species that could be host specific endophytes. However, more data will be necessary to confirm such information. Most importantly, our data highlight critical differences in plant pathogenic fungal clusters. An in-depth investigation into these clusters could make significant contributions toward developing targeted control strategies that are focused on managing the most prevalent phytopathogens in a given region. Considering that the Californian study extended over large regions, it might appear surprising that the species overlap with our data is relatively small. The data therefore suggest that fungal ecosystem diverge very significantly according to region and vineyard, further supporting the idea of a microbial *terroir* of relevance to wine style and wine quality. Impact of farming practices also appears highly relevant in shaping fungal biodiversity, in particular when considering that the composite samples were representative of entire neighboring vineyards of the same age sampled at the same time.

ACKNOWLEDGMENTS

The authors would like to thank Winetech (grant IWBT-MG-11/01) and the National Research Foundation-Technology and Human Resources for Industry Programme (grant TP2011060600013) for funding. The opinions expressed and conclusions arrived at, are those of the authors and are not necessarily to be attributed to the funding agencies. We also thank Mr. Johan Reyneke (Reyneke Wines), Mr. Deon Joubert as well as Mr. and Mrs. Emil and Sonet den Dulk (De Toren winery) for granting us permission to sample their vineyards. Finally, many thanks to Dr. Nicholas Bokulich who provided the data from Californian grape must analyses.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fmicb.2015.01358>

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer Carmen Portillo and handling Editor Alberto Mas declared their shared affiliation, and the handling Editor states that the process nevertheless met the standards of a fair and objective review.

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Hanseniaspora uvarum from Winemaking Environments Show Spatial and Temporal Genetic Clustering

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OPEN ACCESS

Edited by:

Alberto Mas,
University Rovira i Virgili, Spain

Reviewed by:

Maurizio Ciani,
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Specialty section:

This article was submitted to
Food Microbiology,
a section of the journal
Frontiers in Microbiology

Received: 05 November 2015

Accepted: 27 December 2015

Published: 20 January 2016

Citation:

Albertin W, Setati ME, Miot-Sertier C, Mostert TT, Colonna-Ceccaldi B, Coulon J, Girard P, Moine V, Pillet M, Salin F, Bely M, Divol B and Masneuf-Pomarede I (2016) *Hanseniaspora uvarum* from Winemaking Environments Show Spatial and Temporal Genetic Clustering. *Front. Microbiol.* 6:1569. doi: 10.3389/fmicb.2015.01569

Hanseniaspora uvarum is one of the most abundant yeast species found on grapes and in grape must, at least before the onset of alcoholic fermentation (AF) which is usually performed by *Saccharomyces* species. The aim of this study was to characterize the genetic and phenotypic variability within the *H. uvarum* species. One hundred and fifteen strains isolated from winemaking environments in different geographical origins were analyzed using 11 microsatellite markers and a subset of 47 strains were analyzed by AFLP. *H. uvarum* isolates clustered mainly on the basis of their geographical localization as revealed by microsatellites. In addition, a strong clustering based on year of isolation was evidenced, indicating that the genetic diversity of *H. uvarum* isolates was related to both spatial and temporal variations. Conversely, clustering analysis based on AFLP data provided a different picture with groups showing no particular characteristics, but provided higher strain discrimination. This result indicated that AFLP approaches are inadequate to establish the genetic relationship between individuals, but allowed good strain discrimination. At the phenotypic level, several extracellular enzymatic activities of enological relevance (pectinase, chitinase, protease, β -glucosidase) were measured but showed low diversity. The impact of environmental factors of enological interest (temperature, anaerobia, and copper addition) on growth was also assessed and showed poor variation. Altogether, this work provided both new analytical tool (microsatellites) and new insights into the genetic and phenotypic diversity of *H. uvarum*, a yeast species that has previously been identified as a potential candidate for co-inoculation in grape must, but whose intraspecific variability had never been fully assessed.

Keywords: *Hanseniaspora uvarum*, wine, intraspecific diversity, microsatellites, phenotypic screening

INTRODUCTION

Hanseniaspora uvarum (anamorph *Kloeckera apiculata*) is an apiculate yeast species frequently found on mature fruits (Spencer et al., 1992; Morais et al., 1995) and particularly on grapes where it forms part of the grape and fermentation microbiome. Its association with grapes and the first stages of alcoholic fermentation (AF) has been reported repeatedly during the last century (Castelli, 1955; Schütz and Gafner, 1993; Hierro et al., 2006) and for most—if not all—vineyard regions worldwide (Heard and Fleet, 1985; Holloway et al., 1990; Mateo et al., 1991; Comi et al., 2001; Beltran et al., 2002; Jolly et al., 2003; Combina et al., 2005; Li et al., 2010; Zott et al., 2010; Kachalkin et al., 2015). *H. uvarum* is also frequently isolated from other fermented beverages such as cider (Lachance, 1995; Cabranes et al., 1997; Valles et al., 2007; Pando Bedrinana et al., 2012), palm wine and cashew juice (Owuama and Saunders, 1990), tequila (Bilbao et al., 1997), sugar-cane aguardente (Morais et al., 1997), etc. It is part of the natural microbiome of many fermented food processes, including coffee (Masoud et al., 2004) and cocoa (Batista et al., 2015) fermentations. In some biotechnological processes such as yogurt (Kosse et al., 1997), orange juice (Renard et al., 2008), beer (Wiles, 1950), and honey (Pulvirenti et al., 2009) production, *H. uvarum* is considered as a spoilage species. *H. uvarum* also displays industrially relevant antagonistic properties against the development of molds responsible for fruit spoilage. The species is thus extensively assessed as a biocontrol agent against *Botrytis cinerea* (gray mold) on grapes and strawberries (Long et al., 2005; Liu et al., 2010a,b; Cai et al., 2015), *Penicillium* spp. (fruit rot) on citrus (Long et al., 2005), *Colletotrichum capsici* (fruit rot) on chili (Basha and Ramanujam, 2014), etc., while the underlying mechanisms of action are actively studied (Liu et al., 2014; Pu et al., 2014). The ecological extent of *H. uvarum* is large: it has been collected from soils (Capriotti, 1955), plants (Sláviková et al., 2009), insects (Nguyen et al., 2007), birds (Kocan and Hasenclever, 1972), molluscs (de Araujo et al., 1995), and shrimps (Pagnocca et al., 1989), while its occurrence as clinical isolate on humans is rare and considered as opportunistic (Emmanouil-Nikoloussi et al., 1994; Garcia-Martos et al., 1999).

In winemaking, the presence of indigenous apiculate yeasts has long been viewed as undesirable (Velázquez et al., 1991; Ciani, 1998; Comitini and Ciani, 2010), and methods or factors to limit their proliferation during AF have been described (Farías and Manca de Nadra, 2003; Sosa et al., 2008; Comitini and Ciani, 2010). However, the renewed interest in non-conventional yeasts in the wine industry has led to the reassessment of the species suitable—and beneficial—for winemaking purpose. Several studies report on the characterization of the outcome of AF by *H. uvarum* in mixed or sequential inoculation with *Saccharomyces cerevisiae* in grape must, as *H. uvarum* alone is not able to complete AF (i.e., to consume all the sugar contained in grape musts). Wines resulting from mixed or sequential inoculation of *H. uvarum* and *S. cerevisiae* were shown to differ from pure cultures (*S. cerevisiae*) in their chemical composition. Indeed, the concentrations of some organic acids, aldehydes and minor alcohols (Hong and Park,

2013), higher alcohols and volatile metabolites (Zironi et al., 1993; Zohre and Erten, 2002; Moreira et al., 2011), isoamyl acetate (Moreira et al., 2008), butanediol and acetoin (Romano et al., 1993, 2000), and a few other compounds were reported as significantly different. Some of these alterations could be associated with the secretion of extracellular enzymes. Indeed, several enzymatic activities of technological interest have been characterized, such as β -glucosidase, xylosidase, protease, and lipase activities (Charoenchai et al., 1997; Manzanares et al., 1999; Capece et al., 2005). Moreover, some strains of the *H. uvarum* species were shown to be low producers of ochratoxin A (OTA), the main mycotoxin found in wine (Angioni et al., 2007). For all these reasons, the ability of *H. uvarum* to be preserved by lyophilization and cryopreservation was assessed recently, and was found satisfactory enough to maintain its fermentation ability (de Arruda Moura Pietrowski et al., 2015).

The abiotic and biotic factors to which *H. uvarum* is exposed in grape must have also been investigated. The data showed that the growth of *H. uvarum* was significantly affected by temperature, pH, sulfite, and ethanol concentrations (Gao and Fleet, 1988; Heard and Fleet, 1988; Albertin et al., 2014b), with some of these factors having synergistic or buffering effects. Several authors reported the existence of interactions between *H. uvarum* and *S. cerevisiae* during AF (Mendoza et al., 2007; Wang et al., 2014), associated with various underlying mechanisms including production of killer toxin (Radler et al., 1985, 1990; Schmitt and Neuhausen, 1994), and release of yet unidentified metabolites (Wang et al., 2015).

However, most of these studies evaluated single strains of *H. uvarum*. Only a few authors considered several strains to account for potential diversity within the species (Comi et al., 2001; Capece et al., 2005), but even then, the genetic relationships between the different strains remained obscure due to the lack of dedicated tools. Indeed, the molecular approaches available to date allowed intraspecific discrimination, but not establishment of genetic distance: RAPD (randomly amplified polymorphic DNA, Capece et al., 2005) or restriction endonuclease analysis associated with pulse-field gel electrophoresis (REA-PFGE, Versavaud and Hallet, 1995) were described to discriminate *H. uvarum* strains. By contrast, PCR fingerprinting was not able to discriminate *H. uvarum* strains in Aglianico wines (Caruso et al., 2002). More recently, FT-IR (Fourier transform infrared spectroscopy) was successfully applied to the intraspecific discrimination of *H. uvarum* from grape berries and the winery environment (Grangeteau et al., 2015). However, none of these approaches allows the establishment of genetic relationships between the different isolates. Consequently, the extent of the diversity within the *H. uvarum* species remains uncharacterized.

In this study, 115 strains of *H. uvarum* were isolated from winemaking environments in France and South Africa. Their genetic variability was analyzed using two different approaches: microsatellite markers and AFLP (amplified fragment-length polymorphism). Their phenotypic diversity regarding enzymatic activities and response to environmental factors was also investigated.

MATERIAL AND METHODS

Yeast Strains

One hundred and eleven strains of *Hanseniaspora* spp., including mainly *H. uvarum* and a few *Hanseniaspora guillermundii*, were isolated from French and South African winemaking areas between 2003 and 2014 (Table 1). These strains were identified using molecular techniques like rDNA ITS analysis (Granchi et al., 1999), and sequencing of the D1/D2 domain of 26S rDNA (O'Donnell, 1993; Kurtzman and Robnett, 1998) or the ITS sequence (White et al., 1990; Esteve-Zarzoso et al., 1999). D1/D2 and ITS sequences were then blasted against either NCBI database or YeastIP, a curated yeast database (Weiss et al., 2013).

Fifteen strains from other geographical and substrate (nature, cider, etc.) origins were included (Table 1). For phenotypic characterization, several control strains were used: *S. cerevisiae* VIN13 (Mocke, 2005) was used as positive control for killer activity, *S. cerevisiae* ZIM 1859 S6 (Zagorc et al., 2001) was used as killer sensitive yeasts. *Metschnikowia pulcherrima* IWBT Y1123, *Schwanniomyces polymorphus* var. *africanus* CBS 8047, *Saccharomyces paradoxus* RO88 (Redzepovic et al., 2003), *Metschnikowia chrysoperlae* IWBT Y955 were used as positive controls for the acid protease, β -glucosidase, pectinase, and chitinase tests, respectively.

All strains were grown at 24°C in traditional YPD medium containing 1% yeast extract, 1% peptone, and 2% glucose (w/v), supplemented or not with 2% agar (w/v).

Microsatellite analysis was applied to all *Hanseniaspora* spp. strains available, while only a subset of strains was used for both AFLP and phenotyping assays, more time-consuming and less reproducible over large number of experiments. For AFLP approach, 47 strains were selected, and for phenotyping data we used a subset of 30 strains (all included in the AFLP panel) as well as 10 other *Hanseniaspora* spp.

Genome Sequencing, Microsatellite Loci Identification, and Primers Design

A draft genomic sequence was produced using Ion Torrent technology. Briefly, a genomic library of strain CRBO L0551 was produced using the Ion Xpress Plus Fragment Library Kit (Life Technologies, Carlsbad, USA), with an enzymatic shearing of 10 min at 37°C. DNA was sequenced on an Ion Torrent PGM (Life Technologies, Carlsbad, CA). After trimming on quality threshold (Phred-type quality score of Q20, QPhred = 20) and length threshold (50 bp) using CLC GenomicsWorkbench 7.0.3 (CLC bio, Boston, MA), Newbler software (version 2.7, 454 Life Sciences) was used to produce a *de novo* assembly of 1665 contigs of more than 1000 bp. This draft assembly forms a 7.68 Mb sequence for an estimated genome size of 8–9 Mb (Esteve-Zarzoso et al., 2001).

Microsatellites (di- to tetranucleotide repeats) were searched within the *de novo* genome assembly as described previously (Albertin et al., 2014a), and primers were designed using the Design primers' tool on the SGD website (<http://www.yeastgenome.org/cgi-bin/web-primer>) by applying Schuelke's method (Schuelke, 2000) to reduce costs. Amplified fragment

TABLE 1 | *Hanseniaspora* sp. strains used in this study.

Species	Strain	Collection ^a	Country	Year of isolation	Substrate
<i>H. uvarum</i>	CRBO L0638	CRBOeno	France	2006	Grape/wine
<i>H. uvarum</i>	CRBO L0552	CRBOeno	France	2005	Grape/wine
<i>H. uvarum</i>	CRBO L1437	CRBOeno	France	2014	Grape/wine
<i>H. uvarum</i>	CRBO L0531	CRBOeno	France	2005	Grape/wine
<i>H. uvarum</i>	CRBO L1491	CRBOeno	France	2014	Grape/wine
<i>H. uvarum</i>	CRBO L0555	CRBOeno	France	2005	Grape/wine
<i>H. uvarum</i>	CRBO L1468	CRBOeno	France	2014	Grape/wine
<i>H. uvarum</i>	CRBO L1481	CRBOeno	France	2014	Grape/wine
<i>H. uvarum</i>	CRBO L0413	CRBOeno	France	2003	Grape/wine
<i>H. uvarum</i>	CRBO L0414	CRBOeno	France	2003	Grape/wine
<i>H. uvarum</i>	CRBO L1438	CRBOeno	France	2014	Grape/wine
<i>H. uvarum</i>	CRBO L1497	CRBOeno	France	2014	Grape/wine
<i>H. uvarum</i>	CRBO L0430	CRBOeno	France	2003	Grape/wine
<i>H. uvarum</i>	CRBO L1469	CRBOeno	France	2014	Grape/wine
<i>H. uvarum</i>	CRBO L0406	CRBOeno	France	2003	Grape/wine
<i>H. uvarum</i>	CRBO L14124	CRBOeno	France	2013	Grape/wine
<i>H. uvarum</i>	CRBO L1455	CRBOeno	France	2014	Grape/wine
<i>H. uvarum</i>	CRBO L0765	UR Oeno	France	2007	Grape/wine
<i>H. uvarum</i>	CRBO L1474	CRBOeno	France	2014	Grape/wine
<i>H. uvarum</i>	NZ15	CRPR	New-Zealand	2009	Grape/wine
<i>H. uvarum</i>	Gui21	UR Oeno	France	2012	Grape/wine
<i>H. uvarum</i>	NZ234	CRPR	New-Zealand	2009	Grape/wine
<i>H. uvarum</i>	CRBO L0660	CRBOeno	France	2006	Grape/wine
<i>H. uvarum</i>	CRBO L0764	CRBOeno	France	2007	Grape/wine
<i>H. uvarum</i>	CRBO L0557	CRBOeno	France	2005	Grape/wine
<i>H. uvarum</i>	CRBO L0658	CRBOeno	France	2006	Grape/wine
<i>H. uvarum</i>	CRBO L0659	CRBOeno	France	2006	Grape/wine
<i>H. uvarum</i>	CRBO L0763	UR Oeno	France	2007	Grape/wine
<i>H. uvarum</i>	CRBO L0456	CRBOeno	France	2003	Grape/wine
<i>H. uvarum</i>	CRBO L0666	CRBOeno	France	2006	Grape/wine
<i>H. uvarum</i>	CRBO L0554	CRBOeno	France	2005	Grape/wine
<i>H. uvarum</i>	CRBO L0639	CRBOeno	France	2006	Grape/wine
<i>H. uvarum</i>	IWBT Y888	IWBT	South Africa	2011	Grape/wine
<i>H. uvarum</i>	CRBO L14118	CRBOeno	France	2014	Grape/wine
<i>H. uvarum</i>	CRBO L14150	CRBOeno	France	2014	Grape/wine
<i>H. uvarum</i>	CRBO L14144	CRBOeno	France	2014	Grape/wine
<i>H. uvarum</i>	CRBO L1442	CRBOeno	France	2014	Grape/wine
<i>H. uvarum</i>	NZ5	CRPR	New-Zealand	2009	Grape/wine
<i>H. uvarum</i>	CRBO L1433	CRBOeno	France	2014	Grape/wine
<i>H. uvarum</i>	CRBO L1492	CRBOeno	France	2014	Grape/wine
<i>H. uvarum</i>	CRBO L14112	CRBOeno	France	2014	Grape/wine
<i>H. uvarum</i>	CRBO L14136	CRBOeno	France	2014	Grape/wine
<i>H. uvarum</i>	Y-1612	NRRL	Indonesia	NA	Soil
<i>H. uvarum</i>	Y-915	NRRL	NA	NA	Cider
<i>H. uvarum</i>	NZ1	CRPR	New-Zealand	2009	Grape/wine
<i>H. uvarum</i>	CRBO L1461	CRBOeno	France	2014	Grape/wine

(Continued)

TABLE 1 | Continued

Species	Strain	Collection ^a	Country	Year of isolation	Substrate
<i>H. uvarum</i>	CRBO L1441	CRBOeno	France	2014	Grape/wine
<i>H. uvarum</i>	CRBO L1415	CRBOeno	France	2014	Grape/wine
<i>H. uvarum</i>	CRBO L14130	CRBOeno	France	2014	Grape/wine
<i>H. uvarum</i>	DSMZ 70285	DSMZ	Germany	NA	Nature
<i>H. uvarum</i>	CRBO L14113	CRBOeno	France	2014	Grape/wine
<i>H. uvarum</i>	CRBO L0418	CRBOeno	France	2003	Grape/wine
<i>H. uvarum</i>	CLIB 303	CLIB	Ukraine	NA	Grape/wine
<i>H. uvarum</i>	CRBO L0428	CRBOeno	France	2003	Grape/wine
<i>H. uvarum</i>	CRBO L1449	CRBOeno	France	2014	Grape/wine
<i>H. uvarum</i>	CRBO L1448	CRBOeno	France	2014	Grape/wine
<i>H. uvarum</i>	CRBO L14108	CRBOeno	France	2014	Grape/wine
<i>H. uvarum</i>	CRBO L1420	CRBOeno	France	2014	Grape/wine
<i>H. uvarum</i>	516149	MAFF (NIAS)	Japan	NA	Nature
<i>H. uvarum</i>	CRBO L0401	CRBOeno	France	2003	Grape/wine
<i>H. uvarum</i>	CRBO L1462	CRBOeno	France	2014	Grape/wine
<i>H. uvarum</i>	CRBO L0665	CRBOeno	France	2006	Grape/wine
<i>H. uvarum</i>	CRBO L1414	CRBOeno	France	2014	Grape/wine
<i>H. uvarum</i>	CRBO L14149	CRBOeno	France	2014	Grape/wine
<i>H. uvarum</i>	CRBO L14143	CRBOeno	France	2014	Grape/wine
<i>H. uvarum</i>	CRBO L1404	CRBOeno	France	2014	Grape/wine
<i>H. uvarum</i>	CRBO L14129	CRBOeno	France	2014	Grape/wine
<i>H. uvarum</i>	CRBO L0312	CRBOeno	France	2003	Grape/wine
<i>H. uvarum</i>	Gui1	UR Oeno	France	2012	Grape/wine
<i>H. uvarum</i>	IWBT Y1173	IWBT	South Africa	2009	Grape/wine
<i>H. uvarum</i>	CRBO L0756	CRBOeno	France	2007	Grape/wine
<i>H. uvarum</i>	CRBO L14119	CRBOeno	France	2014	Grape/wine
<i>H. uvarum</i>	IWBT Y968	IWBT	South Africa	2014	Grape/wine
<i>H. uvarum</i>	TB Sau 1	UR Oeno	France	2012	Grape/wine
<i>H. uvarum</i>	IWBT Y952	IWBT	South Africa	2014	Grape/wine
<i>H. uvarum</i>	CRBO L1434	CRBOeno	France	2014	Grape/wine
<i>H. uvarum</i>	IWBT Y1097	IWBT	South Africa	2009	Grape/wine
<i>H. uvarum</i>	CRBO L1446	CRBOeno	France	2014	Grape/wine
<i>H. uvarum</i>	CRBO L0743	CRBOeno	France	2007	Grape/wine
<i>H. uvarum</i>	CRBO L0744	CRBOeno	France	2007	Grape/wine
<i>H. uvarum</i>	IWBT Y864	IWBT	South Africa	2011	Grape/wine
<i>H. uvarum</i>	TB Sem 1	UR Oeno	France	2012	Grape/wine
<i>H. uvarum</i>	IWBT Y967	IWBT	South Africa	2013	Grape/wine
<i>H. uvarum</i>	IWBT Y969	IWBT	South Africa	2014	Grape/wine
<i>H. uvarum</i>	CRBO L1427	CRBOeno	France	2014	Grape/wine
<i>H. uvarum</i>	CRBO L1486	CRBOeno	France	2014	Grape/wine
<i>H. uvarum</i>	IWBT Y1044	IWBT	South Africa	2009	Grape/wine
<i>H. guilliermondii</i>	IWBT Y901	IWBT	South Africa	2012	Grape/wine
<i>H. uvarum</i>	YB-783	NRRL	USA	NA	Nature
<i>H. uvarum</i>	CRBO L1430	CRBOeno	France	2014	Grape/wine
<i>H. uvarum</i>	IWBT Y861	IWBT	South Africa	2011	Grape/wine
<i>H. uvarum</i>	IWBT Y1116	IWBT	South Africa	2009	Grape/wine
<i>H. uvarum</i>	IWBT Y1139	IWBT	South Africa	2009	Grape/wine
<i>H. uvarum</i>	CRBO L1487	CRBOeno	France	2014	Grape/wine

(Continued)

TABLE 1 | Continued

Species	Strain	Collection ^a	Country	Year of isolation	Substrate
<i>H. uvarum</i>	CRBO L0551	CRBOeno	France	2005	Grape/wine
<i>H. uvarum</i>	CRBO L1445	CRBOeno	France	2014	Grape/wine
<i>H. uvarum</i>	Gui3	UR Oeno	France	2012	Grape/wine
<i>H. uvarum</i>	CRBO L14125	CRBOeno	France	2014	Grape/wine
<i>H. uvarum</i>	Y-1614	NRRL	Russia	NA	Grape/wine
<i>H. uvarum</i>	CRBO L1426	CRBOeno	France	2014	Grape/wine
<i>H. uvarum</i>	CRBO L0671	CRBOeno	France	2006	Grape/wine
<i>H. uvarum</i>	CRBO L14137	CRBOeno	France	2014	Grape/wine
<i>H. uvarum</i>	NZ148	CRPR	New-Zealand	2009	Grape/wine
<i>H. uvarum</i>	Gui12	UR Oeno	France	2012	Grape/wine
<i>H. uvarum</i>	CRBO L1473	CRBOeno	France	2014	Grape/wine
<i>H. uvarum</i>	YB-3199	NRRL	USA	NA	Fruit
<i>H. uvarum</i>	IWBT Y941	IWBT	South Africa	2013	Grape/wine
<i>H. uvarum</i>	Yq NS2	UR Oeno	France	2012	Grape/wine
<i>H. uvarum</i>	IWBT Y1100	IWBT	South Africa	2009	Grape/wine
<i>H. uvarum</i>	IWBT Y1013	IWBT	South Africa	2009	Grape/wine
<i>H. uvarum</i>	IWBT Y1196	IWBT	South Africa	2009	Grape/wine
<i>H. uvarum</i>	IWBT Y1177	IWBT	South Africa	2009	Grape/wine
<i>H. uvarum</i>	IWBT Y1192	IWBT	South Africa	2009	Grape/wine
<i>H. uvarum</i>	IWBT Y1133	IWBT	South Africa	2009	Grape/wine
<i>H. uvarum</i>	IWBT Y1190	IWBT	South Africa	2009	Grape/wine
<i>H. uvarum</i>	IWBT Y966	IWBT	South Africa	2013	Grape/wine
<i>H. uvarum</i>	CRBO L1418	CRBOeno	France	2014	Grape/wine
<i>H. guilliermondii</i>	113816	MAFF (NIAS)	NA	NA	Fruit
<i>H. guilliermondii</i>	IWBT Y970	IWBT	South Africa	2013	Grape/wine
<i>H. guilliermondii</i>	IWBT Y1035	IWBT	South Africa	2009	Grape/wine
<i>H. guilliermondii</i>	IWBT Y1165	IWBT	South Africa	2009	Grape/wine
<i>H. guilliermondii</i>	Y-1625	NRRL	South Africa	NA	Clinical
<i>H. opuntiae</i>	IWBT Y863	IWBT	South Africa	2011	Grape/wine
<i>H. opuntiae</i>	IWBT Y875	IWBT	South Africa	2011	Grape/wine
<i>H. vineae</i>	IWBT Y907	IWBT	South Africa	2012	Grape/wine
<i>H. vineae</i>	IWBT Y971	IWBT	South Africa	2013	Grape/wine

^aCLIB, CIRM-Levures, INRA/AgroParisTech, Thiverval-Grignon, France; CRBOeno, Centre de Ressources Biologiques Œnologie, Villenave d'Ornon, France; CRPR, Centre de Recherche Pernod-Ricard, Creteil, France; DSMZ, Leibniz-Institut DSMZ, Braunschweig, Germany; IWBT, IWBT, Stellenbosch University, South Africa; MAFF (NIAS), NIAS Genebank, Ibaraki, Japan; NRRL, ARS Culture Collection, Peoria, USA; UR Oeno, Research unit Œnologie, Villenave d'Ornon, France.
NA stands for "Not Available."

sizes varied from 120 to 466 bp, allowing subsequent multiplexing of the amplicons (Table 2).

Microsatellites Amplification

DNA was prepared as followed: yeast cells were diluted in 20 mM NaOH (concentration of 1.10⁸ cells/mL), then heated 10 min at

TABLE 2 | Microsatellite loci for *Hanseniaspora uvarum* genotyping.

Microsatellite name	Motif	Fluorescent dye	Primers	Tm	Alleles size range (bp)	Coding sequence (blastx result)	Ho (observed heterozygosity)
HU292	TCT	FAM	F: CACGACGTTGTAAACGACTTCAAATCCGGCATAGTGGT; R: CCATTGTTGCTTCTGAAACG	55	247–253 [2 alleles]	Unnamed protein product in <i>Kluyveromyces dobzhanskii</i> CBS 2104 [emb]CDO94325.1]	0.017
HU409	ACA	PET	F: CACGACGTTGTAAACGACAGATCAAGCAGAGACACACAAA; R: TTGTCGCTTTC TTGTAAACA	52	429–441 [4 alleles]	Hypothetical protein D499_OZ00400 in <i>H. uvarum</i> DSM 2768 [gb KKA01551.1]	0.087
HU440	ATA	FAM	F: CACGACGTTGTAAACGACACTGCTGTATTGCAATTTTG; R: GGAAAAAGCAGAAAAAAGCG	55	389–451 [9 alleles]	No significant similarity found	0.574
HU467	TGT	NED	F: CACGACGTTGTAAACGACGGGTTAGCAACGTTTTTCGAT; R: TGGATTGGTGAAAAATGTGCA	52	267–291 [6 alleles]	No significant similarity found	0.078
HU508	ACA	NED	F: CACGACGTTGTAAACGACAGAGGTACAGGCGTTATATCCGA; R: TGGTGTGTGATGACGTTGTG	55	146–192 [8 alleles]	No significant similarity found	0.07
HU593	AC	PET	F: CACGACGTTGTAAACGACCTCCTTGACCCCAAAAAA; R: TTCATGGGCTGTATGCTTG	52	261–287 [8 alleles]	No significant similarity found	0.2
HU594	AGA	FAM	F: CACGACGTTGTAAACGACACTTTTGCTTCACTGCTGGTGTC A; R: TGTTTTGATCCATTATCGG	52	120–152 [7 alleles]	No significant similarity found	0.33
HU620	TCTT	NED	F: CACGACGTTGTAAACGACCCCTTGCTGGTAAGGTACCTG; R: GCTTGGAAATGGCTGTTATG	52	428–466 [7 alleles]	Translation initiation factor eIF-2B subunit epsilon in <i>H. uvarum</i> DSM 2768 [gb KKA03659.1]	0.043
HU68	TCT	HEX	F: CACGACGTTGTAAACGACAAAGGCTATGTTGAGCCTGA; R: AAGGCTTGTTTAAAGCAGGT	52	153–180 [6 alleles]	No significant similarity found	0.096
HU853	TCT	PET	F: CACGACGTTGTAAACGACGCTTCAGAAATCAAAGCAGC; R: AGGTGCGGATCTAAATTGA	55	138–166 [5 alleles]	FACT complex subunit HuSPT16 in <i>H. uvarum</i> DSM 2768 [gb KKA03363.1]	0.27

Allele size in pb. Forward primers were tailed on 5'-end with M13 sequence (CACGACGTTGTAAACGAC). Tm is the melting temperature used for microsatellite amplification (see Section Materials and Methods).

94°C. This solution was used as DNA templates for further PCR reactions.

PCR were performed in a final volume of 15 µL containing 1 µL of DNA template, 0.05 µM of forward primer, 0.5 µM of reverse primer and labeled primer, 1X Taq-&GO (MP Biomedicals, Illkirch, France). Universal M13 primers were labeled with either FAM-, HEX-, PET-, or NED-fluorescent dyes (Eurofins MWG Operon, Les Ulis, France).

Touch-down PCR were carried out using iCycler (Biorad, Hercules, CA) thermal cycler. The program encompassed an initial denaturation step of 1 min at 94°C followed by 10 cycles of 30 s at 94°C, 30 s at $T_m + 10^\circ\text{C}$ (followed by a 1°C decrease per cycle until T_m is reached) and 30 s at 72°C, then 20 cycles of 30 s at 94°C, 30 s at T_m and 30 s at 72°C, and a final extension step of 2 min at 72°C.

Amplicons were initially analyzed by a microchip electrophoresis system (MultiNA, Shimadzu) and the optimal conditions for PCR amplifications were assessed. Then, the sizes of the amplified fragments were measured on an ABI3730 DNA analyzer (Applied Biosystems). For that purpose, PCR amplicons were diluted (1800-fold for FAM, 600-fold for HEX, 1200-fold for PET, and 1800-fold for NED-labeled amplicons respectively) and multiplexed in formamide. LIZ 600 molecular marker (ABI GeneScan 600 LIZ Size Standard, Applied Biosystem) was 100-fold diluted and added for each multiplex. Before loading, diluted amplicons were heated 4 min at 94°C. Allele size was recorded using GeneMarker Demo software V2.4.0 (SoftGenetics).

Microsatellite Analysis

Microsatellite analysis, based on allele size, was used to investigate the genetic relationships between isolates. A dendrogram was built using Bruvo's distance (Bruvo et al., 2004) and Ward's clustering, by means of R (R Development Core Team, 2010). Bruvo's distance is particularly well adapted in the case of multiple and/or unknown ploidy levels, which is the case for *H. uvarum* species. Since classical bootstrap resampling is poorly reliable with microsatellite data, we assessed the robustness of the tree nodes using multiscale bootstrap resampling of the loci associated with an approximately unbiased test (Shimodaira, 2002) by means of R and the pvclust package v1.2-2 (Suzuki and Shimodaira, 2006; R Development Core Team, 2010).

Analysis of molecular variance (AMOVA) was performed by means of the pegas package (Paradis, 2010) with $n = 1000$ permutations. We tested whether the genetic distance was significantly explained by geographical localization (i.e., the country of isolation was used as grouping factor) or year of isolation (from 2003 to 2014).

Amplified Fragment Length Polymorphism

For all yeast species and isolates, genomic DNA was extracted using mechanical cell breakage with glass beads (Hoffman, 2001). DNA concentrations were determined using the NanoDrop™ 1000 spectrophotometer. The AFLP reactions were performed according to Esteve-Zarzoso et al. (2010). Briefly, 1.5 µg DNA was digested for 4 h with *EcoRI*

and *MseI* at 37°C followed by ligation of the *EcoRI* (5'-CTCGTAGACTGCGTACC-3' and 5'-AATTGGTACGCAGTC-3') and *MseI* (5'-GACGATGAGTCCTGAG-3' and 5'-TACTCAGGACTCAT-3') adaptors. The primer pair *EcoRI*-0 (5'-GACTGCGTACCAATTC-3') and *MseI*-C (5'-GATGAGTCCTGAGTAAC-3') was used for the non-selective PCR of a 5 µL aliquot of the ligation mix diluted 10× with TE buffer, while the selective primer was performed using *EcoRI*-C (5'-GACTGCGTACCAATTCC-3') and *MseI*-AC (5'-GATGAGTCCTGAGTAAAC-3') primer pair. The bands were resolved on a 2% (w/v) agarose gel with 1× TBE buffer at 80 V. The gel was stained with GelRed and visualized under UV. The presence/absence of AFLP markers was scored against a GeneRuler™ 100 bp Plus DNA ladder (Fermentas Life Sciences, Finland) using GeneTools version 4.01 (SynGene, Synoptics Ltd., Cambridge, England). AFLP fragment sizes were rounded to the closest integer and a binary matrix (presence/absence) of 263 AFLP bands, ranging from 94 to 1865 pb was created. A dendrogram was subsequently built using Euclidean's distance, Ward's clustering and multiscale bootstrap resampling.

Screening for Extracellular Enzyme Activities of Enological Relevance

All yeast species were grown overnight in YPD broth (Biolab-Merck, Wadeville, South Africa) at 30°C on a rotary wheel. In order to standardize the number of cells spotted, the cultures were diluted to an optical density of 0.1 at a wavelength of 600 nm. On each plate, 10 µL of the diluted culture was spotted and incubated for 3 days at 30°C. The following activities were screened on solid agar media as previously reported in literature.

β-Glucosidase Activity

Extracellular β-glucosidase activity was tested on arbutin substrate [1% (w/v) yeast extract, 2% (w/v) peptone, 0.5% (w/v) arbutin, 20 mL 1% ammonium ferric] at pH 3.5 according to the method described by Strauss et al. (2001). *S. polymorphus* var. *africanus* (previously *Debaryomyces polymorphus* var. *africanus*) CBS 8047 was used as a positive control.

Acid Protease Activity

This assay was performed according to the method described by Bilinski et al. (1987). Sixty milliliters of phosphate-sodium buffer (24 g/L KH_2PO_4 + 35 g/L $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$) was microwaved with 70 mL skim milk solution (100 g/L skim milk in 0.05 M citrate phosphate buffer) for ~45 s or until it starts simmering. Four hundred and eighty milliliters agar (20 g/L, pH adjusted to 3.5) was then added and the plates poured. *M. pulcherrima* IWBT Y1123 (Reid et al., 2012) was used as a positive control for protease activity.

Polygalacturonase Activity

The assay was carried out following the method described by van Wyk et al. (van Wyk and Divol, 2010). Polygalacturonic acid [1.25% (w/v)] was dissolved in 0.68% (w/v) potassium phosphate (pH 3.5), together with 0.67% (w/v) YNB, 1% (w/v) glucose, 2% (w/v) agar. Positive activity was measured against the control yeast *S. paradoxus* RO88 (Mocke, 2005).

Chitinase Activity

Colloidal chitin [0.45% (w/v)] was used as substrate to test for chitinase activity according to the method described by Agrawal and Kotasthane (2012). The pH of the medium was adjusted to 4.7. *M. chrysoperlae* IWB T Y955 was used as a positive control (Ghosh, 2015).

Screening for Killer Activity

Hanseniaspora spp. isolates were tested for their potential killer activity against *S. cerevisiae* ZIM1859 S6 previously reported as killer sensitive strains. The so-called “spot-on-the-lawn” technique was used, as described by Mehlomakulu et al. (2014). Briefly, all the strains were cultivated overnight in 5 mL YPD broth on a rotary wheel. The cells were harvested by centrifugation and re-suspended in saline [0.9% (w/v) NaCl] to an OD_{600nm} of 0.1 ($\sim 3 \times 10^7$ cells/mL). To prepare the seeded cultures, 1 mL of the sensitive cells was mixed with 4 mL of a 4% (w/v) pre-autoclaved agar solution and 5 mL of a filter sterilized commercial preservative-free white table grape juice supplemented with 0.5% (w/v) yeast extract, adjusted to pH 4.5. The solution was poured into Petri dishes and allowed to set. Thereafter, 10 μ L of overnight cultures of the potential killer strains in saline were spotted on the surface. The plates were incubated at 20°C until a lawn of seeded yeasts was visible and a zone of inhibition around the killer positive strain *S. cerevisiae* VIN13 was observed.

Sporulation

Sporulation ability of *Hanseniaspora* spp. isolates was assessed on three different media: McClary's acetate agar (10% glucose, 1.8 g/L potassium chloride, 8.2 g/L sodium acetate trihydrate, 2.5 g/L yeast extract, 15 g/L agar), malt extract agar (5% malt extract, 2% agar) as described by Kurtzman et al. (2011), and potassium acetate agar (10 g/L Potassium Acetate, 15 g/L agar) by streaking colonies on these media. The cells were then stained according to the method described by Merritt and Hurley (1972).

Growth Assays under Various Environmental Conditions

Strain ability to metabolize glycerol as sole carbon source was tested as followed: strains were plated on 2% glycerol agar plates (1% yeast extract, 2% peptone, 2% glycerol, 1.5% agar) and incubated at 25°C for up to 7 days.

In order to test the impact of low temperature (12°C), anaerobia and the addition of copper solution, yeast strains were grown for 24 h in YNB (BD Difco) pH 3.5 at 25°C with constant agitation then serially 10-fold diluted and spotted on YNB agar plates (pH 3.5). Ten microliters of serial cellular concentrations were tested (10^3 cells/mL, 10^4 cells/mL, 10^5 cells/mL) and gave similar results. Cellular suspensions were spotted using a Steers multipoint inoculator. Anaerobic conditions were created in sachet by AnaeroGen sachet AN0025 (Oxoid). Actual anaerobia was checked using GasPak™ Dry Anaerobic Indicator Strips (BD). The presence or absence of growth was recorded after 48 h incubation (12°C or 25°C, aerobia or anaerobia).

Susceptibility to copper was estimated by plating the yeast strains on YNB pH 3.5 containing either CuSO₄ (copper

sulfate, the molecule usually contained in Bordeaux mixture) or Cu(OH)₂ (copper hydroxide, as contained in ChampFlo, Nufarm) at concentrations varying from 0.03 to 32 μ g/mL of CuSO₄ or Cu(OH)₂ respectively. After 48 h incubation at 25°C, the minimum inhibitory concentration (MIC) was determined.

RESULTS

Development of Microsatellite Markers for *Hanseniaspora Uvarum*

Next generation sequencing was used to produce a *de novo* assembly of the genome sequence of CRBO L0551, a strain isolated from grape must in Bordeaux region in 2005. Although this *de novo* assembly displayed an important number of contigs (1665 contigs of more than 1000 bp), it was sufficient to locate repeated sequence. Microsatellite loci (dinucleotide to tetranucleotide) were selected on the basis of their location: on different contigs and not within the 5'-end and 3'-end of the contigs (3 kb exclusion in order to exclude possible telomeric or subtelomeric positions). Primers were designed to amplify 11 microsatellite loci, four of them being located within putative coding sequence (Table 2). The amplicons were separated using a microchip electrophoresis system (MultiNA), and the optimal conditions for microsatellites amplifications were assessed on a subpanel of five strains of *H. uvarum* (data not shown). After optimization, the microsatellites markers were tested on other species of the *Hanseniaspora* genus: *H. guillermontii* Y-1625^T, 113816, IWB T Y1035, IWB T Y1165, IWB T Y901, IWB T Y970; *H. opuntiae* IWB T Y863 and IWB T Y875; *H. vineae* IWB T Y907, and IWB T Y971. No amplification was observed for these non-*uvarum* strains (data not shown), except for strain IWB T Y901. Strain IWB T Y901 was identified as *H. guillermontii* by sequencing both ITS and LSU D1/D2 rRNA regions, yet allowed the normal amplification of all 11 microsatellites markers.

The 11 microsatellites markers were then used to genotype 115 strains, including 101 *H. uvarum* strains isolated from various wineries in France near Bordeaux and in South Africa near Stellenbosch (Table 1). A few other isolates from winemaking environments were added: the type strain Y-1614 from Russia, five strains from New-Zealand (NZ1, NZ5, NZ15, NZ148, and NZ234) and CLIB 303 from Ukraine. Six strains from non-enological environments were also genotyped: Y-1612 (soil, Indonesia), Y-915 (cider, DSMZ 70285 (soil), 516149 (maize, Japan), YB-783 (tree, USA), and YB-3199 (fruit, USA). Strain IWB T Y901, identified as *H. guillermontii* but able to amplify all microsatellites, was also added. All microsatellites were polymorphic on this panel of 115 strains, with only two alleles for HU292 and up to 9 alleles for HU440 (Table 2). Although the polymorphism of the microsatellite loci was limited compared to other species (Legras et al., 2005; Albertin et al., 2014a,c; Masneuf-Pomarede et al., 2015), altogether they were discriminant enough to detect 86 different genotypes over the 115 tested. Twenty strains displayed only one allele per locus, while 95 showed heterozygosity for at least 1 upon 10 loci. Heterozygosity was detected for all loci, with observed heterozygosity ranging

from 0.017 for the less polymorphic locus HU292 to 0.574 for HU440, the more polymorphic locus.

Exploring the Genetic Relationships Between *H. uvarum* Isolates Using Microsatellites

The genetic relationships between the 115 isolates of *H. uvarum* were studied using Bruvo's distance (Bruvo et al., 2004) and Ward's clustering. The resulting dendrogram (Figure 1) shows three main clusters: one cluster (group C) contained almost all strains from South African winemaking environments (19 of the 21), and was highly supported (bootstrap value of 96). The two other groups contained mostly wine strains from France, but interestingly, these groups clustered on the basis of the year of isolation: most strains collected before 2009 clustered in group A (19 strains upon 29), with high bootstrap value (91). Group B contained 40 strains, most of them (25) being isolated after 2009 from winemaking environments in France (bootstrap value of 91).

To confirm the genetic clustering based on both geographical distance and year of isolation, we performed AMOVA. When using the country of origin as grouping factor, AMOVA was significant ($p = 0.00099$), and the country explained 8.54% of the total variation of the microsatellite dataset (Table 3). The year of

isolation was also used as a grouping factor, and explained much more variation (20.62%, $p < 10^{-6}$). These results confirmed that year of isolation as well as geographical origin significantly shaped the diversity of *H. uvarum* populations from winemaking environments.

Genetic Diversity of *H. uvarum* Populations in Winemaking Environments

The wine strains used in this study were isolated from several wineries, sometimes from different samples over several years. This is the case of winery G, for which 19 strains were isolated from grape must between 2003 and 2014 (Table 4). These 19 strains displayed 17 different genotypes distributed throughout the dendrogram, indicating that no clone was a specific signature of this winery. The same pattern was observed for all wineries: in most instances, several genotypes from different genetic groups were identified within the same winery, suggesting that the absence of genetic signature at the winery level was common for *H. uvarum* population.

We also studied the genetic diversity at sample level. For example, five strains from grape must were isolated and genotyped from the same sample in winery. The five strains (CRBO L0743, CRBO L0756, CRBO L0763, CRBO L0764, and

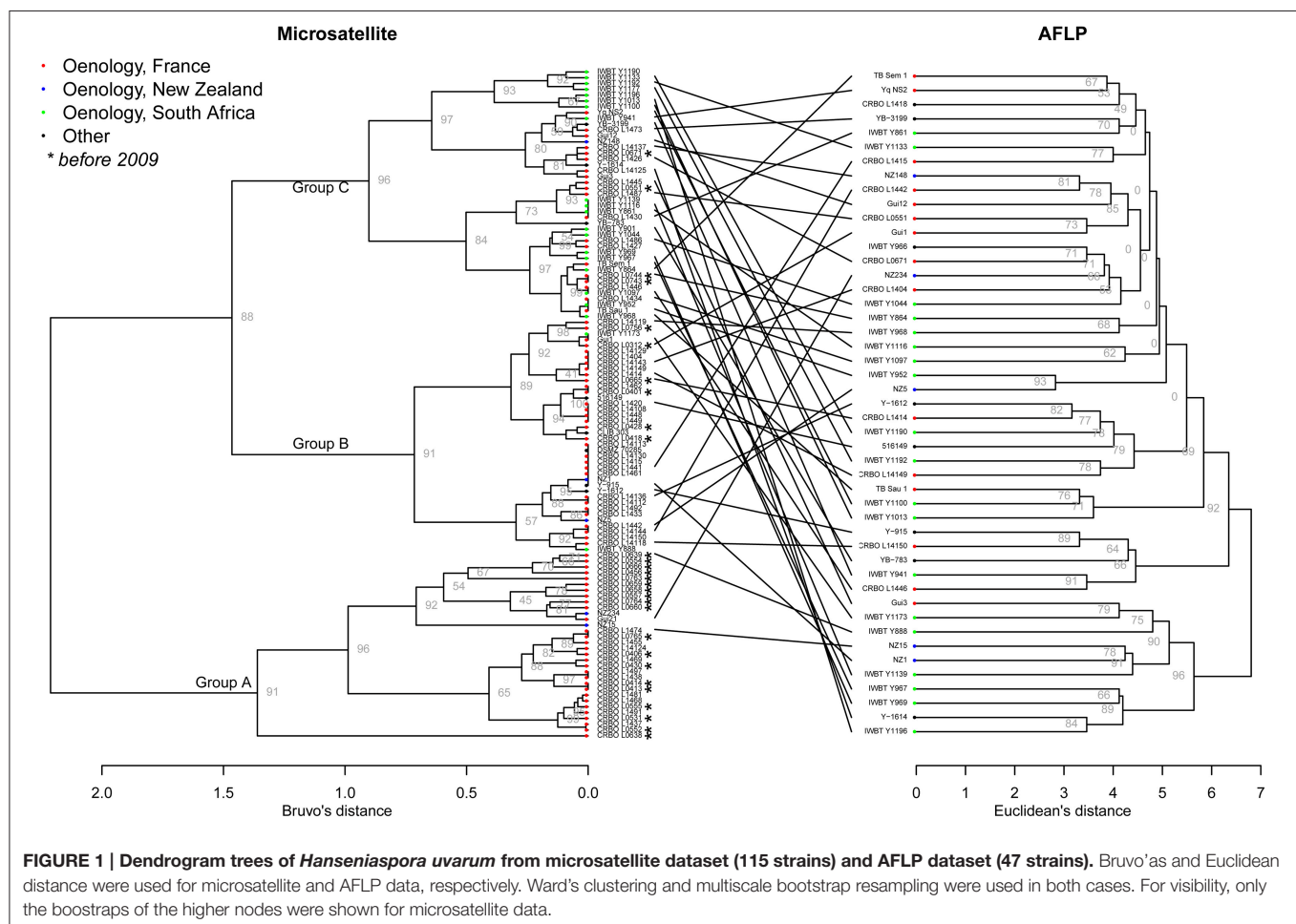


TABLE 3 | AMOVA results using microsatellite or AFLP dataset, for country and year of isolation as grouping factors.

Dataset	Factor	p-value	Variation explained by factor	Modalities (number of strains)
Microsatellite	Country	0.00099	8.54%	France (81); South Africa (21); New Zealand (5)
Microsatellite	Year of isolation	$<10^{-6}$	20.62%	2003 (9); 2005 (6); 2006 (8); 2007 (6); 2009 (17); 2011 (3); 2012 (8); 2013 (3); 2014 (47)
AFLP	Country	0.2258	–	France (16); South Africa (20); New Zealand (5)
AFLP	Year of isolation	0.7323	–	2005 (1); 2006 (1); 2009 (16); 2011 (3); 2012 (6); 2013 (3); 2014 (11)

TABLE 4 | Diversity of *Hanseniaspora uvarum* populations for 20 wineries as detected by microsatellite genotyping.

Winery	Country	Number of genotypes/ Number of strains	Year of isolations	Genetic groups	Strains ID
Winery A	France	3 genotypes/4 strains	2007–2009	Groups A-B	CRBO L0413, CRBO L0414, CRBO L0428, CRBO L0430
Winery B	France	3 genotypes/3 strains	2014–2014	Group C	CRBO L1426, CRBO L1427, CRBO L1430
Winery C	France	5 genotypes/6 strains	2003–2014	Groups A-B	CRBO L1414, CRBO L1415, CRBO L1437, CRBO L1438, CRBO L1441, CRBO L1442
Winery D	France	3 genotypes/3 strains	2005–2007	Group B	CRBO L14108, CRBO L14112, CRBO L14113
Winery E	South Africa	2 genotypes/2 strains	2009–2014	Group C	IWBT Y941, IWBT Y967
Winery G	France	17 genotypes/19 strains	2003–2014	Groups A-B-C	CRBO L0312, CRBO L14143, CRBO L14144, CRBO L14149, CRBO L14150, CRBO L1468, CRBO L1469, CRBO L1473, CRBO L1474, CRBO L1481, CRBO L1486, CRBO L1487, CRBO L1491, CRBO L1492, CRBO L1497, Gui1, Gui12, Gui21, Gui3
Winery H	New-Zealand	5 genotypes/5 strains	2005–2011	Groups A-B-C	NZ1, NZ148, NZ15, NZ234, NZ5
Winery I	South Africa	6 genotypes/6 strains	2003–2014	Groups B-C	IWBT Y861, IWBT Y864, IWBT Y888, IWBT Y952, IWBT Y968, IWBT Y969
Winery J	France	4 genotypes/4 strains	2005–2014	Groups B-C	CRBO L14129, CRBO L14130, CRBO L14136, CRBO L14137
Winery L	France	18 genotypes/19 strains	2003–2014	Groups A-B-C	CRBO L0551, CRBO L0552, CRBO L0554, CRBO L0555, CRBO L0557, CRBO L0638, CRBO L0639, CRBO L0658, CRBO L0659, CRBO L0660, CRBO L0665, CRBO L0666, CRBO L0671, CRBO L0743, CRBO L0744, CRBO L0756, CRBO L0763, CRBO L0764, CRBO L0765
Winery M	France	3 genotypes/3 strains	2005–2014	Groups B-C	CRBO L1404, CRBO L1433, CRBO L1434
Winery N	France	1 genotypes/2 strains	2007–2007	Group B	CRBO L1448, CRBO L1449
Winery O	France	2 genotypes/2 strains	2014–2014	Group C	CRBO L1445, CRBO L1446
Winery P	South Africa	3 genotypes/3 strains	2014–2014	Groups B-C	IWBT Y1013, IWBT Y1173, IWBT Y1177
Winery R	South Africa	2 genotypes/2 strains	2012–2012	Group C	IWBT Y1190, IWBT Y1192
Winery S	South Africa	2 genotypes/2 strains	2014–2014	Group C	IWBT Y1116, IWBT Y1196
Winery T	France	6 genotypes/6 strains	2003–2014	Groups A-B-C	CRBO L14118, CRBO L14119, CRBO L14124, CRBO L14125, TB Sau 1, TB Sem 1
Winery U	South Africa	2 genotypes/2 strains	2012–2014	Group C	IWBT Y1097, IWBT Y1100
Winery X	France	2 genotypes/2 strains	2009–2014	Groups A-B	CRBO L0401, CRBO L0406
Winery Y	France	4 genotypes/4 strains	2006–2014	Groups A-B-C	CRBO L1455, CRBO L1461, CRBO L1462, Yq NS2

CRBO L0765) exhibited five different genotypes distributed on the tree. In addition, in 2005, for winery L, some strains were isolated on days 1, 3, 4, and 5 during the pre-fermentative stage of the same tank. The four corresponding strains (CRBO L0552, CRBO L0554, CRBO L0555, and CRBO L0557) clustered in group A on the dendrogram, but they all displayed different genotypes. Altogether, these results suggest that the diversity of the population of *H. uvarum* is high in winemaking environments and that no specific genetic signature exists in a given winery.

Comparing Microsatellite and AFLP Typing

AFLP techniques are viewed as moderately repeatable over a high number of experiments, so that AFLP analyses are usually

applied to a limited number of strains in order to be reliable. Here, we chose to apply AFLP analysis to a subpanel of 47 strains, in order to compare the results obtained from microsatellite data and AFLP data. The AFLP dendrogram was produced using Euclidean distance and Ward's clustering (**Figure 1**). Comparison between microsatellite and AFLP dendrograms revealed important differences with no obvious clustering for AFLP data for country origin or vintage, even when comparing exactly the same subset of strains (**Supplementary Figure 1**). We subsequently performed AMOVA analysis using the distance matrix produced from AFLP data (**Table 3**). When using the country or the year of isolation as grouping factor, AMOVA was not significant ($p > 0.05$) using AFLP data, indicating that AFLP clustering was not able to detect the genetic structure

depending on geographical origin nor year of isolation. However, it has to be noted that AFLP tool was able to discriminate 47 strains upon 47 on the basis of their AFLP patterns. By contrast, on the same subset, the microsatellite tool identified 37 different genotypes. This indicated that although AFLP tool lacked robustness to assess the genetic relationship of individuals, it was more discriminant than the microsatellite tool.

Phenotyping *Hanseniaspora* sp. Isolates

As phenotyping assays are time-consuming, a subpanel of 30 strains of *H. uvarum* and 10 other *Hanseniaspora* spp. were selected and subjected to various plate assays to assess whether they possessed any extracellular enzyme activity that could be of interest in enology (Table 5). Their killer activity against two strains of *S. cerevisiae* that are sensitive to *S. cerevisiae*'s killer toxins was also investigated. Finally, their ability to grow when exposed to various environmental factors of scientific or enological interest (low temperature, anaerobia, copper presence, glycerol as the only carbon source) was recorded.

With regards to extracellular enzyme activity, all strains showed growth on the arbutin plates, although no distinct halo could be observed. Most *Hanseniaspora* strains showed a slight browning of the colony which might be due to weak activity or even intracellular β -glucosidase activity. Weak acid protease activity was observed for most strains, with YB-783 showing the largest halo (4 mm) and strains Gui21 and CRBO L0551 showing halos of 2 mm after 72-h incubation. No polygalacturonase activity was observed. Finally, weak chitinase activity could be visualized in isolates NZ234 and Y-861 (1-mm halo) while the other isolates showed no growth on the chitin media.

None of the strains was able to sporulate. Regarding their growth ability, most strains were unable to grow on 2% glycerol agar plates after incubation at 30°C for up to 7 days. Their growth ability under various environmental conditions was tested: ability to grow at low temperature (12 and 30°C), under oxic or anoxic, in presence of various copper concentrations. The MIC for copper sulfate and copper hydroxide was either 2 or 4 mg/L with no specific correlation between these 2 factors. All strains tested showed similar ability to grow under these conditions of enological interest, and limited phenotypic variations were recorded. Indeed, only strain Y-1614 did not show any growth at 12°C and anaerobiosis. Finally, no killer activity was observed against *S. cerevisiae*.

DISCUSSION

Comparing Microsatellite and AFLP Genotyping

In this paper, we compared the intraspecific clustering using two different techniques: AFLP and microsatellites. Both approaches allowed discrimination at the strain level: 47 different patterns were scored for AFLP (for 47 strains), while 86 genotypes were evidenced for 115 strains with microsatellite data. Indeed, both methods proved to be discriminant as previously reported (Mariette et al., 2001; Gaudeul et al., 2004), with AFLP having a higher discriminant power in our case. However, it has to

be noted that using AFLP, the amplification of multiple bands in a single run may lead to competition between amplicons and therefore to differences of band intensity that complicate data analysis. In addition, AFLP techniques are usually viewed as moderately repeatable thereby making the technique usually poorly reliable, while the repeatability of microsatellites markers is usually higher (Jones et al., 1997) and can be thus applied to a larger number of individuals.

Moreover, AFLP markers are non-codominant markers, so that homozygosity or heterozygosity is difficult to assess (Gaudeul et al., 2004). By contrast, microsatellites are codominant markers, allowing assessing heterozygosity status. Here, we found that 95 out of 115 strains showed heterozygosity, allowing an unprecedented insight into the genetics of the species. Microsatellites are widely used to estimate relatedness among individuals or differentiation among groups. By contrast, AFLP should be taken with caution due to the lack of complete genotypic information caused by dominance (Parker et al., 1998). Indeed, the dendrogram obtained by both approaches are not comparable and the genetic structure based on year of isolation and geographical origin evidenced using microsatellite was completely missed by AFLP analysis.

As expected, microsatellite genotyping proved to be a better tool for establishing genetic relation between strains and getting new insights within species at genetic level (Ross et al., 1999). By contrast, AFLP is interesting to perform assays where genetic relatedness is not needed, which is usually the case for several biotechnological applications in enology: assessing the global population diversity, testing for the prevalence/implantation of a specific (known) strain, searching for contamination evidence, etc. In these latter instances, the technical simplicity and rapidity of AFLP, associated with low cost, is definitively advantageous compared to microsatellite genotyping.

New Insights into the Genetic Structure of *Hanseniaspora uvarum* from Winemaking Environments

Like many non-conventional yeasts of enological interest, the genetic structure of *H. uvarum* from winemaking environments remained elusive. Here, using microsatellite data, we show that an important number of *H. uvarum* strains (95/115) are heterozygous. This result could be congruent with the hypothesis of a diploid species, although the possibility of aneuploidy could not be excluded. Additional work should be performed to confirm its diploid status, but will be complicated by the absence of sporulation on classical medium. The absence of sporulation could be explained, at least in part, by the weak ability of the species to metabolize glycerol, suggesting poor respiration ability (sporulation being strongly linked to respiration ability in *S. cerevisiae*, Codon et al., 1995). Indeed, all *H. uvarum* strains (except Y1614) showed unperturbed growth under anaerobic conditions, indicating that respiratory metabolism is not necessary for their normal growth.

Interestingly, strain IWBT Y901 –showing 456/456 identities for D1/D2 sequence with *Hanseniaspora guilliermondii* CBS 465^T– was clustered among *H. uvarum* strains in group C.

TABLE 5 | Phenotyping 45 *Hanseniaspora* sp. strains for enzymatic activities and growth ability.

Substrate	Activity					Growth				
	β-glucosidase	Protease	Pectinase	Pectinase	Chitinase	Glycerol	12°C	Anaerobia	CMI CuSO ₄ (μg/mL)	CMI Cu(OH) ₂ (μg/mL)
	Arbutin	Skim milk	PG Agar	PG Agarose	Chitin	–	–	–	–	–
516149	LB	1	0	–	–	w	+	+	4	4
NZ1	LB	1	0	–	–	–	NA	NA	NA	NA
NZ148	LB	0	–	–	–	w	+	+	4	2
NZ15	LB	1	–	–	–	w	+	+	2	2
NZ234	LB	0	w	–	1	G	+	+	2	4
NZ5	LB	1	0	–	–	–	+	+	4	4
TB Sau 1	LB	1	–	–	–	w	+	+	4	2
TB Sem 1	LB	1	0	–	–	w	+	+	4	2
IWBT Y1013	LB	1	–	–	–	w	NA	NA	NA	NA
IWBT Y1044	LB	1	–	–	–	w	NA	NA	NA	NA
IWBT Y1097	LB	1	–	–	–	w	NA	NA	NA	NA
IWBT Y1100	LB	–	–	–	–	w	+	+	2	4
IWBT Y1116	G	–	–	–	–	w	+	+	2	4
IWBT Y1133	LB	1	–	–	–	w	NA	NA	NA	NA
IWBT Y1139	LB	–	–	–	–	w	NA	NA	NA	NA
IWBT Y1173	LB	–	–	–	–	w	+	+	2	2
IWBT Y1177	LB	–	–	–	–	w	+	+	2	2
IWBT Y1190	LB	–	–	–	–	w	+	+	4	4
IWBT Y1192	LB	–	–	–	–	w	NA	NA	NA	NA
IWBT Y1196	LB	–	–	–	–	w	NA	NA	NA	NA
Y-1612	LB	1	–	–	–	w	+	+	2	2
Y-1614	LB	1	0	–	–	w	–	–	2	2
IWBT Y861	LB	1	–	–	1	w	+	+	4	2
IWBT Y864	LB	1	–	–	–	w	+	+	2	4
IWBT Y888	LB	–	–	–	–	w	+	+	2	4
Y-915	LB	1	0	–	–	w	+	+	4	2
IWBT Y941	LB	0	–	–	–	w	NA	NA	NA	NA
IWBT Y952	LB	0	–	–	–	w	+	+	2	4
IWBT Y966	LB	1	–	–	–	w	NA	NA	NA	NA
IWBT Y967	LB	1	–	–	–	w	NA	NA	NA	NA
IWBT Y968	LB	1	–	–	–	w	NA	NA	NA	NA
IWBT Y969	LB	–	–	–	–	w	NA	NA	NA	NA
YB-3199	LB	1	0	–	–	w	+	+	2	2
YB-783	LB	4	0	–	–	w	+	+	2	4
Yq NS2	LB	0	0	–	–	w	+	+	4	4
Other <i>Hanseniaspora</i> sp.										
<i>H. guilliermondii</i> 113816	LB	1	–	–	–	w	+	+	1	2
<i>H. guilliermondii</i> IWBT Y901	G	–	–	–	–	w	NA	NA	NA	NA
<i>H. guilliermondii</i> IWBT Y970	G	–	–	–	–	w	NA	NA	NA	NA
<i>H. guilliermondii</i> IWBT Y1035	LB	1	–	–	–	w	NA	NA	NA	NA
<i>H. guilliermondii</i> IWBT Y1165	LB	–	–	–	–	w	NA	NA	NA	NA
<i>H. guilliermondii</i> Y-1625	LB	1	0	–	–	w	–	+	2	2

(Continued)

TABLE 5 | Continued

Substrate	Activity					Growth				
	β-glucosidase	Protease	Pectinase	Pectinase	Chitinase	Glycerol	12°C	Anaerobia	CMI CuSO ₄ (μg/mL)	CMI Cu(OH) ₂ (μg/mL)
	Arbutin	Skim milk	PG Agar	PG Agarose	Chitin	–	–	–	–	–
<i>H. opuntiae</i> IWBT Y863	LB	0	–	–	–	w	NA	NA	NA	NA
<i>H. opuntiae</i> IWBT Y875	LB	–	–	–	–	w	NA	NA	NA	NA
<i>H. vineae</i> IWBT Y907	LB	–	–	–	–	w	NA	NA	NA	NA
<i>H. vineae</i> IWBT Y971	LB	–	–	–	–	w	NA	NA	NA	NA
CONTROLS										
<i>S. polymorphus</i> CBS 8047	12	0	G	–	6	G	NA	NA	NA	NA
<i>S. paradoxus</i> RO88	g	0	12	W	–	G	NA	NA	NA	NA
<i>M. pulcherrima</i> IWBT Y1123	2	10	G	–	W	G	+	+	4	2
<i>M. pulcherrima</i> IWBT Y1072	2	11	G	–	W	G	NA	NA	NA	NA
<i>S. cerevisiae</i> VIN13	G	–	W	–	–	G	NA	NA	NA	NA
<i>M. chrysoperlae</i> IWBT Y955	2	W	G	–	1	G	NA	NA	NA	NA

Zone sizes are indicated in mm, after subtracting the colony size from the total diameter of the zone; + or – indicates growth or absence of growth under these conditions; NA stands for “Not Available”; G indicates that there was colony growth but no extracellular enzyme activity was observed; W indicates very weak growth on the respective plate (single colonies could be observed within the spotted zone); LB indicates a color change in the colony, but no halo was observed.

While some microsatellite markers can cross the species, most microsatellites are intraspecific. The fact that IWBT Y901 is the sole *H. guillermundii* strain to be amplified by all 11 markers is clearly unusual. The possibility of a contamination of this strain with an actual *H. uvarum* strain can be eliminated as IWBT Y901 differed from all other strains we genotyped: its closest relative IWBT Y1044 differs from two alleles at two different loci. One possible explanation for these unexpected results is that IWBT Y901 could derived from an interspecific hybrid between *H. uvarum* and *H. guillermundii*, a hypothesis that remains to be demonstrated formally.

Microsatellite analysis also reveals a genetic structure related to geographical localization. Since we genotyped mostly strains from France and South Africa, it could be interesting to extend our analysis to strains from other countries in order to assess the extent of relationship between genetic structure and geographical origin. A few wine yeasts were shown to be genetically structured, at least partially, by geography, as it is the case of *Saccharomyces uvarum*, *Candida zemplinina*, or *Torulaspora delbrueckii* (Albertin et al., 2014a; Almeida et al., 2014; Masneuf-Pomarede et al., 2015). More surprisingly, our data show a strong relationship between the year of isolation and the genetic structure. This result indicates that *H. uvarum* populations isolated from winemaking environments show a temporal clustering in addition to a spatial one. More data are required to determine whether this temporal variation exists

only for French and South African strains over the period we tested (2003–2014), or if this trend is also detected for other vineyards and/or larger periods of time. In addition, further investigation is required to determine which factor(s) could be related to this temporal evolution. Factors to be tested include environmental factors such as temperature, pH, sugar concentrations, ability to survive from season to season, etc. Viticultural and enological practices should also be considered, including phytosanitary treatments, sulfite addition, cold pre-fermentation stage, turbidity, or starter culture addition that were shown to impact *Hanseniaspora* populations during the early stage of AF (Albertin et al., 2014b).

Finally, our data failed to identify any specific genetic signature associated with wineries and/or samples. Moreover, we globally identified high level of genetic diversity within all wineries/samples tested, with no evidence for clonal dominance. Such high genetic diversity was previously shown for *Hanseniaspora* populations in grape must and other environments of the winery using FT-IR (Grangeteau et al., 2015). High diversity was also detected for the wine yeast *C. zemplinina* (Masneuf-Pomarede et al., 2015), while other wine species like the spoilage yeast *Brettanomyces bruxellensis* showed clonal populations and maintenance over decades in winemaking environments (Albertin et al., 2014c). *H. uvarum* is known to be insect associated and therefore we can speculate that its diversity may depend on the diversity and frequency of insect occurrence

during ripening (Lam and Howell, 2015). Such hypothesis could be tested by investigating the insect-associated yeast diversity and comparing it with the grape/winery diversity.

H. uvarum Displays Low Phenotypic Variability for the Traits Investigated in this Study

In order to investigate whether the genetic clustering evidenced above reflects a certain phenotypic diversity, a number of phenotypes of enological relevance were tested: secretion of typical enzymes, as well as ability to grow at cold temperature (similar to that occurring at the beginning of the winemaking process and possibly during the fermentation of white wine), in anaerobiosis (typically occurring during wine fermentation) and ability to tolerate copper, a typical anti-fungal treatment used in the vineyard. The ability to grow on glycerol as sole source of carbon was also tested, following a preliminary observation that *H. uvarum* could not utilize glycerol (not shown). Although both microsatellites and AFLP revealed large genetic variation, the phenotypic variability was very low for the factors investigated (Table 4). Indeed, with a few exceptions, most strains exhibited similar extracellular enzyme activity, tolerance to copper and ability to grow at low temperature or poor ability to use glycerol as sole source of carbon. No clear connection could be observed between these phenotypes and the genetic clustering reported above. *H. uvarum* is usually categorized as a good producer of extracellular enzymes (Dizy and Bisson, 2000) and it is typically reported to exhibit all the enzymatic activities investigated in this study, although this seems to be strain dependent. However, most authors did not adjust the pH of their screening media to wine pH. There seems to be a general consensus between our data and previous studies, that when pH is adjusted to 3.5, most strains of *H. uvarum* display β -glucosidase and protease activities (Lagace and Bisson, 1990; Charoenchai et al., 1997), but not polygalacturonase activity (Charoenchai et al., 1997). No study however investigated the actual impact of these extracellular enzymes on wine composition. All the strains investigated in our study except one could grow at 12°C. This is in agreement with literature. Indeed, it has been reported that low temperatures favor biomass production in *H. uvarum* (Ciani et al., 2006; Mendoza et al., 2009). Surprisingly, *H. uvarum* seems to be poorly able to consume glycerol, even in the presence of oxygen. None of the strains investigated in this study were found able to sporulate. They should therefore all be classified as *H. uvarum* (teleomorph) and not *K. apiculata* (anamorph). As reviewed by Jolly et al. (2006), the region of isolation seems to play a role in the distribution of *H. uvarum* and *K. apiculata*. In temperate regions, an equal mixture of teleomorph/anamorph is found, while in warmer climates, only the teleomorph *H. uvarum* is detected. Yet our strains were isolated from both temperate and warm climate regions (France and South Africa, respectively) and none of the strains studied here were found able to sporulate. Another explanation could be the amount of time that these strains spent as freeze cultures. Indeed, some authors have reported

that the time between isolation and analysis plays a role in the ability of *H. uvarum* to sporulate (see review by Jolly et al., 2006).

Overall, with regard to the traits investigated in this study, *H. uvarum* seems to display very little phenotypic variability. In literature, a greater diversity seems to occur in terms of intracellular metabolism. Indeed, several studies report on the influence of *H. uvarum* inoculated in pure or mixed culture with *S. cerevisiae* and describe its production of esters, higher alcohols, and fatty acids (Moreira et al., 2008; Suzzi et al., 2012). These studies are not always in full agreement, as mentioned by the latter authors, and these would point out toward some intraspecific diversity at this level. Nevertheless, our results show that upon inoculation in grape juice, *H. uvarum* could survive the typical cold temperatures applied early in the winemaking process and during the fermentation of white wine as well as the anaerobic conditions also occurring during fermentation. Furthermore, it could potentially release glycosylated compounds and break down proteins through the activity of its extracellular enzymes, both properties being of strong enological interest. Since *H. uvarum* displayed no killer activity against the strains of *S. cerevisiae* tested, these two yeast species could be co-inoculated without threatening the overall proceedings of AF.

In conclusion, we describe in this paper a new analytical tool (microsatellite markers) that allowed estimating the genetic diversity and the genetic relationship between *H. uvarum* from winemaking environments. Our results indicate that *H. uvarum* populations are structured by both geographical origin and the year of isolation from a genetic viewpoint. By contrast, the phenotypic variability was more limited regarding extracellular enzymatic activities and response to environmental factors. Subsequent analysis of a larger number of isolates will help determine the extent of such results in winemaking environments.

AUTHOR CONTRIBUTIONS

WA and MS designed and performed most of the experiments. CM and TM performed the screening experiments. BC, JC, PG, and VM performed microsatellite development. MP and FS performed genome sequencing and microsatellite analysis. WA, MME, MB, BD and IMF conceived the project, wrote and edited the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fmicb.2015.01569>

Supplementary Figure 1 | Comparaison of dendrogram trees of *Hanseniaspora uvarum* obtained from microsatellite and AFLP approaches on the same subset of strains. Bruvo's and Euclidean distance were used for microsatellite and AFLP data, respectively.

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Past and Future of Non-Saccharomyces Yeasts: From Spoilage Microorganisms to Biotechnological Tools for Improving Wine Aroma Complexity

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Specialty section:

This article was submitted to
Food Microbiology,
a section of the journal
Frontiers in Microbiology

Received: 12 January 2016

Accepted: 14 March 2016

Published: 31 March 2016

Citation:

Padilla B, Gil JV and Manzanares P
(2016) Past and Future
of Non-Saccharomyces Yeasts: From
Spoilage Microorganisms
to Biotechnological Tools
for Improving Wine Aroma
Complexity. *Front. Microbiol.* 7:411.
doi: 10.3389/fmicb.2016.00411

It is well established that non-*Saccharomyces* wine yeasts, considered in the past as undesired or spoilage yeasts, can enhance the analytical composition, and aroma profile of the wine. The contribution of non-*Saccharomyces* yeasts, including the ability to secrete enzymes and produce secondary metabolites, glycerol and ethanol, release of mannoproteins or contributions to color stability, is species- and strain-specific, pointing out the key importance of a clever strain selection. The use of mixed starters of selected non-*Saccharomyces* yeasts with strains of *Saccharomyces cerevisiae* represents an alternative to both spontaneous and inoculated wine fermentations, taking advantage of the potential positive role that non-*Saccharomyces* wine yeast species play in the organoleptic characteristics of wine. In this context mixed starters can meet the growing demand for new and improved wine yeast strains adapted to different types and styles of wine. With the aim of presenting old and new evidences on the potential of non-*Saccharomyces* yeasts to address this market trend, we mainly review the studies focused on non-*Saccharomyces* strain selection and design of mixed starters directed to improve primary and secondary aroma of wines. The ability of non-*Saccharomyces* wine yeasts to produce enzymes and metabolites of oenological relevance is also discussed.

Keywords: non-*Saccharomyces* yeasts, enzymes, secondary metabolites, primary aroma, secondary aroma, mixed starters, aroma complexity

INTRODUCTION

Wine fermentation is a complex microbiological process in which yeasts play a fundamental role. Although *Saccharomyces cerevisiae* is the main microorganism involved in the alcoholic fermentation of grape must, winemaking is a non-sterile process. Many other species of yeasts belonging to various non-*Saccharomyces* genera occur in grape juice and contribute to the first stages of fermentation and to the organoleptic characteristics of final wine (Fleet, 2008).

In the past, non-*Saccharomyces* yeasts were considered to be of secondary significance or undesirable spoilage yeasts; nowadays it is widely accepted that selected strains through appropriate screenings can positively impact on the winemaking process. Thus the growing demand for new and improved wine yeast strains adapted to different types and styles of wines can be met by non-*Saccharomyces* wine yeasts. Since these yeasts are in general poor fermenters, the design of mixed starters including selected non-*Saccharomyces* with optimized biotechnological characteristics and *S. cerevisiae* to ensure a complete fermentation has become one of the main challenges of researchers and oenologists. Moreover, proper mixed starter management during fermentation will allow winemakers to tailor wines to the changing demands of consumers.

The production of wines with particular flavor profiles has been one of the main reasons for including non-*Saccharomyces* yeasts in mixed starters. However, promising approaches to lowering alcohol content of wines, to control wine spoilage or to improve oenological properties are being explored, and undoubtedly they represent new opportunities for exploitation in wine production. Here we revisit the contribution of non-*Saccharomyces* yeasts to wine aroma complexity. First we review the ability of these yeasts to produce enzymes and metabolites of oenological relevance and finally we discuss the design of mixed starters directed to improve primary and secondary aroma of wines. Special attention was paid to update the information covered in recent reviews on the impact of non-*Saccharomyces* in wine production.

NON-Saccharomyces YEASTS IN WINE PRODUCTION

In the second half of the 19th century, Louis Pasteur revealed the role of yeasts during the wine fermentation process, demonstrating that yeast is the primary catalyst responsible for the conversion of grape sugars to alcohol and CO₂. He noticed that in fermenting grape musts coexisted a wide variety of microorganisms, including different types of yeasts. His drawings, based on microscopic observations, showed two kinds of yeasts. The first, which was abundant in the early stages of the process, was the small, apically budding, lemon-shaped *Saccharomyces apiculatus* (now *Hanseniaspora uvarum*). The second which became the most abundant as alcoholic fermentation progressed, was a larger yeast with round cells, which Pasteur called either *Saccharomyces pastorianus* or *Saccharomyces ellipsoideus* (probably the current *S. cerevisiae*) (Barnett, 2000).

Despite the complex wine microbial ecology, *S. cerevisiae* became the wine yeast *par excellence* based mainly on its fermentation behavior (Reed and Peppler, 1973; Bely et al., 1990; Fleet, 1993), but also on its important role in the release of aroma precursors (Dubourdieu, 1996; Úbeda and Briones, 2000; Ugliano et al., 2006) and in the formation of secondary aroma (Fleet, 1993; Pretorius, 2003). The other yeast species occurring in musts and wines were considered as a source of potential spoilage problems during wine production. In fact, the presence or overgrowth of some of these species was often related to stuck or sluggish

fermentations, or to the production of detrimental compounds to the sensory properties of wine (du Toit and Pretorius, 2000). In the context of this simplistic view of the wine fermentation process, where the most important objective was the inoculation and dominance of *S. cerevisiae*, the term ‘non-*Saccharomyces*’ yeasts referred to the wide variety of yeast genera, including more than 20 in both Ascomycota and Basidiomycota phyla, present in grape juice.

Yeasts occurring in grape musts at the early stages of fermentation originate from two main sources, the vineyard and the grapes, and the contact surfaces and equipment of the winery (Pretorius et al., 1999). The latter plays a small role as a source of non-*Saccharomyces* yeasts, while *S. cerevisiae* is the predominant yeast in such surfaces (Peynaud and Dumercq, 1959; Rosini, 1984; Lonvaud-Funel, 1996; Pretorius, 2000). However, it has been recently reported for the first time the implantation in grape must of *Hanseniaspora* species present in the winery environment (Grangeteau et al., 2015) opening the possibility, still unexplored, that some of the non-*Saccharomyces* species could persist from 1 year to another in the winery environment and become dominant during fermentation, as usually described for *S. cerevisiae* (Santamaría et al., 2005; Le Jeune et al., 2006; Mercado et al., 2007).

The great quantitative and qualitative variability of non-*Saccharomyces* species found in the early stages of fermentation can be explained by the large number of factors influencing the grape microbiota such as localization, climatic conditions, cultivar, application of pesticides, and other agronomic practices, stage of ripening, health of the grapes, harvesting procedures and the specific weather conditions in each vintage year (Martini et al., 1980; Rosini et al., 1982; Querol et al., 1990; Regueiro et al., 1993; Epifanio et al., 1999; Jolly et al., 2006; Brilli et al., 2015). In spite of this wide variability of yeast species, during the first 3–4 days of a spontaneous fermentation of grape must, yeast population is numerically dominated by apiculate yeasts, *Hanseniaspora/Kloeckera*, and *Candida* species, followed by several species belonging to the genera *Metschnikowia* and *Pichia*, and occasionally to *Brettanomyces*, *Kluyveromyces*, *Schizosaccharomyces*, *Torulaspora*, *Rhodotorula*, *Zygosaccharomyces*, and *Cryptococcus* genera (Goto, 1980; Benda, 1982; Fleet et al., 1984; Heard and Fleet, 1985; Parish and Carroll, 1985; Martínez et al., 1989; Herraiz et al., 1990; Frezier and Dubourdieu, 1992; Schütz and Gafner, 1993; Granchi et al., 1998; Combina et al., 2005; Fleet, 2008). This scenario, with abundance of apiculate yeasts in the 1st days of alcoholic fermentation and varying amounts of other non-*Saccharomyces* yeasts, followed by the progressive dominance of *S. cerevisiae* is a common denominator in the process of elaboration of all wines, including those produced by inoculation with selected wine yeast strains (Heard and Fleet, 1985).

Industrial wine fermentations are currently conducted by starters of selected wine yeast strains of *S. cerevisiae*. The first reported use of a selected yeast starter for wine production dates from 1890, when Müller-Thurgau introduced this technology adapting the techniques developed by Christian Hansen for the Carlsberg Brewery (Pretorius, 2000; Barnett and Lichtenthaler, 2001). Nowadays, the use of active dry yeasts is one of the

most common practices in winemaking and the market offers a wide variety of yeast strains as dehydrated cultures promising a good implantation, specific skills for different types of wines and a great list of other features such as the ability of enhancing varietal and fermentative aromas, glycerol production, tolerance to alcohol, or specific enzymatic activities. However, the main reason of selected starters is to achieve wines with uniform quality through different years avoiding the variability associated with spontaneous fermentations and the risk of spoilage (Beltran et al., 2002; Santamaría et al., 2005). In such cases, dominant growth of the inoculated strain is required. However many factors might affect the implantation/persistence of individual strains within the total population (Fleet, 2008; Blanco et al., 2012), including the variability that exists from one vintage to another at a given winery (Lange et al., 2014).

Despite the advantages of using pure cultures of *S. cerevisiae* with regard to the easy of control and homogeneity of fermentations, wine produced with pure yeast monocultures lacks the complexity of flavor, stylistic distinction and vintage variability caused by indigenous yeasts (Lambrechts and Pretorius, 2000; Romano et al., 2003). This fact is a never-ending debate between researchers and oenologists, and the growth of non-*Saccharomyces* yeasts can still be seen as an uncontrollable risk or as an opportunity of improving the quality of wine. Nevertheless it is worthwhile to note that the world's best quality wines are produced after a fermentation process in which, in a greater or lesser extent, various species of non-*Saccharomyces* yeasts have played a role in the winemaking process and, therefore, have contributed to the final result. It is in this context where the inclusion of non-*Saccharomyces* wine yeast species as part of mixed starters together with *S. cerevisiae* to improve wine quality was suggested as a way of taking advantage of spontaneous fermentations without running the risks of stuck fermentations or wine spoilage (Jolly et al., 2003; Rojas et al., 2003; Romano et al., 2003; Ciani et al., 2006). However this practice is linked to new challenges for researchers and oenologists such as the selection of suitable non-*Saccharomyces* strains, the appropriate modality and time of inoculation, the proportion of yeasts in the culture and the potential microorganism interactions, among others. **Figure 1** shows a schematic outline of spontaneous versus inoculated fermentation and the use of mixed starters of selected non-*Saccharomyces* yeasts with strains of *S. cerevisiae* as an alternative to both approaches.

INFLUENCE OF NON-*Saccharomyces* YEASTS ON WINE AROMA

Undoubtedly, aroma is one of most important characteristics that contribute to the quality of wine. As in many foods, wine aroma is composed by 100s of different compounds with concentrations that can vary between 10^{-1} and 10^{-10} g/kg (Rapp and Mandery, 1986). The balance and interaction of all of them determine the wine aromatic quality.

Wine aroma can be subdivided into three groups: the varietal or primary aroma, determined by the grape variety; the fermentation or secondary aroma; and the bouquet or tertiary

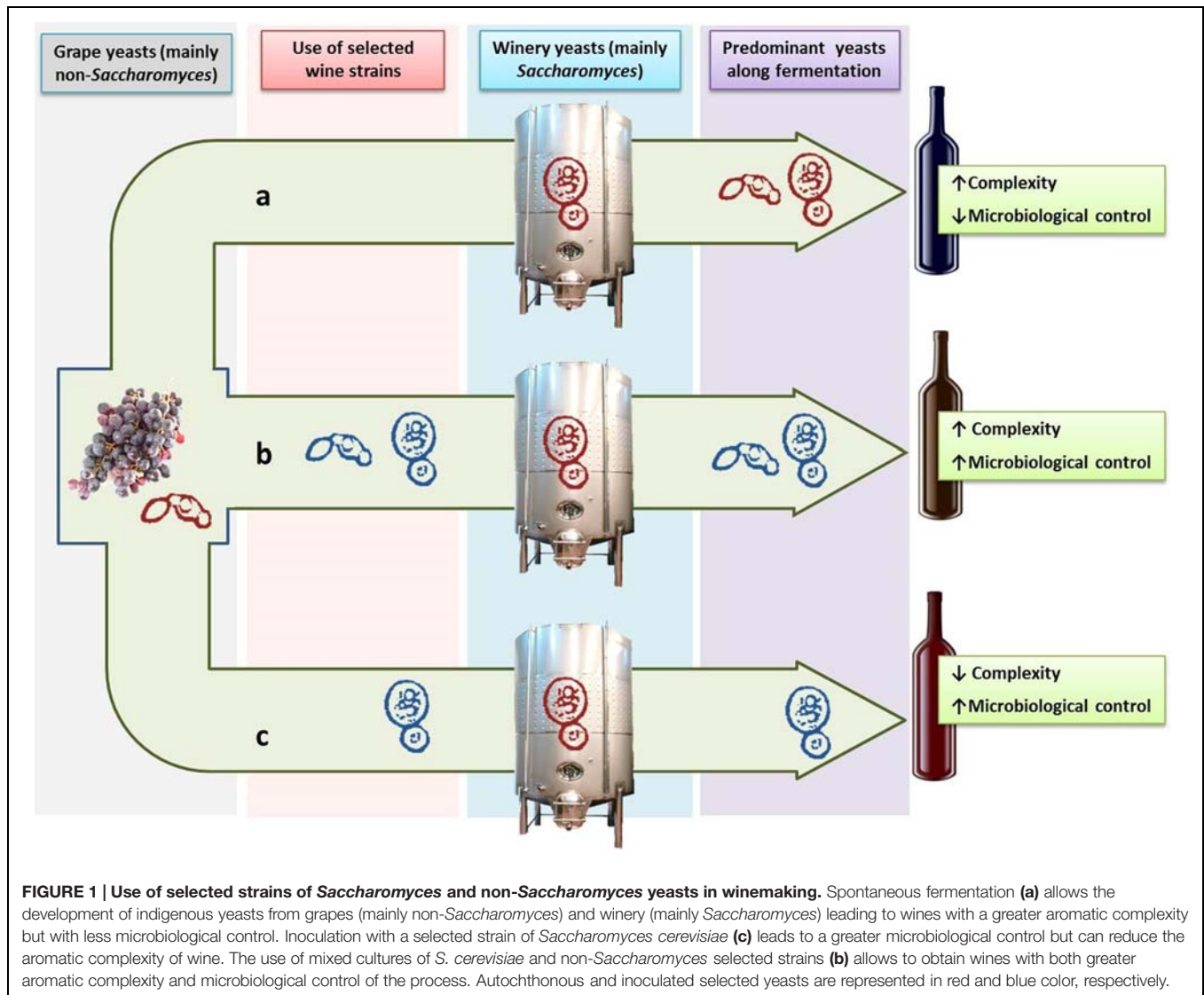
aroma resulting from the transformation of aromas during aging. Non-*Saccharomyces* yeasts can influence both the primary and secondary aroma through the production of enzymes and metabolites, respectively.

Influence on Primary Aroma

Primary or varietal aroma is formed during the ripening of grapes and its contribution to the final wine aroma is considered an appreciated feature. The production of active compounds of primary wine odor takes place in the exocarp of the grape berry and its final concentration in wine is primarily influenced by the vine variety and secondarily by the state of ripeness and the agronomic and oenological practices (Ewart et al., 1985; Spayd et al., 2002; Hernández-Orte et al., 2008, 2015).

Compounds forming primary aroma belong to a limited number of chemical families, including methoxypyrazines, C_{13} -norisoprenoids, volatile sulfur compounds, and terpenes (Ebeler and Thorngate, 2009). Methoxypyrazines are products of amino acid metabolism, and they have been associated to vegetal, green, and herbaceous aromas in certain vine cultivars (reviewed in Sidhu et al., 2015). C_{13} -norisoprenoids derive from carotenoids and particularly β -ionone and β -damascenone are considered impact volatiles of non-floral grapes (Fang and Qian, 2006; Bindon et al., 2007; Pineau et al., 2007; Ristic et al., 2010; Fang and Qian, 2016). Certain organic volatile sulfur compounds such as aromatic thiols make important contributions to Sauvignon Blanc and red cultivars aroma (Darriet et al., 1995; Tominaga et al., 1996, 1998a; Bouchilloux et al., 1998), whereas terpenoids, although present in grapes of all vine varieties, occur in aromatic varieties such as Muscat, Gewürztraminer and Rhine Riesling in the highest concentrations (King and Dickinson, 2000). In grape berries and corresponding wines, approximately seventy terpenoid compounds have been identified (Mateo and Jiménez, 2000). Among them, five monoterpenoid alcohols, namely linalool, geraniol, nerol, citronellol, and α -terpineol are the most abundant and the strongest contributors to wine aroma (Rapp, 1998; Mateo and Jiménez, 2000; Carrau et al., 2005). These compounds provide floral notes and have low odor thresholds (Zalacain et al., 2007).

Interestingly most of primary aroma compounds are found in free or bound forms. The latter are not odorant compounds which hydrolysis can occur during fermentation through the action of wine yeasts (**Figure 2**). Particularly important are aroma precursors linked to sugar molecules, mainly terpenol and C_{13} -norisoprenoid glycosides, and the non-volatile precursor forms of volatile thiols conjugated to cysteine or glutathione. The main yeast enzymes involved in the release of aroma compounds from odorless grape precursors are glycosidases that hydrolyze the non-volatile glycosidic precursors (Gunata et al., 1988), and carbon-sulfur lyases that release volatile thiols from aroma-inactive cysteine-bound conjugates (Tominaga et al., 1998b). Below we focus on the production of these enzymes of oenological relevance by non-*Saccharomyces* wine yeasts. **Table 1** summarizes the yeast species described as producers of glycosidases and carbon-sulfur lyases.



Glycosidases

Since the demonstration that the aromatic components of certain grape varieties are present in the grape berry both in free form and bound to sugars as glycosides (Cordonnier and Bayonove, 1974; Williams et al., 1982), there has been a continuous research to find glycosidases able to release varietal aromas from precursors. The bound aroma fraction comprises glucosides and diglycosides, and compounds such as terpenols, terpene diols, 2-phenylethyl alcohol, benzyl alcohol and C₁₃-norisoprenoids have been shown to be aglycons of such glycosides (Winterhalter and Skouroumounis, 1997). Diglycosides mainly include 6-O- α -L-arabinofuranosyl- β -D-glucopyranosides, 6-O- α -L-rhamnopyranosyl- β -D-glucopyranosides and 6-O- β -D-apiofuranosyl- β -D-glucopyranosides. Due to the important role of monoterpenes in determining the aroma of grapes and wines, hydrolysis of terpene glycosides has been the main focus of research. It is now well established that the enzymatic hydrolysis occurs in two

steps (Gunata et al., 1988). During the first step and depending on the conjugate, the glycosidic linkage is cleaved by either an α -L-arabinofuranosidase, an α -L-rhamnosidase or a β -D-apiosidase, and the corresponding monoterpenyl- β -D-glucosides are released. In the second step, monoterpenes are liberated by the action of a β -D-glucosidase.

Although oenological yeasts may produce glycosidases, the potential effectiveness of enzymes may be hampered by acidic wine conditions or high ethanol concentrations; another limitation of these enzymes is their weak activity in the presence of glucose in the must or wine, making it especially necessary to analyze their inhibition by these wine components. The potential effectiveness of yeast-derived glycosidases is even further reduced in most cases by the fact that some of the enzymes are intracellular and released only in very small amounts into the culture medium. The degree to which these factors inhibit glycosidase production and activity depends on the species and strains of the organisms involved, pointing out the need of enzyme screenings.

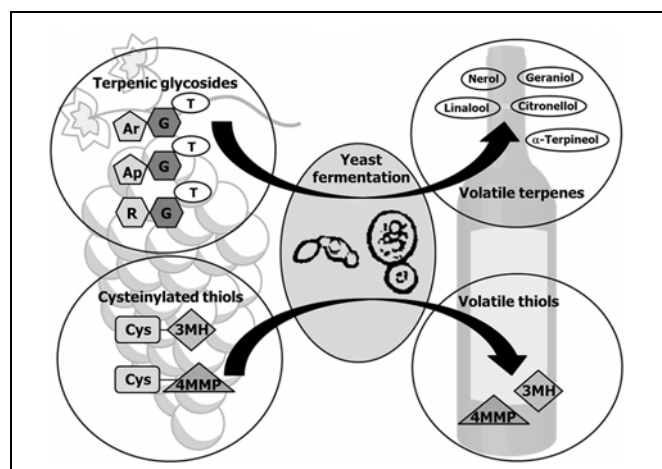


FIGURE 2 | Release of primary aroma compounds by yeasts.

Monoterpenes and volatile thiols occur in grape as odorless precursors which can be released by enzymatic activities of *Saccharomyces* and non-*Saccharomyces* yeasts during fermentation. Monoterpene glycosides are mainly glucosides and diglycosides, in which the glucose moiety (G) has been further substituted mainly with arabinose (Ar), apiose (Ap), or rhamnose (R). A two-step enzyme-catalyzed reaction is the main mechanism proposed for the enzymatic hydrolysis of the diglycosides and subsequent release of the free volatile terpene (T) to wine. First a specific glycosidase cleaves the linkage between both sugars and in a second step the released glucoside is hydrolyzed by a β -D-glucosidase, liberating glucose and the corresponding terpene. Volatile thiols are generated from the odourless cysteinylated precursors cysteine-3-mercaptohexan-1-ol (Cys-3MH) and cysteine-4-mercapto-4-methylpentan-2-one (Cys-4MMP) by the action of carbon-sulfur-lyases.

Widespread occurrence of β -D-glucosidase activity in non-*Saccharomyces* yeasts has been revealed in several screenings. Rosi et al. (1994) showed that yeasts of the genera *Candida*, *Debaryomyces*, *Hanseniaspora/Kloeckera*, *Kluyveromyces*, *Metschnikowia*, *Pichia*, *Saccharomycodes*, *Schizosaccharomyces*, and *Zygosaccharomyces* can produce β -D-glucosidases. Later on this capability was confirmed by other authors (Charoenchai et al., 1997; McMahon et al., 1999; Manzanares et al., 2000; Strauss et al., 2001; Spagna et al., 2002; Cordero-Otero et al., 2003; Fernández-González et al., 2003; Rodríguez et al., 2004; González-Pombo et al., 2008; Sabel et al., 2014; López et al., 2015) and was extended also to the genera *Torulaspora* (Hernández-Orte et al., 2008; Cordero-Bueso et al., 2013), *Brettanomyces* (Cordero-Otero et al., 2003; Fia et al., 2005; Arévalo-Villena et al., 2007), and *Trichosporon* (Wang et al., 2011). Some of these enzymes, selected as a result of their activity with artificial substrates, proved also effective in hydrolyzing either a grape glycoside extract or in releasing terpenols after addition to must or wine. *Debaryomyces hansenii* and *H. uvarum* β -D-glucosidases hydrolyzed terpenic glycosides isolated from grape must (Rosi et al., 1994; Fernández-González et al., 2003). Terpene release was also observed in must and wine treated with β -D-glucosidases from *Hanseniaspora* sp. and *Pichia anomala* (Swangkeaw et al., 2011). Moreover, results suggested that the enzyme from *Hanseniaspora* sp. was more efficient in releasing desirable aromas during an early stage of alcoholic fermentation

while β -D-glucosidase from *P. anomala* was suitable at the final stage. Several yeast β -D-glucosidases have been purified and characterized. Two *Debaryomyces* β -D-glucosidases suitable for enhancing wine aroma have been reported. An intracellular *D. hansenii* β -D-glucosidase, tolerant to ethanol and glucose, efficiently released monoterpenols from the glycosides extracted from Muscat grape must. In addition, when the enzyme was added during Muscat fermentation, a considerably increase in the concentration of mainly nerol and linalool was observed (Yanai and Sato, 1999). By contrast, a *Debaryomyces pseudopolymorphus* strain (Cordero-Otero et al., 2003) produced an exocellular β -D-glucosidase with acidic optimal pH and not inhibited by glucose or ethanol (Arévalo-Villena et al., 2006), although the effectiveness of the purified enzyme in winemaking was not tested. Enzymatic treatment of wine with a purified ethanol tolerant β -D-glucosidase from *Sporidiobolus pararoseus*, a member of oenological ecosystems in the southeastern region of Brazil, considerably increased the amount of free terpenes (Baffi et al., 2011, 2013).

Several strategies for either improving β -D-glucosidase stability or enzyme yield have been described. An extracellular β -D-glucosidase from *Issatchenkia terricola*, active in the presence of glucose, ethanol, and metabisulfite was immobilized for improving acidic pH stability. This strategy increased the amount of monoterpenes and norisoprenoids, showing the potential of the immobilized enzyme for aroma development in wines (González-Pombo et al., 2011). With respect to yield enhancement, the usefulness of response surface methodology for optimizing the production of a *Trichosporon asahii* β -D-glucosidase was reported (Wang et al., 2012). *T. asahii* β -D-glucosidase exhibited better ability than fungal and plant commercial enzymes in hydrolyzing aromatic precursors in young wine. Also, a recombinant *S. cerevisiae* wine yeast expressing the *Candida molischiana bglN* gene encoding a β -D-glucosidase able to release terpenols and alcohols from a glycoside extract has been used to facilitate protein purification (Genovés et al., 2003).

Some of the β -D-glucosidase screenings described above also included the search of less common glycosidases, such as α -L-arabinofuranosidase, α -L-rhamnosidase and β -D-xylosidase. From more than 300 wine yeast strains, only one strain of *P. anomala* showed α -L-arabinofuranosidase activity whereas none of them was positive for α -L-rhamnosidase production (Spagna et al., 2002). Also the potential of certain wine yeasts from the genera *Candida*, *Hanseniaspora*, and *Pichia* to produce β -D-xylosidase activity active at winemaking conditions has been discussed (Manzanares et al., 1999; Yanai and Sato, 2001; Rodríguez et al., 2004; López et al., 2015). Interestingly yeast strains able to display several glycosidase activities have been reported: one strain of *Aureobasidium pullulans* able to hydrolyze grape glycosides displayed β -D-glucosidase, α -L-arabinofuranosidase and α -L-rhamnosidase activities, whereas *Candida guilliermondii* produced both β -D-glucosidase and α -L-rhamnosidase (McMahon et al., 1999). Two *H. uvarum*, one *Hanseniaspora vineae* and one *P. anomala* strains were described as producers of the four glycosidase activities (Mateo et al., 2011), while a *Wickerhamomyces anomalus*

TABLE 1 | Non-Saccharomyces yeast species described as producers of enzymes involved in the release of aroma compounds from grape precursors.

Yeast species	Enzyme ¹					Reference
	BGL	ARA	RHA	XYL	CSL	
<i>A. pullulans</i>	x	x	x			McMahon et al., 1999
<i>B. anomalus</i>	x					Fia et al., 2005
<i>Brettanomyces</i> spp.	x					Cordero-Otero et al., 2003; Arévalo-Villena et al., 2007
<i>C. guilliermondii</i>	x		x	x		McMahon et al., 1999; Cordero-Otero et al., 2003; Rodríguez et al., 2004
<i>C. molischiana</i>	x					Fernández-González et al., 2003; Genovés et al., 2003
<i>C. stellata</i>	x		x	x		Rosi et al., 1994; Strauss et al., 2001; Cordero-Otero et al., 2003
<i>C. utilis</i>				x		Yanai and Sato, 2001
<i>C. zemplinina</i>					x	Anfang et al., 2009
<i>D. castellii</i>	x					Rosi et al., 1994
<i>D. hansenii</i>	x					Rosi et al., 1994; Yanai and Sato, 1999; Fernández-González et al., 2003
<i>D. polymorphus</i>	x					Rosi et al., 1994; Cordero-Otero et al., 2003; Arévalo-Villena et al., 2007
<i>D. pseudopolymorphus</i>	x					Cordero-Otero et al., 2003; Arévalo-Villena et al., 2006, 2007
<i>D. vanriji</i>	x					García et al., 2002
<i>Hanseniaspora</i> sp.	x			x		Swangkeaw et al., 2011
<i>H. guilliermondii</i>	x					Manzanares et al., 2000
<i>H. osmophila</i>	x			x		Manzanares et al., 1999, 2000
<i>H. vineae</i>	x	x	x	x		Mateo et al., 2011; Maturano et al., 2012; López et al., 2015
<i>H. uvarum</i>	x	x	x	x		Rosi et al., 1994; Charoenchai et al., 1997; Manzanares et al., 1999, 2000; Strauss et al., 2001; Fernández-González et al., 2003; Rodríguez et al., 2004; Arévalo-Villena et al., 2007; Mateo et al., 2011; López et al., 2015
<i>I. terricola</i>	x					González-Pombo et al., 2011
<i>K. thermotolerans</i>	x				x	Rosi et al., 1994; Zott et al., 2011
<i>M. pulcherrima</i> /C. <i>pulcherrima</i>	x			x	x	Rosi et al., 1994; Fernández-González et al., 2003; Rodríguez et al., 2004, 2010a; González-Pombo et al., 2008; Zott et al., 2011
<i>P. angusta</i>			x			Yanai and Sato, 2000a
<i>P. anomala</i>	x	x	x	x		Rosi et al., 1994; Charoenchai et al., 1997; Manzanares et al., 1999, 2000; Spagna et al., 2002; Mateo et al., 2011; Swangkeaw et al., 2011
<i>P. capsulata</i>		x				Yanai and Sato, 2000b
<i>P. guilliermondii</i>			x			Rodríguez et al., 2004, 2010b
<i>P. kluyveri</i>					x	Anfang et al., 2009
<i>P. membranifaciens</i>	x			x		López et al., 2015
<i>S. ludwigii</i>	x					Rosi et al., 1994
<i>S. pombe</i>	x					Rosi et al., 1994
<i>S. pararoseus</i>	x					Baffi et al., 2013
<i>T. delbrueckii</i>	x				x	Hernández-Orte et al., 2008; Zott et al., 2011; Maturano et al., 2012; Cordero-Bueso et al., 2013; Čuš and Jenko, 2013
<i>T. asahii</i>	x					Wang et al., 2011
<i>W. anomalus</i>	x	x		x		Sabel et al., 2014; López et al., 2015
<i>Z. bailii</i>	x					Rosi et al., 1994; Cordero-Otero et al., 2003

¹BGL, β -D-glucosidase; ARA, α -L-arabinofuranosidase; RHA, α -L-rhamnosidase; XYL, β -D-xylosidase; CSL, carbon-sulfur lyase.

(alternative names *Hansenula anomala*, *P. anomala* and *Candida pelliculosa*) strain producing β -D-glucosidase, also exhibited α -L-arabinofuranosidase and β -D-xylosidase activities (Sabel et al., 2014). However, the effectiveness of purified glycosidases for terpene releasing from precursors has only been reported for the intracellular α -L-rhamnosidases from *Pichia angusta* (Yanai and Sato, 2000a) and *Pichia guilliermondii* (Rodríguez et al.,

2004, 2010b), an α -L-arabinofuranosidase from *Pichia capsulata* (Yanai and Sato, 2000b) and a β -D-xylosidase from *Candida utilis* (Yanai and Sato, 2001). The latter also increased the concentration of terpenes after addition to Moscatel grape must during fermentation (Yanai and Sato, 2001).

The role of exo-glucanases in the release of aromatic compounds from glycosidically bound precursors in a single

enzymatic step has been discussed (Gil et al., 2005). In this regard, a yeast isolate AS1, identified as a *W. anomalus* strain was selected by its capability to hydrolyze several synthetic and natural glycosides under oenological conditions (Sabel et al., 2014). Later, the enzyme responsible for the hydrolysis of selected glycosides was purified from the culture supernatant of AS1 and characterized as a multifunctional exo- β -1,3-glucanase active under typical wine related conditions (Schwentke et al., 2014).

The feasibility of using β -D-glucosidase-producing yeasts in fermentation instead of adding purified enzymes represents an interesting option. Either alone or in a mixed starter with *S. cerevisiae*, the ability of non-Saccharomyces yeasts to contribute to the aromatic wine profile has been assessed. Different strains of *Torulaspora delbrueckii* contributed to the aroma profile with flowery and fruity aroma (Maturano et al., 2012; Cordero-Bueso et al., 2013). Secreted *H. vineae* and *T. delbrueckii* β -D-glucosidases were detected throughout the fermentation process, although activity diminished with increasing fermentation time, suggesting the adverse effect of ethanol (Maturano et al., 2012). Also, isolates of *Pichia membranifaciens*, *H. vineae*, *H. uvarum*, and *W. anomalus* showing β -D-glucosidase activity provoked a moderated overall terpene increase when inoculated to final wines (López et al., 2015). The first mixed starters based on non-Saccharomyces yeasts able to produce β -D-glucosidase activity were described for *Debaryomyces vanriji* and *D. pseudopolymorphus* (García et al., 2002; Cordero-Otero et al., 2003), and later on for *Candida pulcherrima* (alternative name *Metschnikowia pulcherrima*) (Rodríguez et al., 2010a) and *T. delbrueckii* (Čuš and Jenko, 2013). Detailed information of these mixed starters will be found in next sections.

Carbon-Sulfur Lyases

Some sulfur containing compounds, the so-called volatile or varietal thiols, can contribute to positive fragrances such as tropical, passion fruit and guava-like nuances. These compounds considered to be impact odorants in Sauvignon Blanc wines are 4-mercapto-4-methylpentan-2-one (4MMP), reminiscent of box tree, passion fruit, broom, and black current bud; and 3-mercaptohexan-1-ol (3MH) and 3-mercaptohexyl acetate (3MHA), responsible for the passion fruit, grapefruit, and citrus aroma. Volatile thiols are not unique to Sauvignon Blanc wines. They have been also found to contribute significantly to the aroma profiles of wines made from other varieties such as Riesling, Colombard, Semillon, Cabernet Sauvignon, and Merlot (revised in Coetzee and du Toit, 2012).

Volatile thiols are mostly non-existent in grape juice and they are generated during the fermentation process by yeasts from odorless, non-volatile precursors initially present in must (revised in Cordente et al., 2012). It has been shown that 4MMP and 3MH exist in grapes in their non-volatile precursor form, conjugated to cysteine, or glutathione. The key enzyme for cleaving cysteinylated precursors is the *S. cerevisiae* β -lyase IRC7, with a substrate preference for cys-4MMP over cys-3MH (Roncoroni et al., 2011). The mechanism by which the glutathionated thiol precursors are degraded has not been fully elucidated, but is likely to

involve a multi-step pathway with the production of the cysteinylated form as an intermediate (Grant-Preece et al., 2010). No precursor of 3MHA has been identified in grapes; this compound is formed during fermentation through esterification of 3MH by the alcohol acetyltransferase ATF1 (Swiegers et al., 2006).

Undoubtedly the main factor in volatile thiol release during alcoholic fermentation is the yeast strain (Dubourdieu et al., 2006). It was found that *S. cerevisiae* strains varied significantly in terms of their capabilities to produce volatile thiols and to modulate the varietal characters of Sauvignon Blanc wine (Swiegers et al., 2009). With regard to non-Saccharomyces species, only two screenings have addressed their feasibility to release volatile thiols. The first screening by Anfang et al. (2009) showed that most of the eleven non-Saccharomyces isolates tested were able to produce concentrations of 3MH above the perception threshold, but only two isolates of *Pichia kluyvery* and *Candida zemplinina* (alternative names *Candida stellata* and *Starmerella bacillaris*) produced concentrations of 3MH and 3MHA comparable with those produced by *S. cerevisiae*. In contrast to that found for *S. cerevisiae*, results showed an inverse correlation between the concentrations of 3MH and 3MHA produced by the *P. kluyvery* and *C. zemplinina* isolates, suggesting a decreased ability to convert 3MH to 3MHA, or possibly alternate metabolic routes for its formation (Anfang et al., 2009). In a second screening, the potential impact of 15 non-Saccharomyces strains from seven species on 4MMP and 3MH release in model medium and Sauvignon Blanc must was evaluated after partial fermentation (Zott et al., 2011). In general, non-Saccharomyces strains had greater ability to release 3MH than 4MMP in both media. Only *M. pulcherrima* and *H. uvarum* strains in model medium and *Kluyveromyces thermotolerans* in must were able to produce significant amounts of 4MMP. With respect to 3MH release, *M. pulcherrima* and *T. delbrueckii* strains released large amounts of this compound in model medium whereas *M. pulcherrima* and *K. thermotolerans* stood out as good producers in natural must. *C. zemplinina* isolates included in the screening did not produce volatile thiols, in contrast to previous results (Anfang et al., 2009). This can be explained by the strain dependent capacity to release 3MH as showed for *M. pulcherrima* (Zott et al., 2011). Undoubtedly additional screening experiments including numerous non-Saccharomyces strains are required to obtain a clear image of species-associated behavior or strain effects. Mixed fermentations with volatile thiol releasing yeasts will be discussed in later sections.

Influence on Secondary Aroma

Most of the compounds that determine wine aroma arise from the fermentation process. Their concentrations are mainly dependent on the predominant yeasts and the fermentation conditions (Egli et al., 1998; Henick-Kling et al., 1998; Steger and Lambrechts, 2000). Although ethanol, glycerol, and CO₂ are quantitatively the most abundant of these compounds, their contribution to the secondary aroma is relatively limited. Volatile fatty acids, higher alcohols, esters, and, to a lesser extent, aldehydes, have a greater contribution to secondary aroma (Rapp

and Versini, 1991), although volatiles derived from fatty acids and from nitrogen- or sulfur-containing compounds also contribute (Boulton et al., 1996). The biosynthesis of these compounds has been reviewed in greater detail by Lambrechts and Pretorius (2000). It is worthwhile to note that the biosynthesis of these compounds is species- and strain-dependent, allowing the selection of those strains of biotechnological interest. Moreover, and depending on the concentration reached in wine, those compounds arising from yeast metabolism have a positive or negative impact on wine aroma and quality. Below we describe the contribution of non-Saccharomyces yeast species to wine secondary aroma. **Table 2** shows the yeast species described as high- or low-producers of secondary aroma compounds.

Volatile Fatty Acids

Acetic acid is responsible for 90% of the volatile acidity of wines while the remaining fatty acids, such as propanoic and butanoic acid, are present in small quantities (Radler, 1993). Their production is also associated with bacterial growth

(Ribereau-Gayon et al., 1998). Acetic acid becomes unpleasant at concentrations near its flavor threshold of 0.7–1.1 g/L and usually values between 0.2 and 0.7 g/L are considered optimal (Lambrechts and Pretorius, 2000).

Studies of acetic acid production by non-Saccharomyces yeasts have generated highly variable results. Some non-Saccharomyces genera such as *Hanseniaspora* and *Zygosaccharomyces* have been traditionally described as producers of excessive amounts of acetic acid (du Toit and Pretorius, 2000; Loureiro and Malfeito-Ferreira, 2003; Romano et al., 2003; Mendoza et al., 2007) and, for this reason, they have been considered for long time as spoilage yeasts. Also the species *Schizosaccharomyces pombe* is commonly associated with high levels of acetic acid (Gallander, 1977; Snow and Gallander, 1979). However, this compound is produced with a considerably strain variability. For instance, levels of acetic acid ranging from about 0.6 g/L to more than 3.4 g/L have been described for *H. uvarum* strains (Romano et al., 2003) while a screening of *S. pombe* allowed the selection of strains producing less than 0.4 g/L of acetic acid (Benito et al., 2014a).

TABLE 2 | Secondary aroma compounds produced by non-Saccharomyces wine yeasts.

Compound	High producers	Low producers	Reference
Volatile fatty acids			
Acetic acid	<i>Hanseniaspora</i> <i>Zygosaccharomyces</i> <i>S. pombe</i>	<i>T. delbrueckii</i> <i>K. thermotolerans</i> <i>C. stellata</i> / <i>C. zemplinina</i>	Gallander, 1977; Snow and Gallander, 1979; Ciani and Maccarelli, 1998; du Toit and Pretorius, 2000; Soden et al., 2000; Loureiro and Malfeito-Ferreira, 2003; Romano et al., 2003; Kapsopoulou et al., 2005; Mendoza et al., 2007; Renault et al., 2009; Comitini et al., 2011; Rantsiou et al., 2012; Benito et al., 2014a; Englezos et al., 2015
Higher alcohols			
	<i>M. pulcherrima</i> <i>C. zemplinina</i> <i>L. thermotolerans</i>	<i>Hanseniaspora</i> <i>Zygosaccharomyces</i>	Romano and Suzzi, 1993; Rojas et al., 2003; Clemente-Jiménez et al., 2004; Moreira et al., 2008; Viana et al., 2008; Andorrà et al., 2010; Beckner Whitener et al., 2015
Esters			
	<i>Candida</i> <i>Hansenula</i> <i>Pichia</i> <i>Hanseniaspora</i> <i>Rhodotorula</i> <i>T. delbrueckii</i> <i>K. gamospora</i>		Ough et al., 1968; Suomalainen and Lehtonen, 1979; Nykänen, 1986; Mateo et al., 1991; Sponholz, 1993; Romano et al., 1997; Rojas et al., 2001, 2003; Moreira et al., 2005; Viana et al., 2008; Beckner Whitener et al., 2015
Aldehydes			
Acetaldehyde		<i>K. apiculata</i> <i>C. krusei</i> <i>C. stellata</i> <i>H. anomala</i> <i>M. pulcherrima</i> <i>H. uvarum</i>	Fleet and Heard, 1993; Romano et al., 2003
Volatile phenols			
	<i>Brettanomyces/Dekkera</i> <i>P. guilliermondii</i>	<i>Candida</i> <i>K. lactis</i> <i>T. delbrueckii</i> <i>M. pulcherrima</i> <i>H. guilliermondii</i> <i>H. osmophila</i> <i>P. membranifaciens</i>	Lambrechts and Pretorius, 2000; Shinohara et al., 2000; Dias et al., 2003; Viana et al., 2008; Renault et al., 2009; Beckner Whitener et al., 2015
Sulfur compounds			
	<i>Candida</i> <i>Hanseniaspora</i> <i>T. delbrueckii</i> <i>K. gamospora</i>		Strauss et al., 2001; Moreira et al., 2008; Viana et al., 2008; Renault et al., 2009; Beckner Whitener et al., 2015

By contrast, different screenings of *T. delbrueckii* strains for desirable oenological properties pointed out differences in fermentative capability but always a low production of volatile acidity when compared to *S. cerevisiae* (Ciani and Maccarelli, 1998; Renault et al., 2009; Comitini et al., 2011). This feature is also a characteristic of *Lachancea thermotolerans* (previously known as *K. thermotolerans*) together with the high production of L-lactic acid (Kapsopoulou et al., 2005). *C. stellata*/*C. zemplinina* presents a strong fructophilic character (Soden et al., 2000), which may be an advantage during the fermentation of sweet wines, since this species do not produce excessive levels of acetic acid as a response to the osmotic stress in comparison to *S. cerevisiae* (Rantsiou et al., 2012). Recently the strong fructophilic character of *C. zemplinina* and its ability to produce low quantities of ethanol and acetic acid and high amounts of glycerol were confirmed (Englezos et al., 2015).

Higher Alcohols

They are the largest group of aromatic compounds (Amerine et al., 1980). Higher alcohols contribute to the aromatic complexity of wine at concentrations below 300 mg/L. However when their concentrations exceed 400 mg/L, they are considered to have a negative effect on aroma (Rapp and Mandery, 1986). The importance of higher alcohols is also related to their role as ester precursors (Soles et al., 1982).

In general, studies of higher alcohol production in non-Saccharomyces yeasts highlight the influence that these yeasts can have on the chemical composition and quality of wine (Herraiz et al., 1990; Mateo et al., 1991; Gil et al., 1996). In fermented musts, the total production of higher alcohols by pure cultures of *Hanseniaspora* species is lower than that found with *S. cerevisiae* (Rojas et al., 2003; Moreira et al., 2008; Viana et al., 2008). Also, *Zygosaccharomyces* strains isolated from grape musts have been described as producers of low amounts of higher alcohols (Romano and Suzzi, 1993). By contrast, *C. zemplinina* wines contained huge amounts of higher alcohols, which concentrations clearly exceeded 400 mg/L (Andorrà et al., 2010).

Regarding specific alcohols, increased production of 2-phenylethyl alcohol, compound associated with pleasant aromas, has been described as a characteristic of *M. pulcherrima* (Clemente-Jiménez et al., 2004), *L. thermotolerans* (Beckner Whitener et al., 2015), and *C. zemplinina* (Andorrà et al., 2010).

Esters

Esters are the most abundant compounds found in wine, with around 160 identified to date. Although various esters can be formed during fermentation, the most abundant are those derived from acetic acid (ethyl acetate, isoamyl acetate, isobutyl acetate, and 2-phenylethyl acetate) and ethyl esters of saturated fatty acids (ethyl butanoate, ethyl caproate, ethyl caprylate, and ethyl caprate). The main ester in wine is ethyl acetate, and it can impart spoilage character at levels of 150–200 mg/L (Lambrechts and Pretorius, 2000).

Non-Saccharomyces wine yeasts, known as good producers of esters, have been traditionally associated with the negative effects of high ethyl acetate formation, whereas the levels of ethyl esters produced by these yeasts are generally much lower than those detected in *S. cerevisiae* wines (Rojas et al., 2001, 2003).

Species belonging to the genera *Candida*, *Hansenula*, and *Pichia* were described as having a greater capacity to produce ethyl acetate than wine strains of *S. cerevisiae* (Ough et al., 1968; Nykänen, 1986). Also, in a study where ester production was grouped by yeast genera, *Hanseniaspora* and *Pichia* stood out by the production of ethyl acetate (Viana et al., 2008). Both genera produced similar ethyl acetate levels, but *Hanseniaspora* was also a potent producer of specific fruity acetate esters such as 2-phenylethyl acetate and isoamyl acetate (Rojas et al., 2001; Moreira et al., 2005; Viana et al., 2008), whereas the genera *Pichia* and *Rhodotorula* produced remarkable levels of isoamyl acetate (Suomalainen and Lehtonen, 1979; Viana et al., 2008). Among *Hanseniaspora* species, specifically *H. uvarum* is reported to be a good producer of esters in general (Mateo et al., 1991; Sponholz, 1993; Romano et al., 1997) whereas *Hanseniaspora guilliermondii* and *Hanseniaspora osmophila* are strong producers of 2-phenylethyl acetate (Rojas et al., 2001, 2003; Viana et al., 2008).

Regarding ethyl esters, production of ethyl caprylate seems to be a characteristic of *T. delbrueckii* (Viana et al., 2008). The aroma profile of the newly discovered yeast *Kazachstania gamospora* showed that this species produced more esters than the *S. cerevisiae* control strain, but specially phenylethyl propionate, an ester desirable in wine due to its floral aroma (Beckner Whitener et al., 2015).

Aldehydes

These compounds with apple-like odors are important to the aroma and bouquet of wine due to their low sensory threshold values. Among aldehydes, acetaldehyde constitutes more than 90% of the total content of wines, and its amount can vary from 10 mg/L up to 300 mg/L (Lambrechts and Pretorius, 2000).

Saccharomyces cerevisiae strains usually produce higher acetaldehyde levels (5–120 mg/L) than non-Saccharomyces species (up to 40 mg/L) such as *Kloeckera apiculata*, *Candida krusei*, *C. stellata*, *H. anomala*, and *M. pulcherrima* (Fleet and Heard, 1993). A mean acetaldehyde concentration of around 25 mg/L was described for *H. uvarum* strains, although significant differences in production among strains were observed (Romano et al., 2003).

Volatile Phenols and Sulfur Compounds

Among volatile phenols, the most important are vinylphenols in white wines and ethylphenols in red wines. Their presence is always undesirable, since even at concentrations below the perception threshold they are reported to mask the fruity notes of white wines. These compounds are produced from the non-volatile ferulic and *p*-coumaric acids. Traditionally, ethylphenol producers have been ascribed to the genus *Brettanomyces*/*Dekkera* (Lambrechts and Pretorius, 2000). However, several studies also identified

Candida species, *Kluyveromyces lactis*, *T. delbrueckii*, *M. pulcherrima*, and *P. guilliermondii* strains as volatile phenol producers, although only *P. guilliermondii* displayed the same conversion capacity as *Dekkera* species (Shinohara et al., 2000; Dias et al., 2003; Renault et al., 2009; Beckner Whitener et al., 2015). By contrast *H. guilliermondii*, *H. osmophila*, and *P. membranifaciens* were not able to decarboxylate either ferulic or *p*-coumaric acids (Viana et al., 2008).

The sensory properties of sulfur compounds vary extensively and although most of them are associated with negative aromatic descriptors, they can have a positive contribution to wine aroma through the introduction of fruity notes (reviewed in Swiegers and Pretorius, 2005). The main compound in this group is hydrogen sulfide. Production by non-*Saccharomyces* yeasts includes *Candida* and *Hanseniaspora* species (Strauss et al., 2001; Viana et al., 2008) as well as *T. delbrueckii* (Renault et al., 2009). The contribution of *H. uvarum* and *H. guilliermondii* to the sulfur compound profile of wines was evaluated by Moreira et al. (2008) and it was concluded that the growth of the apiculate yeasts might not have a negative influence. It should also be noted that *T. delbrueckii* and *K. gamospora* are able to produce the sulfur compound 3-methylthio-1-propanol in higher concentration than *S. cerevisiae*, although it was dependent on the must variety (Beckner Whitener et al., 2015). The formation of volatile thiols by non-*Saccharomyces* yeasts has been described in the primary aroma section.

MIXED STARTERS

The use of mixed starters of selected non-*Saccharomyces* yeasts to exploit their positive abilities combined with *S. cerevisiae* to avoid stuck fermentations represents a feasible alternative to both spontaneous and inoculated fermentations (Figure 1). These combinations could be used to produce wines with unique aromatic characteristics. Based on their capability to produce flavor enhancing enzymes or to modify the concentration of secondary metabolites, different mixed starters have been designed and proposed as a tool to enhance wine quality (Table 3). Some of them were designed with the aim of modifying a specific target such as the terpenic profile or final ester concentrations while others show a general impact on wine aroma complexity. The increasing interest in the use of non-*Saccharomyces* yeasts in winemaking has even prompted commercial production of several species including *L. thermotolerans*, *M. pulcherrima*, *T. delbrueckii*, *P. kluyvery*, and *S. pombe*.

When using non-*Saccharomyces* yeasts in mixed starters, there are two general practices of inoculation. The first, known as co-inoculation, involves the inoculation of the selected non-*Saccharomyces* yeasts at high cell concentration together with *S. cerevisiae*, while the second, sequential inoculation, implies that the selected non-*Saccharomyces* yeasts are first inoculated at high levels and allowed to ferment on their own for a given amount of time before *S. cerevisiae* is added to take over

the fermentation. Both are feasible practices although potential interactions between yeasts could determine which inoculation strategy is more appropriate.

Primary Aroma Influence on Terpenes

With the aim of obtaining terpene-enriched wines, *T. delbrueckii*, *M. pulcherrima*, *D. hansenii*, and *D. pseudopolymorphus* strains able to produce β -D-glucosidase activity were combined with *S. cerevisiae*.

Higher concentrations of α -terpineol and linalool were found in Gewürztraminer wine fermented with the combination *T. delbrueckii*/*S. cerevisiae*, although more nerol and geraniol were detected in the control fermentation conducted with *S. cerevisiae* alone (Çuș and Jenko, 2013). Moreover those chemical changes enhanced the overall quality of Gewürztraminer wine.

Metschnikowia pulcherrima is known to produce β -D-glucosidase activity able to increase the α -terpineol, nerol as well as geraniol concentrations in monoculture wines (Rodríguez et al., 2010a). However, in wines obtained by mixed fermentation, in either simultaneous or sequential inoculation, nerol and geraniol concentrations were significantly lower than those observed in grape must, and only α -terpineol concentration was higher. This fact was related to the *S. cerevisiae* capability to transform nerol and geraniol into α -terpineol at must pH (Di Stefano et al., 1992; Mateo and Jiménez, 2000), pointing out the relevance of yeast interactions.

With respect to *Debaryomyces* species, a *D. vanriji* strain isolated from grape berry flora was found to influence wine volatiles of the cv. Muscat of Frontignan when co-cultured with native or selected strains of *S. cerevisiae*. The concentrations of several volatiles including terpenols were significantly different between the control and the wines inoculated with *D. vanriji*. The increase in geraniol concentration was attributed to the hydrolysis of the corresponding glucosidic precursor by *D. vanriji* β -D-glucosidase since musts inoculated with the non-*Saccharomyces* yeast showed higher levels of enzymatic activity throughout the fermentation compared to the control sample. Moreover bound geraniol concentration was found to be lower in *D. vanriji* inoculated wines compared to the control one (García et al., 2002). The high terpene concentrations of wines obtained with mixed cultures *D. vanriji*/*S. cerevisiae* was recently confirmed and associated with the production of pectinase, amylase, and xylanase activities along the fermentation (Maturano et al., 2015). Also, a β -D-glucosidase producing *D. pseudopolymorphus* strain when co-cultured with *S. cerevisiae* VIN13 significantly increased concentrations of citronellol, nerol, and geraniol during the fermentation of Chardonnay juice (Cordero-Otero et al., 2003).

Influence on Thiols

To take advantage of the capability of *C. zemplinina* to produce volatile thiols, mixed starters of commercially available *S. cerevisiae* strains with *C. zemplinina* isolates were employed in Sauvignon Blanc fermentations. Inoculation with equal amounts or with a ratio that initially favored the non-*Saccharomyces*

TABLE 3 | Mixed starters designed to improve primary and secondary wine aroma.

Mixed starter	Impact on wine aroma	Inoculation	Must	Reference
<i>C. zemplinina</i> / <i>S. cerevisiae</i>	3MH increase	Co-inoculation	Sauvignon Blanc	Anfang et al., 2009
	Acetic acid decrease	Co-inoculation, sequential	Erbaluce dried grape must, Pinot Grigio	Ciani and Ferraro, 1998; Rantsiou et al., 2012
<i>D. pseudopolymorphus</i> / <i>S. cerevisiae</i>	Geraniol, nerol and citronellol increase	Co-inoculation	Chardonnay	Cordero-Otero et al., 2003
<i>D. vanriji</i> / <i>S. cerevisiae</i>	Geraniol increase	Sequential	Muscat of Frontignan	García et al., 2002
<i>H. guilliermondii</i> / <i>S. cerevisiae</i>	Acetate ester increase	Co-inoculation	Bobal, natural must	Rojas et al., 2003; Moreira et al., 2008
<i>H. uvarum</i> / <i>S. cerevisiae</i>	Sulfur compound increase	Co-inoculation	Natural must	Moreira et al., 2008
	Acetate ester increase	Co-inoculation	Synthetic must, Macabeo, natural must	Moreira et al., 2008; Andorrà et al., 2010, 2012
<i>H. vineae</i> / <i>S. cerevisiae</i>	Acetate and ethyl ester increase	Co-inoculation, sequential	Bobal, Chardonnay white, Tempranillo	Viana et al., 2009, 2011; Medina et al., 2013
<i>I. orientalis</i> / <i>S. cerevisiae</i>	Wine deacidification	Co-inoculation	Campbell's Early	Kim et al., 2008
<i>K. gamospora</i> / <i>S. cerevisiae</i>	Acetate and ethyl ester increase	Sequential	Ribolla	Dashko et al., 2015
<i>L. thermotolerans</i> / <i>S. cerevisiae</i>	Wine acidification	Co-inoculation, sequential	Pasteurized natural must, sterile grape must	Kapsopoulou et al., 2007; Comitini et al., 2011; Gobbi et al., 2013
<i>M. pulcherrima</i> / <i>S. cerevisiae</i>	α -Terpineol increase	Sequential	Muscat d'Alexandrie	Rodríguez et al., 2010a
	Acetic acid decrease	Co-inoculation	Pasteurized natural must	Comitini et al., 2011
	Ethyl ester increase	Co-inoculation, sequential	Emir, Muscat d'Alexandrie	Zohre and Erten, 2002; Rodríguez et al., 2010a
<i>P. fermentans</i> / <i>S. cerevisiae</i>	Higher alcohol increase	Co-inoculation	Pasteurized natural must	Comitini et al., 2011
	Acetic acid decrease	Sequential	Sterile must	Clemente-Jiménez et al., 2005
	Higher alcohol increase	Co-inoculation	Pasteurized natural must	Comitini et al., 2011
<i>P. kluyveri</i> / <i>S. cerevisiae</i>	3MHA increase	Co-inoculation	Sauvignon Blanc	Anfang et al., 2009
<i>S. pombe</i> / <i>S. cerevisiae</i>	Wine deacidification	Co-inoculation, sequential	Airen, Garnacha	Benito et al., 2013, 2014b
<i>T. delbrueckii</i> / <i>S. cerevisiae</i>	α -Terpineol and linalool increase	Sequential	Gewürztraminer	Čuš and Jenko, 2013
	Acetic acid decrease	Co-inoculation	Botritized Semillon, pasteurized natural must	Bely et al., 2008; Comitini et al., 2011
	Acetate and ethyl ester increase	Co-inoculation, sequential	Sauvignon Blanc, Syrah, Tempranillo	Loira et al., 2014, 2015; Renault et al., 2015
	Higher alcohol increase	Co-inoculation, sequential	Chardonnay, Corvina, Corvinone, Rondinella, pasteurized natural must, Soave, Vino Santo	Comitini et al., 2011; Azzolini et al., 2012, 2015
	Acetate and ethyl ester increase	Sequential	Mazuela	Izquierdo-Cañas et al., 2014
<i>W. anomalus</i> / <i>S. cerevisiae</i>	Acetate ester increase	Co-inoculation	Emir	Erten and Tanguler, 2010, Tanguler, 2012, 2013
<i>Z. bailii</i> / <i>S. cerevisiae</i>	Ethyl ester increase	Co-inoculation	Chardonnay	Garavaglia et al., 2015

isolates, produced wines with the greatest increase of the volatile thiol 3MH when compared with the *S. cerevisiae* single ferment. However, the co-ferments with the *C. zemplinina* isolates had significantly lower concentrations of 3MHA (Anfang et al., 2009). By contrast, an elevation in thiol production, especially 3MHA, was found in Sauvignon Blanc wines co-fermented with *P. kluyveri* isolates and different *S. cerevisiae* strains (Anfang et al., 2009). Although both species were able to produce 3MHA in monoculture, the increase in 3MHA concentration observed in co-fermented wines could not be explained under simple additive assumptions suggesting an interaction between the co-fermenting partners. Moreover the elevation in thiols

was only seen in co-ferments with certain *S. cerevisiae* strains suggesting that the nature of this particular interaction may not be generalized to the species level. However, the mechanism behind this interaction is still unknown.

Nowadays, selected non-*Saccharomyces* strains for the improvement of wine primary aroma are on the market. A *M. pulcherrima* strain selected for its specific property to release enzymes with α -L-arabinofuranosidase activity is now available. *M. pulcherrima*, with a suitably paired *S. cerevisiae* strain sequentially inoculated, impacts on the expression of terpenes and thiols and it is recommended for Riesling and Sauvignon Blanc wines. Also a commercial product based on a

selected strain of *P. kluyveri* is recommended due to its ability to boost fruit flavors through a more efficient conversion of flavor precursors into volatile thiols.

Secondary Aroma Control of Wine Acidity

Different strategies based on non-*Saccharomyces* yeasts have been described for reducing volatile acidity or either for acidifying or deacidifying wines.

To solve the problem of excessive volatile acidity due to high acetic acid concentrations, non-*Saccharomyces* yeasts such as *T. delbrueckii*, *M. pulcherrima*, and *C. stellata*/*C. zemplinina* can be employed. *T. delbrueckii*, often described as a low acetic acid producer under standard conditions, retains this quality even fermenting high-sugar media. A mixed culture of *T. delbrueckii* and *S. cerevisiae* was shown to be the best combination for improving the analytical profile of wines produced from botrytized musts, particularly volatile acidity, and acetaldehyde production. Specifically, the mixed *T. delbrueckii*/*S. cerevisiae* culture produced 53% less in volatile acidity and 60% less acetaldehyde than a pure culture of *S. cerevisiae* (Bely et al., 2008). Interestingly, the mixed culture was only effective in simultaneous inoculation since the sequential one resulted in stuck fermentation. Significant reductions in volatile acidity in mixed fermentations of *T. delbrueckii*/*S. cerevisiae* were also reported by other authors (Comitini et al., 2011), who observed the same effect when using *M. pulcherrima*/*S. cerevisiae* starters independently of the inoculum ratios.

The high fermentative capacity of *C. stellata*/*C. zemplinina* has been explored in mixed starters. In a fermentation conducted by a mixture of *C. stellata* and *S. cerevisiae* in Pinot Grigio must (270 g sugars/L), yeast cells were able to completely consume all glucose and fructose while reducing the levels of acetic acid (Ciani and Ferraro, 1998). Similarly, specific strains of *C. zemplinina* when co-inoculated with *S. cerevisiae* were able to reduce the content of acetic acid while maintaining high glycerol and ethanol levels (Rantsiou et al., 2012).

In grape must, different combinations of *Pichia fermentans* with *S. cerevisiae* produced less acetic acid than *S. cerevisiae* in single cultures. Moreover the decrease in acetic acid was accompanied by a substantial increase in aromatic compounds such as acetaldehyde, ethyl acetate, 1-propanol, n-butanol, 1-hexanol, ethyl caprilate, 2,3-butanediol, and glycerol (Clemente-Jiménez et al., 2005).

The ability of non-*Saccharomyces* yeasts to act as acidifying agents is of increasing interest, as global climate change and variations in viticulture and oenology practices have resulted in a trend toward the reduction of the total acidity of wines. *L. thermotolerans*, through the production of L-lactic acid, is a potential acidifying microorganism during must fermentation which could compensate the insufficient acidity of specific grape varieties (Mora et al., 1990; Kapsopoulou et al., 2007). *L. thermotolerans*, in both simultaneous and sequential inoculations with *S. cerevisiae*, provided an effective acidification during alcoholic fermentation although the production of L-lactic acid was dependent on the time of inoculation of the *S. cerevisiae* strain (Kapsopoulou et al., 2007). Also the

L. thermotolerans/*S. cerevisiae* consortium provoked a pH reduction associated with a significant enhancement in the total acidity and reduction in the volatile acidity, as compared to pure *S. cerevisiae* cultures (Comitini et al., 2011). The mixed fermentations were characterized as well by increases in glycerol and main esters. In agreement with previous results, pH reductions and enhancement of glycerol and 2-phenylethyl alcohol contents were shown in wines fermented with the co-culture. Moreover sensory analysis tests showed significant increases in the spicy notes and in terms of total acidity increases (Gobbi et al., 2013).

Also the deacidifying capacity of *S. pombe* and *Issatchenkia orientalis* (alternative name *Pichia kudriavzevii*) due to the consumption of malic acid has been explored in mixed starters. The combination *S. pombe*/*S. cerevisiae* has proved successful in biological deacidification of white and red wines (Benito et al., 2013, 2014b). In all wines obtained with *S. pombe* either alone or together with *S. cerevisiae*, nearly all the malic acid was consumed, and moderate acetic acid concentrations were formed. Moreover the urea content of these wines was notably lower when compared with those that were made with *S. cerevisiae* alone. White wines obtained with mixed cultures received the best overall scores after sensory evaluation (Benito et al., 2013) but in red wine fermentations, the maximum aroma intensity and quality corresponded to those obtained with *S. pombe* in monoculture (Benito et al., 2014b). Similarly, wines co-fermented by *I. orientalis* and *S. cerevisiae* showed decreased malic acid concentrations and the highest score in sensory evaluation. The co-fermentation also decreased the contents of acetaldehyde, 1-propanol, 2-butanol, and isoamyl alcohol but increased the methanol content (Kim et al., 2008). Recently the capability of *P. kudriavzevii* to degrade malic acid in microvinifications, increasing the pH 0.2–0.3 units, was confirmed (del Mónaco et al., 2014).

Additionally, the combined use of selected *S. pombe* and *L. thermotolerans* strains has been described as a feasible alternative to the traditional malolactic fermentation (Benito et al., 2015). With this approach, malic acid is totally consumed by *S. pombe*, while lactic acid produced by *L. thermotolerans* maintains or increases the acidity of wines produced from low acidity musts. Final wines had more fruity character and they contained less acetic acid and biogenic amines than the traditional malolactic fermentation controls (Benito et al., 2015).

Influence on Esters

The increase of fruity acetate esters has been the main target of mixed starters designed with *Hanseniaspora* species. *H. guilliermondii* and *H. uvarum* grown as mixed cultures with *S. cerevisiae* in grape must increased the 2-phenylethyl acetate and isoamyl acetate content of wines, respectively (Rojas et al., 2003; Moreira et al., 2008). However, the excessive production of ethyl acetate limited the applicability of both mixed starters. Similarly, although a desirable increase of acetate esters in mixed fermentations of Macabeo and synthetic must with *H. uvarum*/*S. cerevisiae* was reported, the high concentration of acetic acid hampered the industrial application of the mixed starter (Andorrà et al., 2010, 2012). However, since a large

strain variability is associated to metabolite production (Plata et al., 2003; Romano et al., 2003; Ciani et al., 2006), the excessive concentration of both ethyl acetate and acetic acid can be avoided by means of specific screenings. In this regard a *H. vineae* strain yielding high levels of 2-phenylethyl acetate while producing levels of acetic acid and ethyl acetate within the optimal ranges described for wine was selected (Viana et al., 2008). Moreover the potential of using that selected strain in a mixed starter with *S. cerevisiae* to increase the levels of 2-phenylethyl acetate in wines without compromising quality was demonstrated (Viana et al., 2009). Besides, the ratio of both yeast strains in the mixed culture modulated ester concentrations leading to wines with a wide range of flavor compounds. Further studies showed that the selected *H. vineae* strain inoculated as a part of a sequential mixed starter was able to compete with native yeasts present in a non-sterile must and modify the wine aroma profile, specifically 2-phenylethyl acetate concentration (Viana et al., 2011). Recently, wines obtained from industrial Chardonnay white grape vinifications conducted by sequential *H. vineae*/*S. cerevisiae* inoculation showed a significant increase in fruity intensity described as banana, pear, apple, citric fruits, and guava, in comparison to spontaneous and pure *S. cerevisiae* fermentations. Fruity intensity was mainly correlated to higher concentrations of acetyl and ethyl esters and relative decreases in alcohols and fatty acids (Medina et al., 2013).

An increase in isoamyl acetate content can also be achieved by fermenting Emir must with *Williopsis saturnus*/*S. cerevisiae* cultures. Furthermore the mixed culture did not produce any off-flavors, although the changes observed in the aromatic profile of mixed wines were inoculum and temperature dependent (Erten and Tanguler, 2010; Tanguler, 2012, 2013). Wines elaborated by sequential fermentation of *W. anomalus* and *S. cerevisiae* presented higher levels of acetate and ethyl esters and of linear alcohols, which contribute to increase the aromatic quality with floral and fruity notes (Izquierdo-Cañas et al., 2014). With the aim of obtaining white wines with an enhanced aromatic complexity, a *Zygosaccharomyces bailii* strain characterized as producer of several esters (Garavaglia et al., 2014) was inoculated together with *S. cerevisiae*. In all trials that contained the non-*Saccharomyces* yeast the production of ethyl esters was increased in comparison to the vinification control (Garavaglia et al., 2015).

In mixed cultures *T. delbrueckii*/*S. cerevisiae* the formation of specific esters was reported. For instance, the mixed culture produced Tempranillo wines with larger quantities of 2-phenylethyl acetate and ethyl lactate than single *S. cerevisiae* fermentations (Loira et al., 2014), or larger amounts of isoamyl acetate, hexyl acetate, ethyl hexanoate, and ethyl octanoate in Syrah wines (Loira et al., 2015). Also, ethyl propanoate, ethyl isobutanoate, ethyl dihydrocinnamate, isobutyl acetate, and isoamyl acetate concentrations were increased in wines obtained by mixed fermentations *T. delbrueckii*/*S. cerevisiae*, either in sequential or simultaneous inoculation (Renault et al., 2015). Favoring *T. delbrueckii* development when performing sequential inoculation enhanced the concentration of the above mentioned ethyl esters, which were linked to *T. delbrueckii* activity. On the contrary, simultaneous inoculation restricted the growth of *T. delbrueckii*, limiting the production of its activity

markers. However, simultaneous inoculation involved a high production of numerous esters due to more important positive interactions between yeast species. These results suggested that the ester concentration enhancement via interactions during mixed modalities was due to *S. cerevisiae* production in response to the presence of *T. delbrueckii* (Renault et al., 2015). A pure culture of *T. delbrueckii* selected for its properties to enhance wine aromatic and mouthfeel complexity is available on the market. When used in sequential inoculation with compatible selected *S. cerevisiae*, it favors the perception of certain esters without overwhelming the wines.

Finally, aromatic complexity of Ribolla wines can be improved by sequential fermentation with *K. gamospora* and *S. cerevisiae* due to the enhanced production of esters such as 2-phenylethyl acetate and ethyl propionate and also of 2-phenylethyl alcohol (Dashko et al., 2015).

Influence on Higher Alcohols

The presence of *T. delbrueckii* in mixed starters has been associated with increases in the production of 2-phenylethyl alcohol in different kinds of wine. Mixed starters *T. delbrueckii*/*S. cerevisiae* were proposed for the production of Amarone wine, a high-alcohol dry red wine obtained from withered grapes. The most significant changes caused by the presence of *T. delbrueckii* were observed among alcohols, specifically benzyl alcohol and 2-phenylethyl alcohol, but also in fermentative esters, fatty acids and lactones, which are important in the Amarone wine flavor (Azzolini et al., 2012). Interestingly the increase in the levels of 2-phenylethyl alcohol seemed to be related to the β -glucosidase activity of the *T. delbrueckii* strain employed, although other factors could not be discarded. Also in the fermentation of dry and sweet wines, mixed cultures *T. delbrueckii*/*S. cerevisiae* affected the content of several important volatile compounds, including 2-phenylethyl alcohol, isoamyl acetate, fatty acid esters, C₄–C₁₀ fatty acids and vinylphenols (Azzolini et al., 2015). In addition to *T. delbrueckii* mixed starters, *M. pulcherrima*/*S. cerevisiae*, *L. thermotolerans*/*S. cerevisiae*, and *K. gamospora*/*S. cerevisiae* fermentations resulted in higher productions of 2-phenylethyl alcohol (Comitini et al., 2011; Dashko et al., 2015).

Triple Mixed Cultures

Finally, and with the aim of mimicking the complex yeast microbiota present in fermenting musts, wine mixed cultures composed by more than one non-*Saccharomyces* species in combination with *S. cerevisiae* have been also developed. However, the number of studies focused on wine aroma development is still low and results somehow controversial.

In this regard, fermentations of natural grape musts with a *Saccharomyces* strain together with a *C. zemplinina* and/or a *H. uvarum* strains showed the preferential use of some groups of amino acids (aliphatic, aromatic, and sulphur amino acids) in the mixed fermentations compared with the pure cultures. These results suggested that the presence of several yeast species might improve the uptake or consumption of some amino acids by some kind of synergistic mechanism (Andorrà et al., 2010). However the preferential use of amino acids did not

have a clear consequence on aroma production as it would be expected: fermentations with the triple mixed culture only stood out for ethyl lactate production and significant differences were only observed with the pure *S. cerevisiae* fermentation, while fermentations including one or two non-*Saccharomyces* strains were comparable to each other. Moreover the amount of acetic acid was well above the admissible levels and thus compromising the immediate application of these mixed cultures (Andorrà et al., 2010). Later on, the same authors reported significant differences in the above results when a synthetic grape must was used (Andorrà et al., 2012).

Production of white wine by sequential inoculation of *H. anomala*, *T. delbrueckii*, and *S. cerevisiae* has been proposed (Izquierdo-Cañas et al., 2011). Resulting wines were chemically different to those produced by *S. cerevisiae* alone, by *H. anomala*/*S. cerevisiae*, or by *T. delbrueckii*/*S. cerevisiae*. Surprisingly, the two last combinations produced wines exhibiting more complexity than the one including the two non-*Saccharomyces* species, which in fact was comparable to the pure *S. cerevisiae* fermentation.

Nowadays, a blend of three yeasts, *S. cerevisiae*, *K. thermotolerans*, and *T. delbrueckii*, is commercialized. The mixture gives tropical fruitiness and an overall aromatic intensity in white wines and more pronounced fruity and spicy notes in red wines.

FINAL CONSIDERATIONS

Based on numerous studies showing the positive influence of non-*Saccharomyces* yeasts in winemaking, the wine industry has been directed toward the use of controlled mixed fermentations. Indeed designed mixed starters with selected non-*Saccharomyces* strains and *S. cerevisiae* can enhance, as pointed out in this review, primary and secondary wine aroma, but also they are involved in reductions of the ethanol content of wine (González et al., 2013; Contreras et al., 2015; Morales et al., 2015), control of the spoilage wine microflora (Oro et al., 2014), release of mannoproteins (Domizio et al., 2014) or wine color stabilization (Morata et al., 2012; Loira et al., 2015). Moreover they can exert a positive effect in base wines for sparkling wine production improving foaming properties (González-Royo et al., 2015). Remarkably, a new red winemaking technology based on the combined use of two non-*Saccharomyces* yeast strains has been developed as an alternative to the traditional malolactic fermentation (Benito et al., 2015).

In addition to the mandatory strain selection, the benefits of mixed cultures should be tested in different grape musts since different nutritional characteristics and limitations might modify the impact of the individual components of the starter on the final wine. Moreover mixed cultures should be tested at industrial or semi-industrial scales because it has been reported that the production of different metabolites can vary depending on the fermentation volume and the oxygen conditions (Beltran et al., 2008; Viana et al., 2009). Certainly the study of the impact of common oenological practices on the dynamics of non-*Saccharomyces* yeasts will be also useful for

a better management of mixed fermentations (Albertin et al., 2014).

Considering that the main reason for re-evaluating non-*Saccharomyces* yeasts and for introducing mixed cultures in the winemaking process was to get differentiated wines reflecting the characteristic of a given wine region, the commercial assortment of non-*Saccharomyces* cultures is still reduced. In this context the continuous ecological studies as well as the oenological and sensory characterization of autochthonous non-*Saccharomyces* and even *S. cerevisiae* isolates will provide appropriate candidates to be included as a part of commercial mixed starter cultures for the production of typical wines (Canonico et al., 2015; Teixeira et al., 2015).

Finally, rational design of mixed cultures should take into account not just results from smart screenings that allow exploiting positive features of non-*Saccharomyces* yeasts but also potential interactions among microorganisms. Some yeast interactions reported to occur in mixed starters have been briefly discussed in this review, but little is known about the mechanisms involved. In fact, positive, negative, and neutral interactions in mixed fermentations of non-*Saccharomyces* and *Saccharomyces* yeasts for the formation of aromatic compounds have been identified (Sadoudi et al., 2012). These interactions seem to be strain-dependent for both non-*Saccharomyces* and *S. cerevisiae* strains (Anfang et al., 2009; Canonico et al., 2015) and might affect the entire metabolic pathway. Current knowledge on wine yeast interactions has been recently revised (Ciani and Comitini, 2015) but it is an area that requires in-depth studies. Undoubtedly the application of high throughput techniques will offer a powerful approach for unraveling microbial interactions and thus it will allow a better design of mixed cultures and also an increased control over mixed culture fermentations.

AUTHOR CONTRIBUTIONS

BP, JG, and PM made contributions to conception and design of the review. BP and JG draft the manuscript. PM supervised and edited the manuscript. All authors commented on the manuscript at all stages.

FUNDING

Our work on non-*Saccharomyces* yeasts was supported by grants VIN03-007-C2-1, AGL2003-01295, AGL2004-00978, Consolider-Ingenio 2010 CSD2007-0063, AGL2010-21009, and AGL2014-58205-REDC from the Spanish Government-FEDER.

ACKNOWLEDGMENTS

We extend special thanks to our collaborators and laboratory members for help and thoughtful discussions. We apologize to all the investigators whose research could not be appropriately cited because of space limitations.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Effects of new *Torulaspora delbrueckii* killer yeasts on the must fermentation kinetics and aroma compounds of white table wine

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OPEN ACCESS

Edited by:

Alberto Mas,
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Reviewed by:

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Specialty section:

This article was submitted to
Food Microbiology,
a section of the journal
Frontiers in Microbiology

Received: 04 September 2015

Accepted: 19 October 2015

Published: 03 November 2015

Citation:

Velázquez R, Zamora E, Álvarez ML, Hernández LM and Ramírez M (2015) Effects of new *Torulaspora delbrueckii* killer yeasts on the must fermentation kinetics and aroma compounds of white table wine. *Front. Microbiol.* 6:1222. doi: 10.3389/fmicb.2015.01222

Torulaspora delbrueckii is becoming widely recommended for improving some specific characteristics of wines. However, its impact on wine quality is still far from satisfactory at the winery level, mostly because it is easily replaced by *Saccharomyces cerevisiae*-like yeasts during must fermentation. New *T. delbrueckii* killer strains were here isolated and selected for winemaking. They killed *S. cerevisiae* yeasts and were able to dominate and complete the fermentation of sterile grape must. Sequential yeast inoculation of non-sterile white must with *T. delbrueckii* followed by *S. cerevisiae* did not ensure *T. delbrueckii* dominance or wine quality improvement. Only a single initial must inoculation at high cell concentrations allowed the *T. delbrueckii* killer strains to dominate and complete the must fermentation to reach above 11% ethanol, but not the non-killer strains. None of the wines underwent malolactic fermentation as long as the must had low turbidity and pH. Although no statistically significant differences were found in the wine quality score, the *S. cerevisiae*-dominated wines were preferred over the *T. delbrueckii*-dominated ones because the former had high-intensity fresh fruit aromas while the latter had lower intensity, but nevertheless nice and unusual dried fruit/pastry aromas. Except for ethyl propanoate and 3-ethoxy-1-propanol, which were more abundant in the *T. delbrueckii*-dominated wines, most of the compounds with fresh fruit odor descriptors, including those with the greatest odor activity values (isoamyl acetate, ethyl hexanoate, and ethyl octanoate), were more abundant in the *S. cerevisiae*-dominated wines. The low relative concentrations of these fruity compounds made it possible to detect in the *T. delbrueckii*-dominated wines the low-relative-concentration compounds with dried fruit and pastry odors. An example was γ -ethoxy-butyrolactone which was significantly more abundant in these wines than in those dominated by *S. cerevisiae*.

Keywords: *Torulaspora delbrueckii*, yeast, killer, must fermentation, winemaking, white table wine, aroma compounds

INTRODUCTION

The non-*Saccharomyces* yeasts which are usually present in spontaneous must fermentations have been receiving ever more attention by the part of wine microbiologists because some of them can improve wine complexity. The yeasts which have lately been investigated for wine quality improvement belong to *Candida*, *Kloeckera*, *Hanseniaspora*, *Zygosaccharomyces*, *Schizosaccharomyces*, *Torulaspora*, *Brettanomyces*, *Saccharomycodes*, *Pichia*, and *Williopsis* genera (Jolly et al., 2006). Among them, *Torulaspora delbrueckii* is probably the most commonly used in winemaking. Controlled inoculation with this yeast is widely recommended for improving the complexity and for enhancing certain specific characteristics of wines (Jolly et al., 2006; Bely et al., 2008; Renault et al., 2009; Azzolini et al., 2012, 2015). This yeast can also be used to increase glycerol (Contreras et al., 2015) and mannoproteins (Comitini et al., 2011; Belda et al., 2015), or to reduce ethanol (Contreras et al., 2015) in the wine. However, its commercial impact on wine quality is still far from satisfactory, mostly because of the difficulty in reliably controlling the desired participating proportion of *T. delbrueckii* with respect to the other wine yeast species involved in the same must fermentation process, mainly *Saccharomyces cerevisiae*-like yeasts. It has been reported that the mixed inoculation of *T. delbrueckii* and *S. cerevisiae* reduces such off-flavor compounds as volatile acidity, acetaldehyde, and acetoin (Herraiz et al., 1990; Ciani et al., 2006; Bely et al., 2008), and leads to a systematic increase of 2-phenylethanol, terpenols, and lactones (Herraiz et al., 1990; Comitini et al., 2011; Azzolini et al., 2012; Sadoudi et al., 2012). However, results concerning ester production remain confusing. It has been reported that mixed inoculation can increase the total ester concentration (in particular that of isoamyl acetate and ethyl hexanoate, octanoate, and 3-hydroxybutanoate) relative to pure-culture inoculation (Herraiz et al., 1990). But the contrary has also been reported, i.e., that the total ester concentration of mixed inoculations was less than that of a pure *S. cerevisiae* culture, with a significant reduction in acetate esters, in particular of isoamyl acetate (Comitini et al., 2011; Sadoudi et al., 2012). Similarly, no difference in the overall ester concentrations was found between mixed *T. delbrueckii*/*S. cerevisiae* and single *S. cerevisiae* inoculation, although the level of some esters (ethyl 3-hydroxybutanoate, for instance) was higher in the mixed culture while that of others (such as isoamyl acetate) was lower (Azzolini et al., 2012). These apparently contradictory results concerning ester concentrations may depend on the proportion of each yeast species during must fermentation, or also on the eventual occurrence of malolactic fermentation, neither of which possibilities were discussed in any depth by those authors. Additionally, it has been shown that ester production by *T. delbrueckii* is strain dependent, and that the aromas resulting from this yeast can differ when it is associated with *S. cerevisiae* in mixed cultures (Renault et al., 2009).

As most non-*Saccharomyces* yeasts, *T. delbrueckii* has less fermentation vigor and a slower growth rate than *S. cerevisiae* under usual wine fermentation conditions, being quickly overcome by wild or inoculated *S. cerevisiae* strains (Mauricio

et al., 1998; González-Royo et al., 2014). Thus, knowledge about the interactions between *Saccharomyces* and *Torulaspora* wine yeasts during wine fermentation needs to be improved to better predict the relative participation of each yeast species (Ciani et al., 2010). The availability of good-fermenting killer *T. delbrueckii* strains, able to kill the omnipresent wild *Saccharomyces* yeasts or to control the excessive growth of inoculated *S. cerevisiae* strains, could be an interesting tool with which to attain the desired domination of each inoculated yeast during must fermentation, and thus result in improved quality of the wine. The isolation of *T. delbrueckii* killer strains has been described previously (Sangorin et al., 2007), but they have not been used and analyzed in depth for winemaking as it has been *S. cerevisiae* K2 strains (Pérez et al., 2001). The effect of *S. cerevisiae* killer strains on the growth of sensitive strains during must fermentation was seen to depend on the initial proportion of killer yeasts, the susceptibility of sensitive strains, and the treatment of the must. An initial proportion of 2–6% killer yeasts was enough to suppress isogenic sensitive strains in sterile filtered must, although a greater initial proportion of killer yeasts may be needed to get the same effect against non-isogenic strains. The suspended solids that remain in the must after cold-settling were seen to reduce the killer toxin effect due to inactivation by absorption onto the grape particles (Pérez et al., 2001).

The objective of the present work was to evaluate the use of new killer *T. delbrueckii* strains (Kbarr) for white wine making. We addressed the following issues: (i) capacity of Kbarr strains to dominate and complete must fermentation in the presence of *S. cerevisiae* yeasts; (ii) influence of must treatment on this Kbarr-1 strain domination; (iii) influence of Kbarr strains on malolactic fermentation; and (iv) analysis of the aroma profile of *T. delbrueckii* white wine as compared with *S. cerevisiae* white wine. The usefulness of killer *T. delbrueckii* strains for winemaking will be discussed.

MATERIALS AND METHODS

Yeast Strains and Culture Media

EX85, EX85R, and E7AR1 are prototrophic and homothallic *S. cerevisiae* wine yeasts previously isolated from Spanish wineries, selected for winemaking (Regodón et al., 1997; Ramírez et al., 1998), and sold by Heral Enología SL (Almendralejo, Spain). EX85 is K2-killer, EX85R is virus-free killer-sensitive cycloheximide-resistant (cyh^R), and E7AR1 is K2-killer cyh^R. The *S. cerevisiae* K2-killer strains kill other killer-sensitive *S. cerevisiae* strains but do not kill *T. delbrueckii* yeasts. The new *T. delbrueckii* Kbarr wine yeasts are prototrophic strains isolated from spontaneous fermentations of grapes from vineyards of the Albarregas (*Barraecas* in Latin) river valley in Spain. They kill all *S. cerevisiae* killer and non-killer strains and the non-killer *T. delbrueckii* strains. The industrial use of these Kbarr yeasts is under patent application. The yeast strains used in this work are summarized in **Table 1**.

YEPA + cycloheximide (cyh) is YEPA-agar (1% Bacto-yeast extract, 2% Bacto-peptone, 2% glucose, 2% Bacto-agar) supplemented with cyh, prepared in a concentrated ethanol

TABLE 1 | Yeast strains used.

Strain	Genotype/Relevant phenotype	Origin
Sc EX85	<i>MAT a/α HO/HO</i> L-A M-2 [K2 ⁺]	M. Ramírez ^a (from wine)
Sc EX85R	<i>MAT a/α HO/HO</i> <i>CYH2^R/cyh2^S</i> [cyh ^R K2 ⁰]	M. Ramírez ^a
Sc E7AR1	<i>MAT a/α HO/HO</i> <i>CYH2^R/cyh2^S</i> [K2 ⁺]	M. Ramírez ^a
Td EX1180	<i>wt</i> L-A M-barr-1 [Kbarr-1 ⁺]	This study (from wine)
Td EX1180-11C4	<i>cyh^R</i> L-Abarr M-barr-1 [cyh ^R Kbarr-1 ⁺]	This study (from EX1180)
Td EX1180-2K ⁻	<i>cyh^R</i> L-Abarr M-barr-0 [cyh ^R Kbarr ⁰]	This study (from EX1180)
Td EX1257	<i>wt</i> L-Abarr M-barr-2 [Kbarr-2 ⁺]	This study (from wine)
Td EX1257-CYH5	<i>cyh^R</i> L-Abarr M-barr-2 [cyh ^R Kbarr-2 ⁺]	This study (from EX1257)

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solution to a final concentration of 2 µg/mL (Pérez et al., 2000). Standard yeast genetics procedures were used for sporulation (Kaiser et al., 1994). Cells were grown on YEPD plates for 2 days at 30°C, transferred to sporulation plates (1% potassium acetate, 0.1% Bacto-yeast extract, 0.05% glucose, 2% Bacto-agar) and incubated for 7–30 days at 25°C.

Determination of Yeast Killer Activity

Killer activity was tested on low-pH (pH 3.3 or 4.0) methylene blue plates (3.3MB or 4.0MB; Kaiser et al., 1994) seeded with 100 µL of a 48-h grown culture of the sensitive strain (Ramírez et al., 2004). Depending on the experiments, the strains being tested for killer activity were either loaded as 4 µL aliquots of stationary phase cultures, patched from solid cultures, or replica plated onto the seeded MB plates. Then the plates were incubated for 4–8 days at 12 or 20°C.

Laboratory Must Fermentation

Must fermentation was carried out in 5-L Erlenmeyer flasks with 3.5 L of Cigüente grape must (18.0°Brix, pH 3.5, 50 mg/L SO₂, and 0.3 g/L Actimax nutrients from Productos Agrovín S.A.) sterilized by membrane filtration through a Millipore system (0.45-µm membrane). Yeast cells were cultured in YEPD broth for 2 days at 30°C, washed twice (by centrifugation) with sterile water, and suspended in the must at the desired concentration. Fermentations were conducted at 18°C for 20 days. Yeast growth (determination of total yeast cells by counting with a Neubauer chamber, and viable cells by counting the yeast colonies that arose on YEPD-agar plates), and the °Brix were monitored. All experiments were done in triplicate.

Winery Vinification Trials

The yeast inocula were obtained in a pilot plant of the company Heral Enología SL following its industrial procedure. Cells were

cultured in beet molasses broth [5% beet molasses, 0.2% Bacto-yeast extract, 0.075% (NH₄)₂HPO₄, 0.1% MgSO₄·7H₂O, adjusted to pH 3.5 with HCl] for 18 h at 30°C with strong aeration, washed twice (by centrifugation) with sterile distilled water, and inoculated in 350-L stainless steel tanks with cold-settled white Cigüente (19.0–19.8°Brix, pH 3.42, 80–250 NTU, 50 mg/L SO₂, and 0.3 g/L Actimax) or Macabeo (20.4–20.8°Brix, pH 3.29–3.55, 80–250 NTU, 50 mg/L SO₂, and 0.3 g/L Actimax) grape must to a final concentration of 2–4 × 10⁶ cells/mL for *S. cerevisiae* and 2–4 × 10⁷ cells/mL for *T. delbrueckii*. The vinification process was conducted at 16–18°C. The density, °Brix, and yeast growth (total and viable yeast cells) were monitored throughout fermentation. The tanks were hermetically closed when reducing sugars reached around 1% to avoid oxidation problems. At the end of fermentation, the settled solids were discarded. An 800-mL centrifuged sample of each wine was taken for the analytical assays. The wines were stored at 12°C. After 30 days following the beginning of fermentation, settled solids were again discarded, a 2-L sample of each wine was taken for the first aroma compounds and organoleptic assays, and the wines were returned to store at 12°C. At 60 days, settled solids were discarded once again and the second aroma compounds and organoleptic assays were carried out. The organoleptic characteristics (flavor, color, and odor) of the wines were tested by a panel of 12 experts. Wines were presented in clear tulip-shaped wine glasses covered with glass Petri dishes. A sample of 50–70 mL of wine was poured into each glass immediately before being analyzed by each judge. The temperature of the samples was from 10 to 13°C. Sensory profiles of wines were evaluated for overall aromatic complexity, and fresh fruit and dried fruit/pastry aroma intensities. The judges scored the quality of the wines on a six-point scale (0 = very poor, 1 = deficient, 2 = acceptable, 3 = good, 4 = very good, and 5 = excellent). The maximum score possible (60 points) was considered 100% preference. All experiments were done in duplicate.

Determination of the Amount of Inoculated Yeasts during Must Fermentation

Determination of the percentage of genetically marked yeasts was done by the replica-plating method (Pérez et al., 2000). Samples from fermenting musts were diluted and plated onto YEPD-agar to obtain 100–300 colonies per plate. The detection of the *cyh^R* mutants was accomplished by replica-plating these plates to either YEPD + *cyh* (2 µg/mL) plates using sterile velvets and then to other plates of YEPD-agar to detect wild yeasts sensitive to *cyh*. The time needed to easily observe growth of resistant yeasts on YEPD + *cyh* at 30°C varied between 1 and 3 days depending on the yeast strain.

The percentage of wild parent yeasts, or genetically marked yeasts for the replica-plating results validation, was mostly determined by analyzing the mtDNA restriction pattern as previously described (Maqueda et al., 2010).

The yeast spore (after yeast growth on sporulation medium for 7–30 days at 25°C) or vegetative cell morphology were also eventually analyzed for validation of the previous results obtained

by the replica-plating or mtDNA restriction pattern analyses. This morphology analysis was done by microscopic observation in a Nikon Eclipse 600 microscope equipped with a Nomarski 60× objective.

Analytical Methods

Density, °Brix, pH, total acidity, volatile acid, reducing sugars, alcohol, and malic acid were determined according to the EC recommended methods (E.C, 1999). Lactic acid was determined using the EEC recommended method (E.E.C, 1990). Glycerol was determined with an enzymatic test (Roche, Germany). Mannoprotein content was measured as previously described (Quiros et al., 2012). T15 is the time needed to ferment 15% of the total sugars present in the must, and T100 is the time needed to ferment 100% of the total sugars (Ramírez et al., 1999).

The wine aroma compounds were analyzed by gas chromatography coupled to a mass detector. The minor aroma compounds were isolated and pre-concentrated following a solid-phase extraction (SPE) procedure (García-Carpintero et al., 2011). The analyses were carried out with an Agilent 6890 N gas chromatograph coupled to a Model 5973 mass detector and equipped with an autosampler. The column was a DB WAXETR (60 m × 0.25 mm, i.d; 0.25 μm film thickness). Quantitative data were obtained by calculating the peak area of each compound relative to that of the internal standard, interpolating with the corresponding calibration plot which had been constructed from the analysis of known amounts of the volatile aroma standards. For those compounds for which the authenticated standards were unavailable (ethyl 9-decenoate, diethyl 2-hydroxyglutarate, ethyl 2-hydroxy-3-phenylpropanoate, and γ-ethoxy-butyrolactone), the identification was based on spectral comparison with the Wiley A library data, and quantification was done using the calibration curves of standards with similar chemical structures obtained in the TIC mode. A total of 75 compounds were detected in the wines elaborated (Supplementary Table S1). The odor descriptor and the odor threshold concentration for each volatile compound were taken from the literature (Etievant and Maarse, 1991; Guth, 1997; Ferreira et al., 2000; Moyano et al., 2002; Zea et al., 2007; Muñoz et al., 2008; Pino and Queris, 2011). The odor activity value (OAV) is the ratio between the concentration of each individual aromatic compound and its odor threshold concentration (the minimal concentration that can be detected by the human nose). As no odor threshold concentration was available for some compounds, 1 mg/L was taken as the value for the ethyl 9-decenoate, ethyl 4-hydroxybutyrate, and 9-decenoic acid OAV calculations, and the value for γ-butyrolactone (0.035 mg/L) was taken for the γ-ethoxy-butyrolactone OAV calculation.

Statistical Analysis

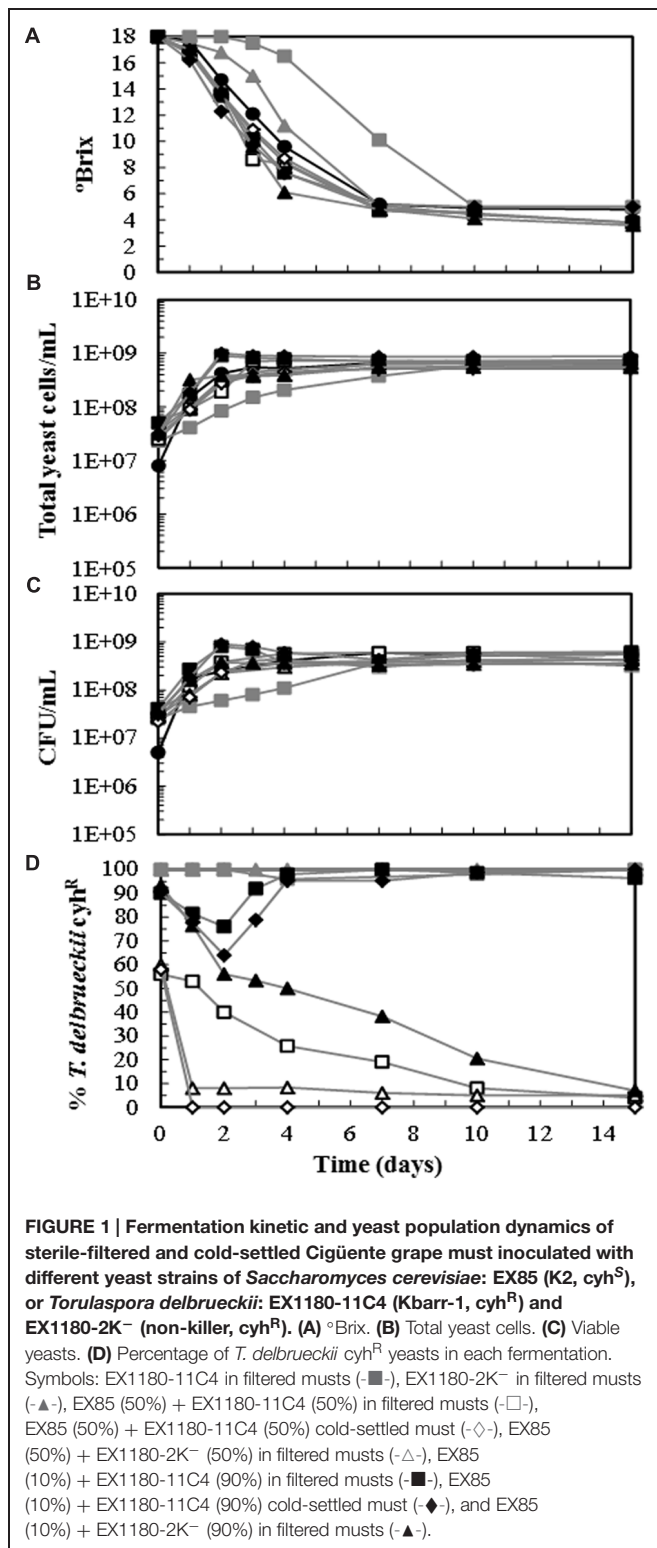
Data were analyzed for statistical significance by a one-way analysis of variance (ANOVA, $p < 0.05$) with the software package SPSS version 20.0 for Windows (Chicago, IL, USA).

RESULTS

Effect of *T. delbrueckii* Killer Yeasts on the *S. cerevisiae* Population during Sterile-must Fermentation

The influence of any given yeast on winemaking will mostly depend on its ability to dominate the must fermentation while reducing the influence of the other participating yeasts. Complementary and reliable methods to monitor the different wine yeasts in the fermenting must are required to accurately determine the degree of domination of each yeast strain. We isolated and characterized new spontaneous cyh-resistant (cyh^R) mutants from new *T. delbrueckii* killer yeasts that had previously been isolated and selected for winemaking (Regodón et al., 1997; Ramírez et al., 2015). Some of these mutants, such as EX1180-2K[−] for instance, had lost the killer virus to become killer sensitive yeasts, but others, such as EX1180-11C4, retained the virus and the Kbarr-1 phenotype (Table 1). Both of these cyh^R mutant types had good must fermentation capabilities, and were easily monitored by simple replica-plating on YEPD-CYH agar. Additionally, these *T. delbrueckii* killer yeasts can also be distinguished from the always present *Saccharomyces* by analyzing their cell morphology, spore forming process, or mtDNA RFLPs (Supplementary Figure S1). Moreover, the killer phenotype or viral dsRNA analysis can also be used for this same purpose (not shown). These alternative techniques were satisfactorily used to validate the results obtained from the simple replica-plating assay on YEPD-CYH agar plates.

To determine whether the *T. delbrueckii* killer yeasts can dominate the must fermentation in the presence of *S. cerevisiae* wine strains, sterile-must laboratory micro vinifications were inoculated with both yeasts. Each yeast species was monitored through the process by replica-plating on YEPD-CYH and by the aforementioned complementary methods (mostly mtDNA RFLP analysis) in at least two samples for each vinification. The results of the different methods showed full agreement, supporting the utility of cyh^R as genetic marker to monitor *T. delbrueckii* in winemaking, as it was previously found for *S. cerevisiae* (Pérez et al., 2000; Ambrona et al., 2005, 2006). The must fermentation inoculated with *S. cerevisiae* alone or with two-yeast mixtures of *S. cerevisiae* + *T. delbrueckii* strains (one of them containing the cyh^R genetic marker) showed faster kinetics than those single-inoculated with a *T. delbrueckii* strain, although all fermentations were completed after 10 days (Figure 1A). The total yeast cell concentration increased to above 2×10^8 cells/mL after 3–4 days from the start of fermentation, except for *T. delbrueckii* killer EX1180-11C4 which reached 2×10^8 cells/mL after 7 days (Figure 1B). The number of viable cells increased in parallel with the number of total cells, except for the vinification of filtered must inoculated with EX85 (10%) + EX1180-11C4 (90%), and for that of cold-settled must inoculated with EX85 (10%) + EX1180-11C4 (90%). In both cases, a decrease in viable cells was observed between days 2 and 4 of fermentation (Figure 1C), indicating that the *S. cerevisiae* yeasts were killed by the *T. delbrueckii* killer yeasts. The *S. cerevisiae* EX85 strain dominated the must fermentation when initially combined with 50% of the non-killer



T. delbrueckii EX1180-2K⁻ strain, that fell to 7% after 1 day of fermentation in filtered must (no grape particles present). This time required for *S. cerevisiae* EX85 domination was extended in filtered must fermentation when it was combined with the

same initial proportion (50%) of the *T. delbrueckii* killer EX1180-11C4, which remained above 20% after 7 days. But this time was reduced again in cold-settled non-filtered must, where EX1180-11C4 disappeared after just 1 day (Figure 1D). A plausible explanation for this behavior is the presence of grape particles through the fermentation, which might adsorb and inactivate the toxin produced by *T. delbrueckii*, as it was previously shown for toxins produced by *S. cerevisiae* (Pérez et al., 2001). The *S. cerevisiae* EX85 strain also dominated the must fermentation when initially combined with 90% of the non-killer *T. delbrueckii* EX1180-2K⁻, although this latter strain remained at above 40% for 7 days in filtered must fermentation. On the contrary, the *S. cerevisiae* EX85 strain almost disappeared when initially combined with 90% of the killer *T. delbrueckii* EX1180-11C4, which was the dominating yeast throughout fermentation in filtered and in non-filtered grape must (Figure 1D).

Winemaking with *T. delbrueckii* Killer and *S. cerevisiae* Yeasts

Once we had determined the *T. delbrueckii* cell concentration required to get its domination during must fermentation, new vinification trials were done using the commonest commercial recommendations: sequential yeast inoculation involving *T. delbrueckii* at the beginning ($2\text{--}4 \times 10^7$ CFU/mL) followed by *S. cerevisiae* ($2\text{--}4 \times 10^6$ CFU/mL) after 2 days of fermentation. In most vinifications, the *T. delbrueckii* viable population decreased to less than 10% of total viable yeast cells after around 1 day following *S. cerevisiae* inoculation. The wine obtained with these sequential mixed-yeast inoculations showed no relevant aromatic differences from those single-inoculated with a *S. cerevisiae* strain. This is probably because *S. cerevisiae*, which became the dominating yeast for most fermentation time, abolished the effect of *T. delbrueckii* on the wine aromatic compounds during the first two fermentation days. Sometimes, the *S. cerevisiae* domination was slower and less efficient, remaining more than 30% of *T. delbrueckii* killer yeasts at the end of a very slow fermentation. These fermentations were frequently not fully completed, mainly in those wines with ethanol concentrations greater than 11.5%. Therefore, in these cases, the wines obtained were not dry since they contained more than 6 g/L of reducing sugars.

In view of these disappointing results, new vinification trials were performed using single inoculation with *T. delbrueckii* ($2\text{--}4 \times 10^7$ CFU/mL). The *S. cerevisiae* yeasts present in these fermentations were only those coming into the fresh cold-settled white must (less than 10^5 CFU/mL). This must was very well clarified (less than 100 NTU turbidity) and its pH was corrected to 3.3 by the addition of tartaric acid. As controls for comparison, vinifications were also performed using only a single initial inoculation with *S. cerevisiae* ($2\text{--}4 \times 10^6$ CFU/mL). All the grape musts contained around 11°Be, but less than 11.5°Be to avoid the toxic effect of ethanol on *T. delbrueckii* yeasts and to facilitate the completion of fermentation. The fastest fermentations were always those inoculated with *S. cerevisiae*, while those inoculated with *T. delbrueckii* started quickly but slowed down as the ethanol concentration increased, and were very slow by the

end of fermentation. Non-inoculated fermentations, performed mostly by wild *Saccharomyces* yeasts from the must, were the slowest in starting, but they finished the fermentation before the vinifications single-inoculated with *T. delbrueckii* yeasts (Figures 2A,B; Table 2). The inoculated *S. cerevisiae* dominated the fermentations (100%) from the beginning to the end. The *T. delbrueckii* killer strains also dominated the fermentation, although sometimes their proportion decreased to 75% at the end of fermentation (wine density less than 995 g/L, Figures 2C,D). These wines contained a certain amount of reducing sugars (5.98 ± 2.15), especially when this *T. delbrueckii*-domination was 100% throughout fermentation and no *S. cerevisiae* ethanol-resistant wild yeasts were present at the end of the process. *T. delbrueckii* non-killer strain did not dominate the fermentation. Sometimes its proportion decreased quickly to less than 1% or, after decreasing, it remained at a proportion of around 10%. All these *T. delbrueckii*-non-dominated fermentations were completed, and they were faster than those dominated by *T. delbrueckii* because *S. cerevisiae* ethanol-resistant wild yeasts were always present in increasing proportions (Figure 2). The main fermentation aroma of these *T. delbrueckii*-non-dominated vinifications was fresh fruit, while it was cooked/dried fruit and pastry for the vinifications dominated by *T. delbrueckii* killer yeasts. The main aroma of the non-inoculated control and the non-killer *T. delbrueckii* inoculated vinifications was very similar to those single-inoculated and dominated by *S. cerevisiae*, although the latter had greater fresh-fruit odor intensities.

None of these wines underwent malolactic fermentation, even those inoculated with *T. delbrueckii* killer strains that had slow

TABLE 2 | Must fermentation parameters and white wine analysis results of independent winery vinifications made with Cigüente and Macabeo musts and of an ANOVA to study the effect of single initial inoculation with *S. cerevisiae* or *T. delbrueckii* yeasts.

Parameter	Yeast species		<i>p</i> ^a
	<i>S. cerevisiae</i>	<i>T. delbrueckii</i>	
T15 (days)	1.75 ± 0.23	5.13 ± 0.60	0.000
T100 (days)	10.0 ± 3.87	20.7 ± 3.08	0.043
Preference (%)	64.4 ± 4.67	56.7 ± 3.79	0.128
Frequency in TF (%)	100 ± 0.00	96.1 ± 2.23	0.180
Frequency in EF (%)	100 ± 0.00	86.8 ± 7.49	0.172
Alcohol (% v/v)	11.3 ± 0.58	11.2 ± 0.56	0.967
Glycerol (g/L)	6.1 ± 0.20	5.65 ± 0.37	0.315
pH	3.07 ± 0.07	3.20 ± 0.05	0.165
Total acidity (g/L)	7.21 ± 0.23	6.89 ± 0.23	0.362
Volatile acidity (g/L)	0.26 ± 0.05	0.36 ± 0.07	0.366
Density (g/L)	990.7 ± 0.41	994.6 ± 1.25	0.026
Reducing sugars (g/L)	1.24 ± 0.15	5.98 ± 2.15	0.091
Mannoproteins (mg/L)	58.8 ± 4.74	123.3 ± 32.6	0.086
Malic acid (g/L)	1.47 ± 0.12	1.45 ± 0.16	0.926
Lactic acid (g/L)	0.07 ± 0.01	0.14 ± 0.06	0.363

The data are the mean ± standard error of 11 independent experiments done with *S. cerevisiae* and 12 with *T. delbrueckii*. ^a*p*-values obtained by ANOVA for the wines made with each yeast species. TF, tumultuous fermentation; EF, end of fermentation; T15, time needed to ferment 15% of the total sugars present in the must; T100, time needed to ferment 100% of the total sugars or to get a non-fluctuating level under 8 g/L.

fermentation kinetics and more than 5 g/L of reducing sugars (Table 2), conditions that usually favor the growth of lactic acid bacteria. However, the wines elaborated with the same grape must but of greater turbidity (around 250 NTU) and higher pH (3.55) did undergo malolactic fermentation (malic acid decreased, while lactic acid increased) when single-inoculated with *T. delbrueckii* killer yeasts, but not when single-inoculated with *S. cerevisiae* yeasts. Although both inoculated yeasts dominated the fermentation (100%) throughout the process, the fermentation inoculated with *S. cerevisiae* was faster than that inoculated with *T. delbrueckii* killer yeast (taking 7 and 14 days to complete fermentation, respectively; Figure 3).

Organoleptic and Physicochemical Analysis of the Wines

The wines made with *S. cerevisiae* or *T. delbrueckii* for which these yeasts dominated all or most of the fermentation process were compared. In particular, the wines inoculated with non-killer *T. delbrueckii* yeast that became replaced by wild *S. cerevisiae* yeast and those that underwent malolactic fermentation were not considered for this analysis. The wine parameter values were consistent with both wine types being non-defective, good-quality products. Significant differences were only found for the fermentation kinetics parameters (T15 and T100), wine density, and marginally significant differences for the amount of reducing sugars and mannoproteins (Table 2). However, although no statistically significant differences were found in the organoleptic quality score, the *S. cerevisiae*-dominated

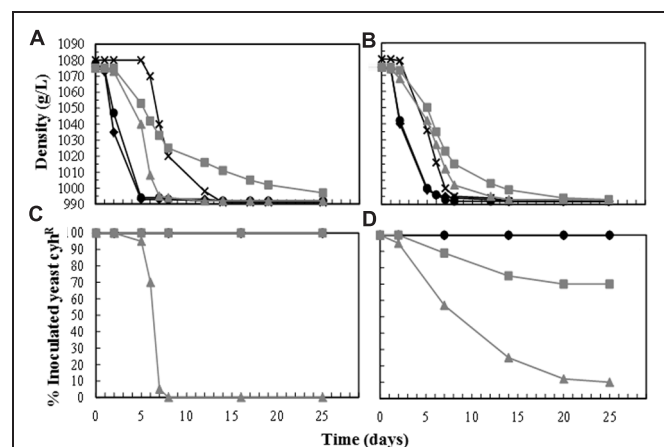


FIGURE 2 | Must fermentation kinetics and yeast population dynamics of two independent sets of vinification trials done with two Macabeo grape musts (<100 NTU, pH < 3.5) in 2011 (A,C) and 2012 (B,D). Each yeast was single inoculated in the fresh must at a cell concentration of $2-4 \times 10^6$ CFU/mL for the *S. cerevisiae* strains E7AR1 (K2, *cyh*^R) or EX85R (non-killer, *cyh*^R), and $2-4 \times 10^7$ CFU/mL for *T. delbrueckii* strains EX1180-11C4 (Kbarr-1, *cyh*^R) or EX1180-2K⁻ (non-killer, *cyh*^R). (A,B) Evolution of must-wine density. (C,D) Evolution of the percentage of each inoculated yeast (*cyh*^R) during the must fermentation. Symbols: Non-inoculated control (x), E7AR1 (◆), EX85R (●), EX1180-11C4 (■), and EX1180-2K⁻ (▲).

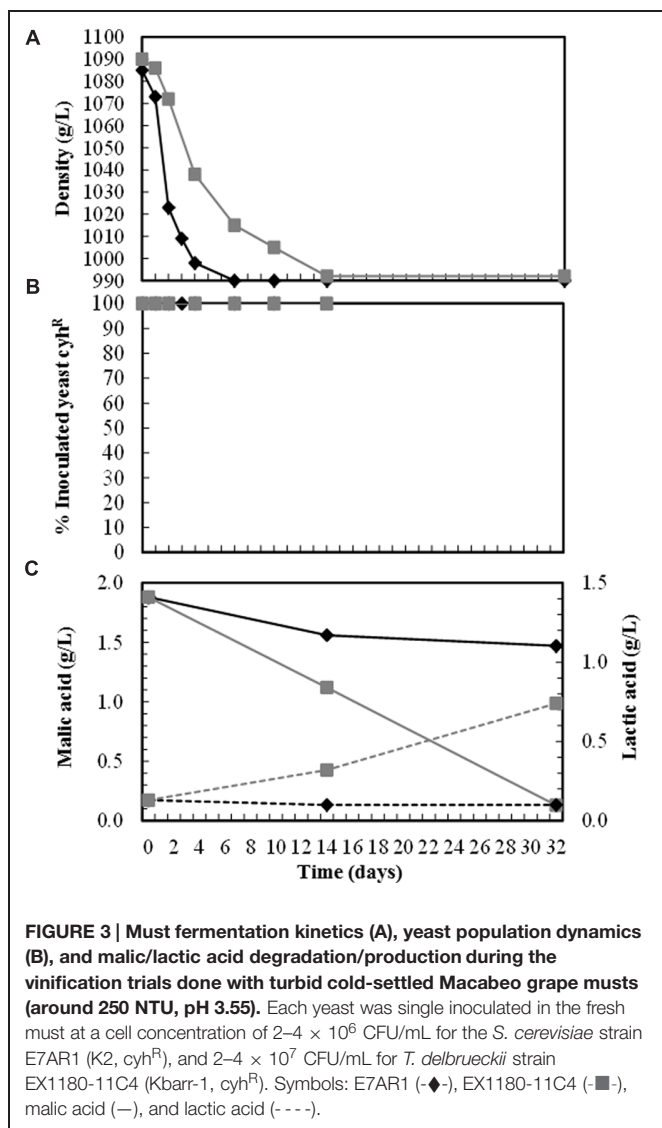


FIGURE 3 | Must fermentation kinetics (A), yeast population dynamics (B), and malic/lactic acid degradation/production during the vinification trials done with turbid cold-settled Macabeo grape musts (around 250 NTU, pH 3.55). Each yeast was single inoculated in the fresh must at a cell concentration of $2-4 \times 10^6$ CFU/mL for the *S. cerevisiae* strain E7AR1 (K2, cyh^R), and $2-4 \times 10^7$ CFU/mL for *T. delbrueckii* strain EX1180-11C4 (Kbarr-1, cyh^R). Symbols: E7AR1 (—◆—), EX1180-11C4 (—■—), malic acid (—), and lactic acid (---).

wines were preferred over the *T. delbrueckii*-dominated wines because the former had high-intensity fresh fruity aromas. The *T. delbrueckii*-dominated wines had low-intensity fresh fruit aroma, better flavor complexity, nice but unusual dried fruit (cooked fruit, pastry, and candy) aromas, a little sourness, and some aged/evolved taste. These unusual wine aromas were very similar to the aromas detected during the respective must fermentations of the same wines (see above), but less intense.

The total (summatory) amount of ethyl esters, acetate esters, organic acids, alcohols, monoterpenes, lactones, and carbonyl compounds was greater in the *S. cerevisiae* than in the *T. delbrueckii* wines, while the contrary was the case for the amount of furans + volatile phenols and norisoprenoid compounds. However, only the difference found for the amount of organic acids was statistically significant (Figure 4). Nevertheless, significant differences were found for 25 of the 75 volatile compounds analyzed independently (Figure 5). Only the amounts of ethyl propanoate (odor descriptor: banana, apple),

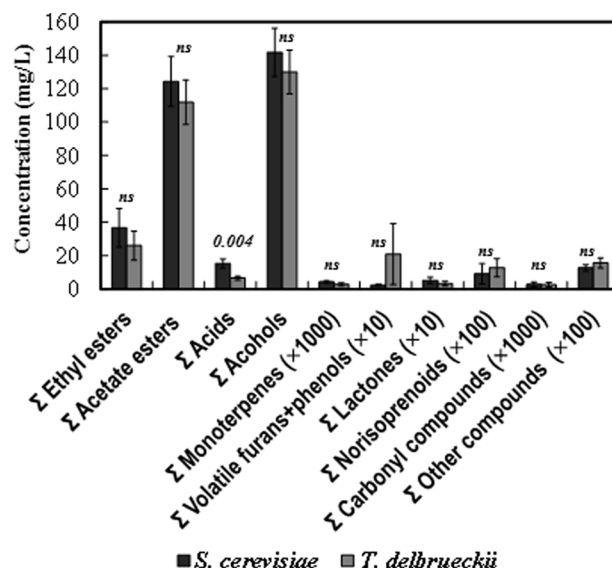


FIGURE 4 | Aroma compound composition of the *S. cerevisiae*-dominated or *T. delbrueckii*-dominated wines. The amounts for the similar chemical compounds were pooled as summatory. The data are the mean \pm standard error of 23 independent vinifications made in duplicate, 11 inoculated with *S. cerevisiae* and 12 with *T. delbrueckii*. Statistically significant difference (p) is stated in the top of the bars. ns, no significant difference.

3-ethoxy-1-propanol (fruity), γ -ethoxy-butyrolactone (as with other lactones, probably cooked peach, coconut, caramel, or toasty odor notes), and isobutyric acid (cheese, sour, butter) were significantly greater in *T. delbrueckii* than in *S. cerevisiae* wines. In contrast, most compounds were more abundant in *S. cerevisiae* than in *T. delbrueckii* wines. These were principally ethyl esters (e.g., ethyl butyrate, ethyl hexanoate, ethyl octanoate, ethyl 3-hydroxybutyrate, ethyl decanoate, ethyl 9-decenoate, ethyl 4-hydroxybutyrate, ethyl laurate, ethyl palmitate) or acetate esters (e.g., isobutyl acetate, isoamyl acetate, hexyl acetate, and phenylethyl acetate), all with fresh fruit odors (Figure 5A). Taking the detection thresholds of these 25 aromatic compounds into account, the greatest OAVs corresponded to three compounds with fresh fruit odor descriptors that were more abundant in the *S. cerevisiae* than in the *T. delbrueckii* wines: isoamyl acetate (banana), ethyl hexanoate (banana, green apple), and ethyl octanoate (banana, pineapple, pear, floral; Figure 5B). No significant differences were found for the 75 compounds analyzed between the wines from *T. delbrueckii*-non-dominated and *S. cerevisiae*-dominated fermentations (data not shown).

DISCUSSION

Influence of *T. delbrueckii* Killer Yeasts on the Must Fermentation Process

The new *T. delbrueckii* killer yeasts were reliably monitored during must fermentation by using spontaneous cyh^R mutants, with the results being validated by complementary methods

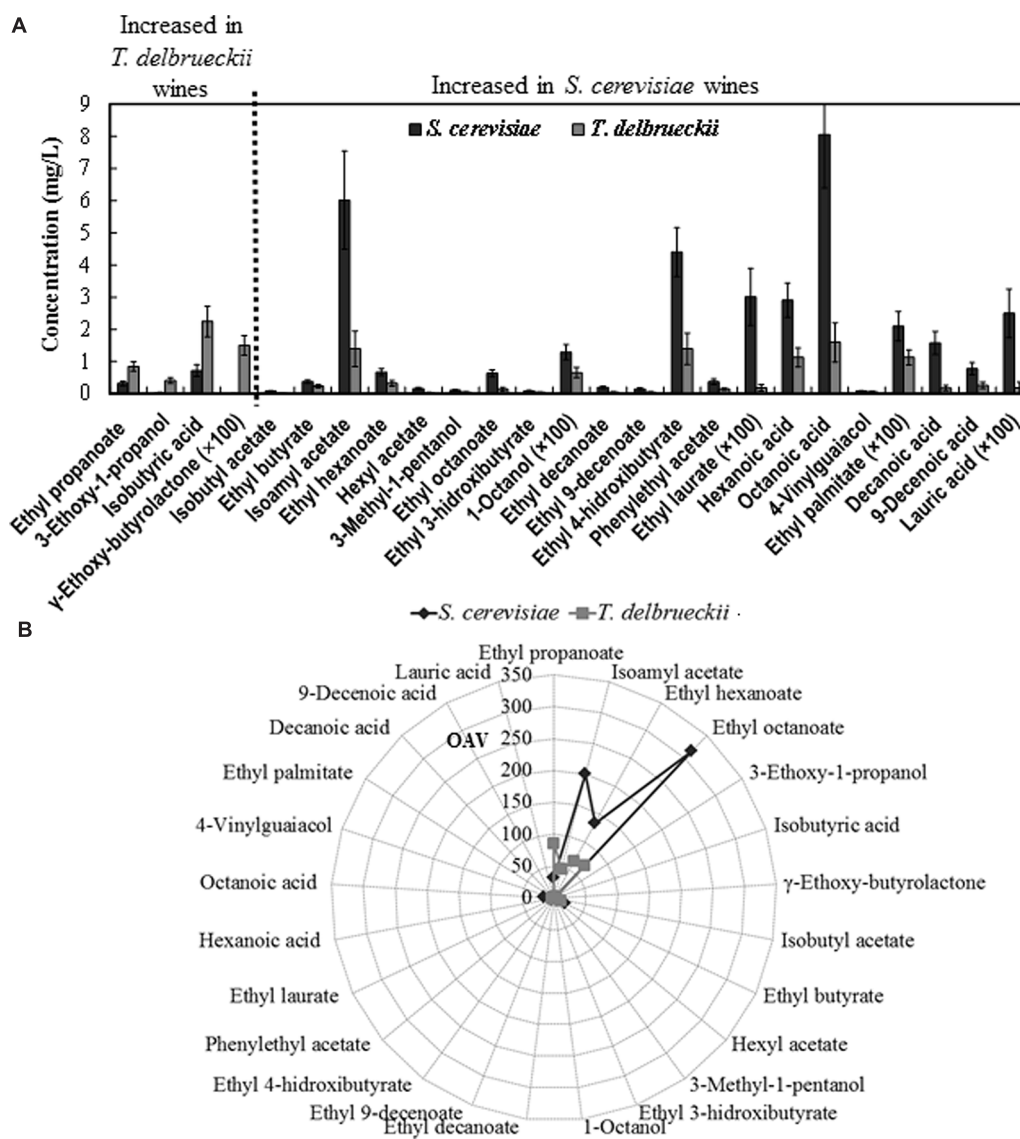


FIGURE 5 | (A) Aromatic compounds from which statistically significant difference ($p < 0.05$) were found between the *S. cerevisiae*-dominated and *T. delbrueckii*-dominated wines. The data are the mean \pm standard error of 23 independent vinifications made in duplicate, 11 inoculated with *S. cerevisiae* and 12 with *T. delbrueckii*. **(B)** Mean values of the odorant activity values (OAV) for the same compounds in *S. cerevisiae*-dominated and *T. delbrueckii*-dominated white wines.

based on molecular polymorphisms or yeast cell morphology. In particular therefore, the results showing that the *T. delbrueckii* Kbarr-1 strain dominated the low-turbidity (<100 NTU) sterile must fermentation when co-inoculated in a 90% initial proportion with 10% of *S. cerevisiae* wine strains are reliable. This initial proportion was much greater than that required for the *S. cerevisiae* killer K2 strain to dominate must fermentation (Pérez et al., 2001), probably because of the faster growth and fermentation rates of *S. cerevisiae* relative to *T. delbrueckii* (Mauricio et al., 1998). Increased must turbidity to values that are frequent in industrial wineries (100–250 NTU) had no relevant inhibitory effect on this *T. delbrueckii* Kbarr-1 domination, and, in particular, much less than the inhibitory

effect that had been found previously using *S. cerevisiae* killer-K2 strains (Pérez et al., 2001). This is probably because the *T. delbrueckii* Kbarr-1 strains had a more intense killer phenotype than the *S. cerevisiae* killer-K2 strains (data not shown), and the proportion of the Kbarr-1 toxin that remained unabsorbed onto the grape particles in the turbid must was active enough to kill the 10% of inoculated *S. cerevisiae* yeast. This *T. delbrueckii* Kbarr-1 domination decreased or disappeared when the initial proportion was reduced to 50%, or when the *T. delbrueckii* strain became non-killer. Thus, although the Kbarr-1 killer toxin kills *S. cerevisiae* and helps *T. delbrueckii* Kbarr-1 yeasts to dominate must fermentation, a high initial proportion of *T. delbrueckii* (90%) is required to overcome the greater growth

rate of *S. cerevisiae* in the environmental conditions of the present study.

Sequential yeast inoculation with *T. delbrueckii* followed by *S. cerevisiae* did not ensure that the *T. delbrueckii* domination would continue beyond the first 2 days of fermentation. Most often the viable *T. delbrueckii* population quickly fell to less than 10% of total viable yeast cells, the aromatic wine profile was similar to those wines which were single-inoculated with *S. cerevisiae*, and the wines were often not fully dry. As has been shown for assimilable nitrogen limitation (Taillandier et al., 2014), the interference of the growths of the two yeasts could make any given yeast nutrient critically scarce, with the result that the *S. cerevisiae* population is unable to complete must fermentation under this limiting situation. Therefore, this sequential inoculation strategy does not seem appropriate for winemaking because it does not guarantee any relevant and reproducible effect of *T. delbrueckii* on wine quality.

Single *T. delbrueckii* inoculation allowed killer strains to dominate fresh-must fermentation (100–75%), but not the non-killer strains. The *T. delbrueckii*-dominated fermentations were rather slow at the end, and the resulting wines usually contained some reducing sugars. This was not a relevant issue, however, because part of this sugar was metabolized to reach wine dryness after 20–30 days of wine maturation (data not shown). The presence of low amounts of viable *S. cerevisiae* ethanol-resistant wild yeasts seems to ensure completion of the fermentation to give dry wines. This could be because there is none of the aforementioned two-yeast-growth interference at this maturation stage since most of the *T. delbrueckii* cells are dead and cannot secrete the required amount of active killer toxin to kill the ethanol-resistant *S. cerevisiae* cells. None of these wines presented malolactic fermentation as long as the musts were thoroughly clarified and their pH was 3.3 or lower. However, the *T. delbrueckii*-dominated wine from the same musts containing more grape particles and pH above 3.5 underwent malolactic fermentation, which is usually undesirable in white table wines. This was probably because of the larger wild bacteria population associated with the solid particles of the turbid must, and because that a pH above 3.5 did not greatly restrict the growth of lactic acid bacteria.

Influence of *T. delbrueckii* Killer Yeasts on the Organoleptic Quality and Aroma Compounds of the Wines

The main fermentation aroma of the *T. delbrueckii*-dominated fermentations and the resulting wines, dried/cooked fruit and pastry/candy, did not appear in the wines from *T. delbrueckii*-non-dominated fermentations, which were very similar to those from *S. cerevisiae*-dominated fermentations, fresh fruit aroma, as usual for young white wines. These results were coherent with the significant differences in the content of 25 aroma compounds found in the two wine types. Most of the compounds with fresh fruit odor descriptors were more abundant in the *S. cerevisiae*-dominated wines, including those with the greatest OAVs: isoamyl acetate, ethyl hexanoate, and ethyl octanoate (Figure 5B). However, no significant differences were found

for the sum of compounds believed to be responsible for a dried/cooked fruit aroma, such as lactones (Hernandez-Orte et al., 2008; Azzolini et al., 2012; Figure 4), although a significantly greater amount of γ -ethoxy-butyrolactone was found in the *T. delbrueckii*-dominated than in the *S. cerevisiae*-dominated wines (Figure 5). However, a greater amount of ethyl 4-hydroxybutanoate (meringue) was detected in the *S. cerevisiae*-dominated wines. While this can potentially be responsible for some pastry odor, no such odor was detected in these wines by the trained judges. An explanation for these apparently contradictory results could be that, in the *S. cerevisiae*-dominated wines, the main compounds which had fresh-fruit-odor descriptors overcame the possibility of detecting the minor compounds which had dried fruit or pastry odor descriptors. On the contrary, the relative low concentrations of fresh-fruit-odor compounds in the *T. delbrueckii*-dominated wines made it possible to detect the dried fruit and pastry odors. Additionally, the slightly sour and evolved/aged flavor detected in the *T. delbrueckii*-dominated wines but not in the *S. cerevisiae*-dominated wines may have been due to the greater isobutyric acid concentration in the former (Figure 5), and which would be coherent with previous findings (Herraiz et al., 1990).

Overall, our results are partially in agreement with those previously reported for the influence of *T. delbrueckii* on the wine quality and aroma compound concentrations. The appearance of dried fruit/coconut aromas associated with the increase in some lactones and the decrease in some ethyl and acetate esters has also been observed in *T. delbrueckii* wine from synthetic white must (Hernandez-Orte et al., 2008). Similarly, the decrease in isoamyl acetate and ethyl esters of C₄–C₁₀ fatty acids has also been noted in *T. delbrueckii* dry white wine from Soave and Chardonnay grape musts, as well as in sweet “Vino Santo” wine from dried Nosiola grapes (Azzolini et al., 2015), although increases in lactones were found only in this last case. Also similarly to our results, that work’s *T. delbrueckii* dry wine had significantly lower freshness and acidity but higher flavor intensity, complexity, and persistence than the *S. cerevisiae* wines. The increased amount of lactones in the “Vino Santo” dessert wine was assumed to improve its organoleptic quality, although this point was not confirmed (Azzolini et al., 2015). Most esters were also found at much lower concentrations in *T. delbrueckii* than in *S. cerevisiae* Sauvignon Blanc dry wines (Renault et al., 2015), although some “minor” esters were considered as produced preferentially by *T. delbrueckii*, in particular ethyl propanoate (in agreement with our findings), ethyl isobutanoate, and ethyl dihydrocinnamate. Additionally, but contrary to our findings, isobutyl acetate and isoamyl acetate concentrations were systematically greater with mixed *T. delbrueckii*/*S. cerevisiae* inoculation although this increase did not correlate with the growth of either species, suggesting that this ester concentration enhancement was due to *S. cerevisiae* production in response to the presence of *T. delbrueckii* (Renault et al., 2015). This increase in isoamyl acetate (banana note) related to *T. delbrueckii* inoculation is rather unexpected given that the contrary has been reported several times (Comitini et al., 2011; Azzolini et al., 2012; Sadoudi et al., 2012), and there has also been a report of greater hydrolytic activity of isoamyl acetate

(via esterase) with *T. delbrueckii* than with *S. cerevisiae* (Plata et al., 2003). In contrast, the increase in ethyl propanoate, ethyl isobutanoate, and ethyl dihydrocinnamate is in agreement with previous findings (Herraiz et al., 1990; Moreno et al., 1991; Plata et al., 2003; Hernandez-Orte et al., 2008; Renault et al., 2009; Sadoudi et al., 2012) and with this present work for the case of ethyl propanoate. One can find additional apparent disagreements in the literature for the relative amounts of other compounds produced by *T. delbrueckii* relative to *S. cerevisiae*, but those compounds are not thought to be as relevant for wine aroma as the aforementioned esters and lactones. Although these disagreements could be due to the different yeast strains inoculated in the winemaking (Renault et al., 2009), we did not find any significant differences among the *T. delbrueckii* strains used in this present study (data not shown). Therefore, we think that other vinification parameters are responsible for the disagreements, especially the degree of dominance of the inoculated yeasts because the *S. cerevisiae*-dominated wines had similar aroma profiles independently of whether or not they had previously been inoculated and partially fermented with *T. delbrueckii*. Only the wines from those vinifications inoculated and clearly dominated by *T. delbrueckii* had a differentiated aroma profile. We cannot evaluate the possible influence of the occurrence of malolactic fermentation on the *T. delbrueckii* wine because this aspect has as yet to be taken into account in any depth in previous studies.

In sum, it seems that *T. delbrueckii* has some common effects on wine quality and aroma composition independently of the winemaking condition as long as it is the most relevant yeast species during fermentation. These effects are reduction of the main ester concentrations, increase of some minor ethyl esters and lactone concentrations, and reduction of fresh fruit aromas. However, this yeast can lead to the production of some interesting wine aromas depending on the must type, the yeast inoculation procedure, the degree of the inoculated yeast's dominance, yeast strain, etc. This variable behavior may determine the wine quality score given by the judges in the sensory evaluation. Therefore, further research on this topic is required to determine the best procedure for the use of *T. delbrueckii* at winery level in order to ensure the expected effect of this yeast on commercial wines' complexity.

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Notwithstanding this finding of variability in the *T. delbrueckii* wine aroma composition, a clear conclusion that can be drawn from this work is that the new *T. delbrueckii* killer strains had the additional advantage of dominating must fermentation in the presence of *S. cerevisiae* relative to the non-killer strains. They significantly decreased the amounts of the main ethyl and acetate ester compounds responsible for a fresh fruit wine aroma, while increasing some minor ethyl ester and lactone compounds that may be responsible for an improved wine complexity. These killer yeasts can be easily and reliably monitored during must fermentation by the incorporated *cyh^R* genetic marker, cell/spore morphology, or molecular polymorphism analyses. Also, they were able to complete the must fermentation of white wines with less than 11.5% ethanol when single inoculated in low-turbidity low-pH must without favoring the growth of lactic acid bacteria.

AUTHOR CONTRIBUTIONS

MR conceived the project. MR, RV, MÁ, and EZ designed and performed the experiments. MR, RV, EZ, and LH analyzed the data. MR wrote and edited the manuscript.

ACKNOWLEDGMENTS

This work was funded by grants GR10088 from the Extremadura Regional Government and AGL2011-25711 from the Spanish Ministry of Education and Science. RV was the recipient of a studentship from the Extremadura Regional Government. We are grateful for the technical and human support provided by the SAIUEX Facility of Elemental and Molecular Analysis (financed by UEX, Junta de Extremadura, MICINN, FEDER, and FSE) for the analyses of the wine aroma compounds.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fmicb.2015.01222>

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Comparison of Fermentation and Wines Produced by Inoculation of *Hanseniaspora vineae* and *Saccharomyces cerevisiae*

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OPEN ACCESS

Edited by:

Sandra Torriani,
Università degli Studi di Verona, Italy

Reviewed by:

Matthias Sipiczki,
University of Debrecen, Hungary
Patrizia Romano,
University of Basilicata, Italy

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Specialty section:

This article was submitted to
Food Microbiology,
a section of the journal
Frontiers in Microbiology

Received: 02 January 2016

Accepted: 03 March 2016

Published: 16 March 2016

Citation:

Lleixà J, Martín V, Portillo MC, Carrau F, Beltran G and Mas A (2016)
Comparison of Fermentation and Wines Produced by Inoculation of *Hanseniaspora vineae* and *Saccharomyces cerevisiae*.
Front. Microbiol. 7:338.
doi: 10.3389/fmicb.2016.00338

Interest in the use of non-*Saccharomyces* yeasts in winemaking has been increasing due to their positive contributions to wine quality. The non-*Saccharomyces* yeast *Hanseniaspora vineae* is an apiculate yeast that has been associated with the production of wine with good aromatic properties. However, little is known about the fermentation dynamics of *H. vineae* in natural must and its interaction with autochthonous yeasts. In the present study, we performed semi industrial fermentations of Macabeo and Merlot musts inoculated with either *H. vineae* or *S. cerevisiae*. The yeast population dynamics were monitored by plate culturing, PCR-DGGE and massive sequencing techniques. The results obtained with these techniques show that *H. vineae* was able to dominate the autochthonous microbiota in Macabeo must but not in Merlot must, which exhibited a larger, more diverse yeast population. The presence of *H. vineae* throughout most of the Macabeo fermentation resulted in more fruity and flowery wine, as indicated by the chemical analysis of the final wines, which demonstrated a strong presence of phenyl ethyl acetate at concentrations higher than the threshold of perception and approximately 50 times more than that produced in wines fermented with *S. cerevisiae*. This compound is associated with fruity, floral and honey aromas.

Keywords: non-*Saccharomyces*, *Hanseniaspora*, alcoholic fermentation, PCR-DGGE, massive sequencing

INTRODUCTION

Wine is the result of the alcoholic fermentation of grape must. Alcoholic fermentation is driven by yeasts, and it consists of the transformation of sugars present in the must, glucose and fructose, into ethanol and carbon dioxide. The yeast species *Saccharomyces cerevisiae* is considered to be primarily responsible for this process. *S. cerevisiae* is known for only metabolizing sugars via the fermentative pathway when the sugar concentration is high, even in the presence of oxygen. This phenomenon is known as the Cabtree effect (Cabtree, 1929).

Winemaking is currently changing because of an emerging interest in the use of non-*Saccharomyces* yeasts during alcoholic fermentation to increase wine complexity and differentiation. Non-*Saccharomyces* yeasts are commonly found on the grape surfaces, and these

yeasts have been associated with spontaneous and unpredictable fermentation, which can result in arrested or sluggish fermentation and wine spoilage. Nevertheless, several recent studies have shown that these yeasts positively affect wine fermentation and the final wine. The positive role of non-*Saccharomyces* ranges from a better fermentation performance to improve wine quality and complexity (Fleet, 2008; Jolly et al., 2014).

Non-*Saccharomyces* yeasts can contribute to the sensorial profile of wine as a result of the production of various metabolites and the activity of certain enzymes that interact with the precursors of aromatic compounds, such as β -glucosidases, which are present in many non-*Saccharomyces* yeast but not in *S. cerevisiae*. β -glucosidases hydrolyze aromatic glycosylated precursors into free volatile compounds to improve the final wine flavor (Swangkeaw et al., 2011; Jolly et al., 2014). Many other enzymes of technological relevance are also secreted by non-*Saccharomyces* yeasts, such as pectinases. Enzymes with proteolytic activity are of key interest in enological fields because they facilitate the clarification process in wine and improve protein stability (Strauss et al., 2001; Maturano et al., 2012).

These yeasts have garnered interest in winemaking due to their beneficial effects and because consumers are demanding new wine styles. Many commercial yeast companies have also begun to promote mixed and sequential wine fermentations in order to satisfy consumer and producer demands. Therefore, companies have begun to thoroughly study and commercialize non-*Saccharomyces* strains, like *Torulaspora delbrueckii* or *Metschnikowia pulcherrima* (Jolly et al., 2014). Moreover, some of the yeast species that are being evaluated belong to *Hanseniaspora* spp., the main non-*Saccharomyces* yeasts in grape must that are considered apiculate yeasts due their cell morphology. Specifically, the yeast *Hanseniaspora vineae* (anamorph *Kloeckera africana*) of this genus has been of great interest because it produces several key aromatic compounds (Viana et al., 2011; Medina et al., 2013).

The strain of *H. vineae* used in this study was isolated from Uruguayan vineyards and selected due to its positive effect on wine fermentation and good contribution to the aroma profile of the final wine. *H. vineae* has been demonstrated to increase fruity aromas and produce a high amount of acetate esters, such as 2-phenylethyl acetate and ethyl acetate, in wines elaborated by sequential fermentation with *S. cerevisiae* (Viana et al., 2011; Medina et al., 2013).

In summary, the use of non-*Saccharomyces* yeasts to produce new wine styles has been increasing due to the different aromatic profiles obtained. The aim of this work was to compare the fermentation dynamics of *H. vineae* and *S. cerevisiae* and the different obtained wines after the inoculation of these two species. To this end, we used natural must from two grape varieties, Macabeo and Merlot, inoculated either with *H. vineae* or *S. cerevisiae* fermented in semi-industrial conditions. The yeast population dynamics were monitored by plate culturing, PCR-DGGE and 18S rRNA gene massive sequencing techniques. To confirm the differences between the two species, the final wines underwent a sensory evaluation, and the aromatic profile was determined.

MATERIALS AND METHODS

Yeast Strains

The commercial wine yeast strain used in this study was *Saccharomyces cerevisiae* QA23 (Lallemand®). The apiculate yeast strain used in this work, *H. vineae* T02/5AF, was isolated from Uruguayan vineyards. Strain QA23 of *S. cerevisiae* was obtained in active dry yeast (ADY) form and rehydrated according to the manufacturer's instructions (Lallemand®). The *H. vineae* strain T02/5AF was obtained in fresh paste form and rehydrated in the same manner as QA23 using warm water. The inoculation was in both cases 2×10^6 cells/ml of must.

Fermentation Conditions

The Macabeo and Merlot grape varieties were fermented at the experimental cellar of the Faculty of Enology (Mas dels Frares, Tarragona Spain). The Macabeo musts were fermented in triplicate in 100 l tanks at 18°C, and 6 kg of Merlot grapes were fermented in 8 l submerged cap fermentation tanks at 26°C. The Macabeo must was submitted to a vacuum filtration process, whereas the Merlot grapes were selectively handpicked in the vineyard.

Fermentation activity was followed by daily density monitoring using a portable densimeter (Mettler Toledo). Samples were taken once a day from each fermenter and studied as described in the following sections.

Cell Growth Measurements

Samples were taken once a day, diluted in sterile MilliQ water (Millipore Q-POD™ Advantage A10), plated on YPD medium (Glucose 2%, Peptone 2%, Yeast Extract 1%, Agar 1.7%) and lysine agar medium (Oxoid, England) plates using an automated spiral platter WASP II (Don Whitley. Scientific Limited, England), and incubated at 28°C for 48 h. The YPD medium provided the total yeast counts, whereas the lysine agar medium only provided the non-*Saccharomyces* cell counts because *S. cerevisiae* cannot grow using lysine as a unique nitrogen source. Appropriate dilution plates were counted, and 20 colonies from the must before the inoculation and the beginning (density 1070 for Macabeo and 1090 for Merlot, both of them at day 1), middle (density between 1050 and 1040) and end (density below 1000, and residual sugars below 5 g/l) of the fermentation were randomly selected and purified on YPD plates for yeast identification.

Yeast Identification

The yeasts were identified based on the RFLPs of the PCR-amplified ITS-5,8S rDNA region from the isolated colonies as described by Esteve-Zarzoso et al. (1999). The RFLP patterns of the yeast isolates were compared with those of the www.yeast-id.org (<https://www.yeast-id.org/>) based on the method described by Esteve-Zarzoso et al. (1999) and grouped to a known yeast species. Yeast identification was confirmed by sequencing the amplified D1/D2 domain of the 26S rDNA of representative colonies of each identified group as described by Kurtzman and Robnett (1998) and comparing this sequence with those of the type strains included in GenBank®. Identification

was considered appropriate with similarities higher than 99%. The sequencing was performed by Macrogen.

Saccharomyces cerevisiae cells from the isolated colonies identified as *S. cerevisiae* were further characterized by Interdelta PCR analysis as described by Legras and Karst (2003).

Massive Sequencing Analysis

DNA (5–100 ng) was extracted from 1 ml samples taken at the beginning, middle and end of the fermentation using the recommended procedure for the DNeasy Plant Mini kit (Qiagen, Hilden, Germany), including three bead-beating steps for 3 min in a FastPrep-24 bead beater (MP Bio, Solon, OH) to homogenize the samples. The extracted DNA was stored at -20°C until further processing. A 350 bp (on average) 18S rRNA gene fragment was amplified in triplicate from each DNA sample with the universal primers FR1 (5'-ANCCATTCAATCGGTANT-3') and FF390 (5'-CGATAACGAACGAGACCT-3') (Chemidlin Prévost-Bouré et al., 2011). All primers had an Ion Torrent tag, and the universal primer included a 10-bp barcode unique to each amplified sample. The PCR reactions contained 5–100 ng DNA template, 1× GoTaq Green Master Mix (Promega), 1 mM MgCl_2 , and 2 pmol of each primer. The reaction conditions consisted of 94°C for 3 min, followed by 35 cycles of 1 min at 94°C , 1 min at 52°C and 1 min at 72°C , and a final extension phase for 10 min at 72°C . The PCR products were pooled by sample and cleaned using a GeneRead Size Selection kit (Qiagen, Hilden, Germany). The cleaned PCR products were submitted to the Centre for Omic Sciences (Reus, Spain), where their quality was assessed with a Bionalyzer and their quantity was adjusted for sequencing. The raw sequences were demultiplexed and quality filtered using QIIME v1.8.0 (Caporaso et al., 2010a). Reads were discarded if the average quality score of the read was <25 , if the length of the read was <200 or >400 and they contained one or more ambiguous base calls. Operational taxonomic units (OTUs) were assigned using QIIME's uclust-based (Edgar, 2010) open-reference OTU-picking workflow with a threshold of 97% pairwise identity. The OTU sequences were aligned using PYNAST (Caporaso et al., 2010b) against the SILVA 119 reference database (Pruesse et al., 2007). Taxonomic assignments were made in QIIME against the SILVA 119 database using the naive Bayesian classifier rdp (Wang et al., 2007). The template alignment of the Greengenes core set filtered at 97% similarity. The OTU taxonomy was determined using the RDP classifier retrained toward the GreenGenes bacterial 16S rRNA database (13_8 release) (DeSantis et al., 2006) at 97% similarity. Chimeric sequences were identified and removed using ChimeraSlayer (Haas et al., 2011), and a phylogenetic tree was generated from the filtered alignment using FastTree (Price et al., 2009). A final OTU table was created that excluded excluding unaligned sequences and singletons (sequences observed just once). To avoid biases generated by differences in sequencing depth, the OTU table was rarified to an even depth of 20,000 sequences per sample in comparisons of all sample types in this study.

PCR-DGGE

The U1GC/U2 primers were used to amplify the specific U1/U2 domain of the 28S ribosomal region of yeast (Meroth et al.,

2003). The PCR amplifications were performed on a Gene Amp PCR System 2700 (Applied Biosystems, Fosters City, USA) using EcoTaq DNA Polymerase (Ecogen, Spain). The Dcode universal mutation detection system (Bio-Rad, Hercules, Calif.) was used to run the DGGE analysis. The amplification of the fragments and denaturing electrophoresis were performed according to Meroth et al. (2003). The bands were excised from the gels, and the DNA was eluted overnight in 40 μl of 10 mM Tris pH 8 and 1 mM EDTA (TE) at 4°C . The DNA was re-amplified with the same pair of primers without the GC-clamp and sequenced by Macrogen. The BLASTN algorithm was applied to the GenBank database to identify sequences (<http://www.ncbi.nlm.nih.gov/BLAST/>). We considered appropriate the identification of the sequences with the corresponding type strains sequences when the sequence identity was higher than 98%.

Analysis of Volatile Compounds

The aromatic compounds were extracted using adsorption and separate elution from an isolate ENV+ cartridge packed with 1 g of highly crosslinked styrene-divinyl benzene (SDVB) polymer (40–140 mm, cod. no. 915- 0100-C), as previously reported by Boido et al. (2003). The cartridges were sequentially equilibrated with methanol (15 mL) and distilled water (20 mL). A sample of 50 mL of wine diluted with 50 mL of distilled water and containing 0.1 mL of internal standard (1-heptanol at 230 mg/L in a 50% hydroalcoholic solution) was applied with an appropriate syringe (4–5 mL/min), and the residue was washed with 15 mL of distilled water. The aromatic compounds were eluted with 30 mL of dichloromethane. The solution was dried with Na_2SO_4 , concentrated to 1.5 mL on a Vigreux column, stored at 10°C , and, immediately prior to GC-MS analysis, further concentrated to 150 μL under a gentle nitrogen stream. The GC/MS analyses were conducted using a Shimadzu-QP 2010 ULTRA (Tokyo, Japan) mass spectrometer equipped with a Stabilwax (30 m \times 0.25 mm i.d., 0.25 μm film thickness) (Restek) capillary column. The components of the wine aromatic compounds were identified comparing their linear retention indices with those of pure standards. (Aldrich, Milwaukee, 194 WI). The mass spectral fragmentation patterns were also compared with those stored in databases. GC-FID and GC-MS instrumental procedures using an internal standard (1-heptanol) were applied for quantitative purposes, as described previously by Boido et al. (2003). Ethanol and residual sugars were quantified using Winescan FT 120 (WineScan FT120 Type 77110, Foss Analytical, Denmark).

Sensory Analysis

A specialized panel (13 panelists) analyzed the sensorial attributes of Macabeo and Merlot wines fermented with *H. vineae* and *S. cerevisiae*. The wines were analyzed by means of a triangle test and descriptive analysis. The aim of the triangle test was to distinguish the wine fermented with *H. vineae* from the wine fermented with *S. cerevisiae*. The descriptive test emphasized the aroma and flavor attributes: Reduction, fresh fruit, candied fruit, flowery, aromatic plant, yeast, toasted (phenolic), herbaceous, aroma, sourness, structure, bitterness, volume and global impression.

Statistical Analysis

The variance the aromatic compounds was analyzed using the Statistica 7.1 software (StatSoft, Tulsa, OK, 1984–2005). The sensory analysis results were submitted to Student's *t*-test. The results were considered significant when the associated *p*-value was below 0.05.

RESULTS

H. vineae and *S. cerevisiae* Fermentations

The changes in the density and yeast populations during the alcoholic fermentations of both Macabeo and Merlot grapes are presented in **Figure 1**. The Macabeo must (**Figure 1A**) inoculated with *H. vineae* required a longer fermentation process (19 days) than those inoculated with *S. cerevisiae* (14 days) due to slower fermentation kinetics and a longer latency phase. However, Merlot grapes (**Figure 1B**) inoculated with *H. vineae* and with *S. cerevisiae* showed a similar fermentative progress, completing the fermentation in 9 days. This fact could be explained by the early presence of non-inoculated *S. cerevisiae* in the first stages of the fermentation.

No significant differences were observed in the ethanol concentration obtained at the end of the fermentation of both varieties (10.75 ± 0.20 for Macabeo and 12.75 ± 0.10 for Merlot wines). Although all the wines were considered as “dry” (sugar concentration below 2 g residual sugars /L), a small difference was observed in the residual sugars in the Macabeo fermentation because the musts fermented with *H. vineae* left 1.7 ± 0.3 g fructose/L, while all the other wines each of the residual sugars (glucose or fructose) were below 1 g/L.

The yeast population was quantified based on the colony growth on YPD and lysine agar medium. The total yeast population (YPD) was similar for the Macabeo and Merlot fermentations. The non-*Saccharomyces* yeasts counts (lysine agar) were slightly lower than the total yeast population counts in tanks inoculated with *H. vineae* for both grape musts. The Macabeo must was submitted to a vacuum filtration, which reduced the initial yeast population and resulted in yeast counts of 8.8×10^4 cfu/ml on YPD and 5.8×10^4 on lysine agar in must before inoculation.

Yeast Biodiversity in Merlot and Macabeo Musts

We identified only three yeast species in Macabeo must (**Figures 2A,B**), with *Candida zemplinina* being the main yeast species representing more than 80% of the yeast population. The other two yeast species identified were *Hanseniaspora uvarum* and *Torulaspora delbrueckii*. Of these, *H. uvarum* represented 12.50% of the total yeast population, whereas *T. delbrueckii* represented only 3.13% of the population. This distribution significantly differed in the yeast population recovered from Merlot must (**Figures 2C,D**). We identified up to eleven yeast species, with *C. zemplinina* and *H. uvarum* being the main species representing a percentage of 41 and 39% of the total yeast population, respectively. The low yeast diversity in Macabeo must may be due to the prefermentative filtration protocol, which reduces the yeast population. Moreover, during Merlot

fermentation the must maintains contact with grape skins, which releases yeasts during the whole process. In both musts, only non-*Saccharomyces* yeasts were detected.

Yeast Population Dynamics during Fermentation

The yeast population dynamics during fermentation after the inoculation of *S. cerevisiae* or *H. vineae* was followed in yeast colonies grown on YPD plates based on PCR-DGGE, and the massive sequencing of the 18S rRNA gene at the beginning, middle and end of the fermentation.

For the Macabeo fermentation, the inoculated strain accounted for 80–90% of the yeast population recovered from the plates 1 and 2 days after inoculation with *H. vineae*, whereas *C. zemplinina* had completely disappeared by the second day. *S. cerevisiae* was present at the beginning of fermentation, but at a very low proportion (**Figure 2A**). Nevertheless, the *S. cerevisiae* population began to increase from the middle to the end of the fermentation, accounting for 60% of the population at the middle of the fermentation and 100% at the end of the fermentation. From this population, up to three different *S. cerevisiae* strains could be identified by interdelta analysis, although QA23 was the most abundant at the end of the fermentation (more than 90%, results not shown). Cross contamination between cellar vats is common in commercial cellars during vintage. Nevertheless, *H. vineae* represented 40% of the yeast population at the middle of the fermentation, which demonstrated the capacity of this yeast to dominate the native microbiota, and a high proportion of its population remained active after the middle of the fermentation. However, this yeast was not able to overcome the initial microbiota in Merlot fermentations inoculated with *H. vineae* (**Figure 2C**) and exhibited very low recovery on plates at the beginning and middle of the fermentation (≈ 5 –7%). Other non-*Saccharomyces* yeasts (*H. uvarum*, *C. zemplinina*, and *Pichia occidentalis*) outgrew *H. vineae* at these stages. The predominant yeast throughout the fermentation was a non-inoculated *S. cerevisiae*, which was recovered from plates and represented 50% of the total population at the beginning of the fermentation.

Macabeo and Merlot fermentations inoculated with *S. cerevisiae* showed similar yeast population patterns (**Figures 2B,D**). In both cases, the inoculated *S. cerevisiae* was able to rapidly dominate the fermentation because it was the only cultivable yeast recovered throughout the fermentation.

The PCR-DGGE profiles obtained for the DNA extracted directly from the wine during alcoholic fermentation identified the detected yeasts as *S. cerevisiae*, *H. uvarum*, *H. vineae*, and *C. zemplinina* (**Table 1**). *S. cerevisiae* was detected in all fermentations after the first day of inoculation, including in fermentations not inoculated with the commercial *S. cerevisiae*. Nevertheless, the *S. cerevisiae* in these latter fermentations appeared to be a different strain, as evidenced different migration patterns on DGGE gels. *S. cerevisiae* QA23 shows a particularity in PCR-DGGE because it produces a double band, which is not observed in other *S. cerevisiae* strains. All the bands excised from the gel migrating to the same height resulted in at least 99.9% sequence similarity to *S. cerevisiae* type strain. Merlot

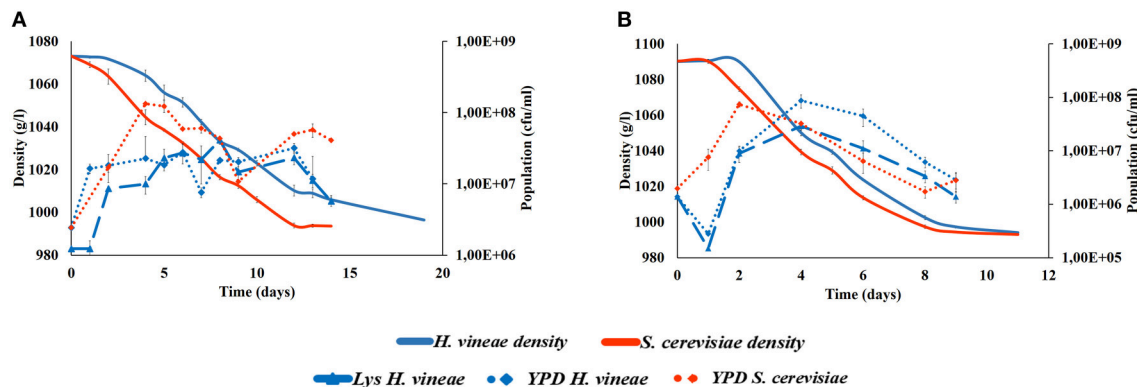


FIGURE 1 | Density measures of fermentations kinetics (—) and yeast population (cfu/ml) growth in YPD (◆) and lysine agar (▲) medium of Macabeo (A) and Merlot (B) tanks inoculated with *H. vineae* (Blue) or *S. cerevisiae* (Red).

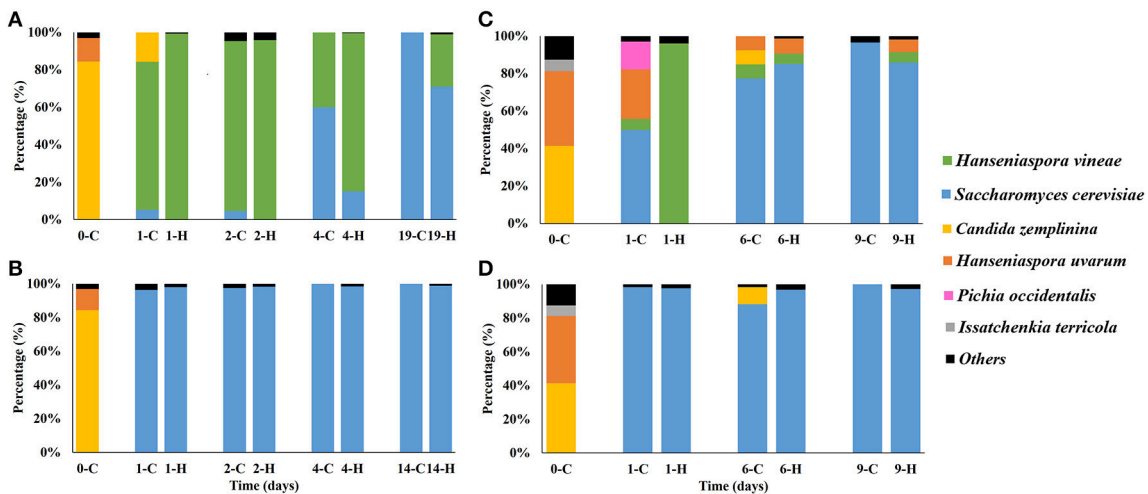


FIGURE 2 | Comparison of the percentage of yeast species grown in YPD medium and the main genera detected by HTS along the fermentation from Macabeo tanks inoculated with *H. vineae* (A) or *S. cerevisiae* (B) and Merlot tanks inoculated with *H. vineae* (C) or *S. cerevisiae* (D). The letters C (Culture) and H (HTS) correspond to the different techniques used. The results included as *others* refer to the yeast species that represent less than 5% of the total yeast population.

and Macabeo musts inoculated with *H. vineae* exhibited more yeast diversity at the beginning of the fermentations than musts inoculated with *S. cerevisiae*, and *H. vineae* was detected until the end of these fermentations.

A high-throughput sequencing (HTS) approach was also used to assess the fermented wine yeast biodiversity. After the removal of low quality sequences and those failing alignment, 642,105 18S rRNA amplicon sequences were generated from 9 Macabeo and 6 Merlot wine samples. The average number of sequences per sample was 42,807, with an average length of 299 bp, and these sequences clustered into 16,302 operational taxonomic units (OTUs; 97% nucleotide identity). To avoid diversity overestimation, singletons (sequences observed only once) were eliminated, and each sample was rarefied to an even depth of 20,000 sequences to avoid biases generated by differences in sequencing depth. The number of different

OTUs was then reduced to 634, and 34 genera were identified. Good's coverage index was 99.7% on average, indicating that the global yeast diversity was mostly covered. The numbers of observed OTUs did not differ between Macabeo or Merlot wine samples inoculated with *S. cerevisiae* or *H. vineae* (Figure 3A). However, the number of genera was significantly higher at the beginning of the Merlot fermentation and tended to decrease toward the end of the fermentation, whereas the number of genera in Macabeo fermentation samples was lower than that in Merlot fermentations and relatively constant throughout the fermentation (Figure 3B). Most of the yeast population in all fermentations (97.7% on average) was represented by the inoculated *S. cerevisiae* and *H. vineae* strains (Tables 1, 2), whereas other non-*Saccharomyces*, such as *H. uvarum* and *Zygosaccharomyces*, accounted for only 1.9% of the sequences, and the remaining genera represented less than 0.5% of the

TABLE 1 | The most abundant yeast genera detected by each technique in the different fermentations.

Yeast species	Macabeo with <i>H. vineae</i>				Macabeo with <i>S. cerevisiae</i>				Merlot with <i>H. vineae</i>				Merlot with <i>S. cerevisiae</i>			
	Day 1	Day 2	Day 4	Day 19	Day 1	Day 2	Day 4	Day 14	Day 1	Day 6	Day 9	Day 1	Day 6	Day 9	Day 1	Day 6
YPD Culture (%)	<i>S. cerevisiae</i>	5,26	<	60,00	100,00	96,30	97,50	100,00	50,00	77,36	96,61	98,33	88,14	100,00		
	<i>H. vineae</i>	78,95	90,70	40,00	—	—	—	—	5,88	7,55	<	—	<	—		
	<i>H. uvarum</i>	—	—	—	—	—	—	—	26,47	7,55	—	<	—	—		
	<i>C. zemplinina</i>	15,79	—	—	—	<	—	—	—	7,55	<	—	10,17	—		
	<i>P. occidentalis</i>	—	—	—	—	—	—	—	14,71	—	—	—	—	—		
HTS (%)	<i>S. cerevisiae</i>	<	<	15,20	70,91	97,97	98,23	98,40	<	85,25	85,93	97,67	96,78	97,20		
	<i>H. vineae</i>	99,28	95,81	84,39	28,00	<	<	<	96,09	5,51	5,53	<	<	<		
	<i>H. uvarum</i>	<	<	<	<	<	<	<	<	7,94	6,77	<	<	<		
	<i>Candida</i>	<	<	<	<	<	<	<	<	<	<	<	<	<		
	<i>S. cerevisiae</i>	—	+	+	++	+	++	++	+	++	++	++	++	++		
DGGE-PCR	<i>H. vineae</i>	++	++	+	+	—	—	—	++	+	+	—	—	—		
	<i>H. uvarum</i>	—	—	—	—	—	—	—	+	+	+	—	—	—		
	<i>C. zemplinina</i>	+	+	+	+	+	++	++	—	—	—	—	—	—		

The YPD-Culture and HTS results are expressed as percentages of the selected yeast specie from the total yeast population. The symbol "<" indicates that the correspondent yeast specie is present but represents less than 5% of the total yeast population. The symbol "—" indicates that the correspondent yeast specie was not detected with this technique. In case of DGGE-PCR, "++" indicates that the band is very strong, "+" indicates that the band is weak and "—" indicates that the band is non-existent.

sequences (Table 2). Some of the detected fungi were not related to alcoholic fermentation (p.e. *Aerobasidium*, *Aspergillus*, *Sporobolomyces*); however, they were mainly detected at the beginning of the fermentation, and their populations quickly decreased or disappeared (Table 2). Interestingly, *Dekkera* was only detected in Merlot samples, and we were able to observe a small but distinct increase during the fermentations with both inocula.

Volatile Compound Composition

Fifty volatile compounds produced during alcoholic fermentations of natural Macabeo musts inoculated with *H. vineae* and *S. cerevisiae* were identified and quantified in the Macabeo wines. These compounds were classified into 10 groups, (acetates, acids, alcohols, C6 compounds, carbonyl compounds, esters, phenols, lactones, unusual compounds (named here as “rars”) and terpenes). Table 3 shows the mean concentration of the identified volatile compounds. To assess the possible contribution of the different components to the wine aroma, the detection threshold and aroma descriptor reported in the literature are included for each compound.

Significant differences between yeasts were only observed in three of the 10 groups of compounds (Acetates and rars in Figure 4A and alcohols in Figure 4B).

Both yeasts primarily produced alcohols and esters, and three (isobutanol, isoamyl alcohol, and phenyl ethanol) of the eleven identified alcohols reached the threshold of perception reported in the literature. Of these three alcohols, phenyl ethanol provides good aromas that are described as rose and honey-like. Among the identified esters, ethyl hexanoate reached the threshold of perception and contributes a green apple aroma. The compounds constituting the next most abundant group produced by *H. vineae* are classified as rare and included N-acetyl tyramine and 1H-indole-3-ethanol acetate ester. These compounds were not found in the wine fermented with *S. cerevisiae*.

As shown in Table 3, a total of 7 acids were identified; hexanoic, decanoic and octanoic acid showed the highest concentration, and octanoic acid exceed the odor threshold reported in the literature.

Four acetates were identified, and phenethyl acetate was the most interesting. Specifically, this compound was 50 times more abundant in wines fermented with *H. vineae* than in those fermented by *S. cerevisiae*. This compound endows wine with floral, fruity and honey-like aromas.

Six phenolic compounds were identified, as shown in Table 3. These compounds did not reach the threshold of detection, and their contribution to wine aroma is consequently expected to be insignificant. One of these compounds, 4-ethylguaicol, is generally attributed to the presence of *Brettanomyces*, although it was identified in wines fermented with *H. vineae*.

Six terpenes were identified, as shown in Table 3. The concentrations of these compounds were lower than the threshold, and they are consequently not expected to contribute to the wine flavor profiles.

Sensory Analysis

To evaluate the ability of *H. vineae* to produce a wine with attributes that differ from those of a wine fermented with *S.*

TABLE 2 | Percentage of main genera and species detected by HTS after the inoculation of *H. vineae* or *S. cerevisiae* on Merlot and Macabeo wines.

Days from inoculation	Macabeo <i>H. vineae</i>					Macabeo <i>S. cerevisiae</i>				Merlot <i>H. vineae</i>			Merlot <i>S. cerevisiae</i>		
	1	2	4	14	19	1	2	4	14	1	6	8	1	6	8
<i>Saccharomyces cerevisiae</i>	0.47	3.68	15.20	60.32	70.91	97.97	98.23	98.40	98.87	0.44	85.25	85.93	97.67	96.78	97.20
<i>Hanseniaspora vineae</i>	99.28	95.81	84.39	38.75	28.00	0.64	0.48	0.57	0.24	96.09	5.51	5.53	0.53	1.69	1.60
<i>Hanseniaspora uvarum</i>	0.07	0.07	0.01	<	0.01	0.05	0.02	0.01	<	0.33	7.94	6.77	0.24	0.40	0.15
<i>Zygosaccharomyces</i>	0.01	0.05	0.23	0.47	0.60	0.67	0.66	0.52	0.39	0.41	0.49	0.49	0.53	0.51	0.47
<i>Saccharomyces (others)</i>	0.01	0.03	0.08	0.31	0.39	0.55	0.54	0.43	0.44	<	0.33	0.36	0.36	0.37	0.36
<i>Aureobasidium</i>	<	<	—	0.01	—	—	—	—	—	1.83	0.03	0.02	0.33	0.05	0.05
<i>Candida</i>	0.09	0.14	0.03	0.06	0.07	0.11	0.07	0.06	0.05	0.31	0.18	0.46	0.19	0.18	0.12
<i>Pichia</i>	0.05	0.17	0.05	<	0.01	—	—	<	—	0.03	0.26	0.26	0.01	0.01	—
<i>Dekkera</i>	—	—	—	—	—	—	—	—	—	—	<	0.17	—	—	0.03
<i>Aspergillus</i>	—	0.01	—	—	—	—	—	—	—	0.11	<	0.01	0.03	<	—
<i>Sporobolomyces</i>	—	—	—	—	—	—	—	—	—	0.08	<	—	0.02	—	—
<i>Issatchenkia</i>	0.01	0.02	0.01	0.01	0.01	0.01	—	<	—	0.02	0.01	—	0.01	—	—
<i>Cryptococcus</i>	—	<	—	<	—	—	—	—	—	0.07	—	—	0.01	—	—
<i>Diplodia</i>	—	—	—	—	—	—	—	—	—	0.07	—	—	<	—	—
<i>Zygoascus</i>	—	—	—	—	—	<	—	—	—	0.03	—	—	0.02	0.01	0.01
<i>Rhizina</i>	—	—	—	—	—	—	—	—	—	0.04	—	—	0.01	—	—
<i>Catenulostroma</i>	—	—	—	—	—	—	—	—	—	0.05	—	—	—	—	—
<i>Bensingtonia</i>	—	—	—	0.04	—	—	—	—	—	<	—	—	—	—	—
<i>Saccharomycodes</i>	—	—	<	0.02	0.01	—	—	—	<	—	—	—	—	—	0.01
<i>Scheffersomyces</i>	—	—	—	—	—	—	—	—	—	0.02	<	<	0.01	—	—
<i>Wickerhamomyces</i>	0.01	0.01	<	—	—	—	—	—	—	0.01	—	—	—	—	—
<i>Cladosporium</i>	—	—	—	—	—	—	—	—	—	0.01	—	—	0.01	—	—
<i>Sugiyamaella</i>	0.01	0.01	<	—	—	—	—	—	—	—	—	—	—	—	—
<i>Trigonopsis</i>	0.01	0.01	—	—	<	—	—	—	—	—	—	—	—	—	—
<i>Lipomyces</i>	—	—	—	—	—	—	—	—	—	—	0.01	0.01	—	—	—
<i>Phillipsia</i>	—	—	—	—	—	—	—	—	—	0.01	—	—	—	—	—
<i>Wallemia</i>	0.01	—	—	—	—	<	—	—	—	<	—	—	—	—	—
<i>Vanderwaltozyma</i>	—	—	—	—	—	—	—	—	<	—	<	—	—	—	<
<i>Cochliobolus</i>	—	—	—	—	—	—	—	—	—	0.01	—	—	—	—	—
<i>Malassezia</i>	—	—	—	—	—	<	—	—	—	<	—	<	—	—	—
<i>Bispora</i>	—	—	—	—	—	—	—	—	—	<	—	—	<	—	—
<i>Rhodotorula</i>	—	—	—	—	—	—	—	—	—	<	—	—	<	—	—
<i>Metschnikowia</i>	—	—	—	—	—	—	—	—	—	<	<	—	—	—	—
<i>Phoma</i>	—	—	—	—	—	—	—	—	—	<	—	<	—	—	—
<i>Agaricostilbum</i>	—	—	—	—	—	—	—	—	—	<	—	<	—	—	—
<i>Baudoinia</i>	—	—	—	—	—	—	—	—	—	<	—	—	—	—	—

The symbol "<" indicates percentages values lower than 0.01 and bigger than 0. The symbol "—" indicates not detected by HTS.

cerevisiae, the produced wines were analyzed with triangle and descriptive tests.

In the triangle test of Macabeo wine (**Figure 5**), wine-tasters easily distinguished the wine fermented with *H. vineae* from that fermented with *S. cerevisiae*, and the majority selected the wine fermented with *H. vineae* as their preference. In the descriptive test, the wine fermented with *H. vineae* received the best rating. Notably, wine fermented by *H. vineae* showed a significantly stronger flowery aroma profile ($p = 0.037$) than wine produced with *S. cerevisiae*.

The Merlot must could not be evaluated as a consequence of a powerful reduction note that could not be corrected for the tasting.

DISCUSSION

In this work, we performed semi-industrial fermentations with *H. vineae* and *S. cerevisiae* using Macabeo and Merlot musts in order to evaluate the differences in yeast populations during fermentation and the wines produced. We followed the yeast population dynamics in both grape musts inoculated with *H. vineae* and *S. cerevisiae* by plate culturing on YPD medium, PCR-DGGE with yeast general primers, as well as yeast general primers and the HTS of 18S rRNA gene.

As expected, the fermentation of musts inoculated with *H. vineae* required more time than fermentations inoculated with *S. cerevisiae*, demonstrating the high fermentative ability of this

TABLE 3 | Average concentrations of the two fermentations (\pm Standard Deviation) in $\mu\text{g/l}$.

	<i>H. vineae</i> Average SD	<i>S. cerevisiae</i> Average SD	Odor descriptor	Odor threshold ($\mu\text{g/l}$)
ACETATES				
Isobutyl acetate	11 \pm 1	0 \pm 0*	N/A	N/A
Isoamyl acetate	222 \pm 20	218 \pm 93	Banana ^a	30
1,3-Propanediol, diacetate	99 \pm 18	160 \pm 7	N/A	N/A
Phenethyl acetate	2322 \pm 50	47 \pm 13**	Fruity, honeyed, floral ^a	250
Acetate sum	2653 \pm 89	425 \pm 100**		
ACIDS				
Isobutyric acid	74 \pm 40	0 \pm 0	Acid, fatty ^b	230
Heptanoic acid	231 \pm 28	304 \pm 35*	N/A	N/A
Hexanoic acid	330 \pm 35	777 \pm 70*	Fatty, cheese ^a	420
Octanoic acid	734 \pm 12	1757 \pm 335	Fatty ^a	500
Decanoic acid	979 \pm 31	389 \pm 212	Rancid, fat ^a	1000
9-Hexadecenoic acid	479 \pm 11	72 \pm 57	N/A	N/A
Acids sum	2825 \pm 48	3299 \pm 708		
ALCOHOLS				
Isobutyl alcohol	2388 \pm 277	1895 \pm 165	Fusel oil, chemical ^b	0,5
1-Butanol	58 \pm 9	84 \pm 38	Like wine, medicine ^a	150.000
Isoamyl alcohol	36361 \pm 4127	61355 \pm 5063*	Alcoholic, fruity at low concentration ^b	0,3
3-Methyl-1-pentanol	36 \pm 1	69 \pm 7	Like wine, nail polish ^a	40.000
3-Ethoxy-1-propanol	28 \pm 0	108 \pm 12	Fruity ^b	
Furfuryl alcohol	12 \pm 2	0 \pm 0	N/A	N/A
3-(Methylthio), 1-Propanol	321 \pm 35	599 \pm 281	Sweet, potato ^a	1000
Benzyl alcohol	37 \pm 7	0 \pm 0	Floral, rose, phenolic, balsamic ^a	200.000
Phenyl ethanol	8099 \pm 158	16830 \pm 957	Rose, honey ^a	10000
Tyrosol	1855 \pm 156	5274 \pm 3149	N/A	N/A
Tryptophol	1365 \pm 95	0 \pm 0**	N/A	N/A
Alcohols sum	50557 \pm 4276	86214 \pm 897*		
C6 COMPOUNDS				
1-Hexanol	386 \pm 7	328 \pm 50	Grass just cut ^a	2500
Trans 3-Hexen-1-ol	7 \pm 1	129 \pm 19	Green ^a	1000
Cis 3-Hexen-1-ol	120 \pm 1	0 \pm 0**	Green, kiwi ^a	400
C6 compounds sum	513 \pm 9	457 \pm 31		
CARBONYL COMPOUNDS				
Acetoin	15 \pm 13	56 \pm 59	Creamy, butter, fat ^b	0,15
Furfural	9 \pm 2	0 \pm 0	Fusel alcohol, toasted bread ^a	770
Carbonyl compounds sum	23 \pm 16	56 \pm 59		
ESTERS				
Methyl butyrate	9 \pm 4	14 \pm 7	N/A	N/A
Ethyl butyrate	62 \pm 15	158 \pm 38	N/A	N/A
Ethyl hexanoate	81 \pm 4	241 \pm 24	Green apple ^a	14
Ethyl lactate	8285 \pm 378	3071 \pm 1915	Strawberry, raspberry ^a	60.000
Ethyl octanoate	79 \pm 33	225 \pm 9	Sweet, banana, pineapple ^a	500
Ethyl 3-hydroxybutyrate	119 \pm 8	52 \pm 16	N/A	N/A
Ethyl decanoate	143 \pm 46	76 \pm 6	Sweet, hazelnut oil ^a	200
Ethyl succinate	1240 \pm 47	1775 \pm 836	Toffee, coffee ^a	1.000.000
Diethyl malate	88 \pm 6	428 \pm 165	Green ^a	760.000
Diethyl 2 hydroxy glutarate	233 \pm 6	268 \pm 67	Grape, green apple, marshmallow ^a	20.000
Diethyl succinate	4012 \pm 255	15671 \pm 6792	Overripe melon, lavender ^a	100000
Ester sum	14348 \pm 509	21979 \pm 9334		

(Continued)

TABLE 3 | Continued

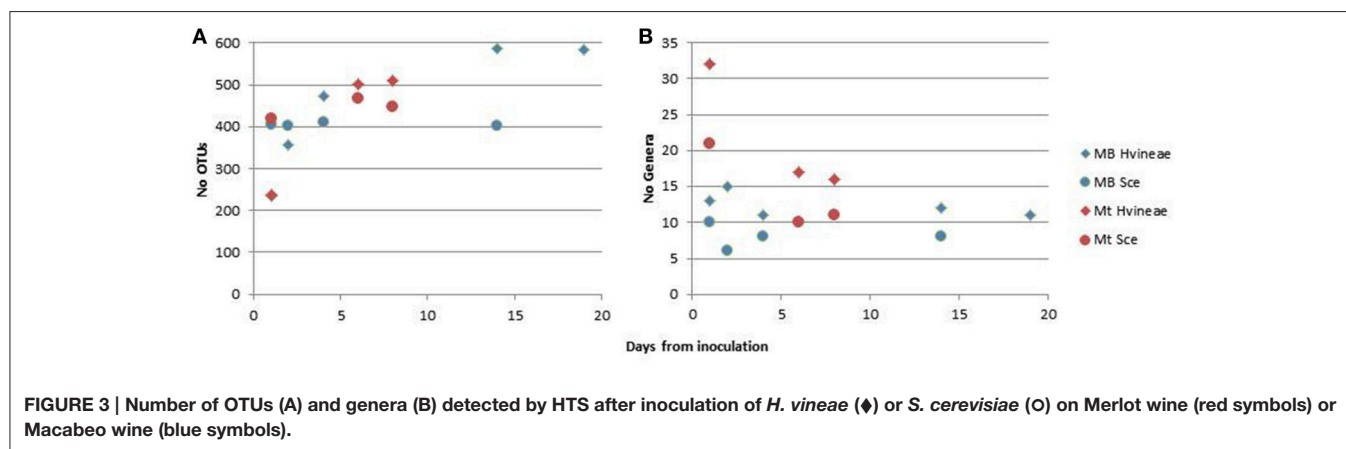
	<i>H. vineae</i> Average SD	<i>S. cerevisiae</i> Average SD	Odor descriptor	Odor threshold ($\mu\text{g/l}$)
PHENOLS				
Guaiacol	6 \pm 1	0 \pm 0	Smoky, hospital ^a	9,5
4-ethylguaiacol	73 \pm 66	0 \pm 0	Bretty flavors ^a	110
4-vinylguaiacol	33 \pm 21	28 \pm 14	Clove, curry ^a	40
Phenyl lactate	53 \pm 10	128 \pm 32	N/A	N/A
Ethyl vanillate	5 \pm 0	17 \pm 20	N/A	N/A
Acetovainillone	14 \pm 5	15 \pm 13	N/A	N/A
Phenol sum	183 \pm 41	188 \pm 11		
LACTONES				
Butyrolactone	223 \pm 1	251 \pm 6	Toasted burned ^a	1000
5-carboethoxy-gamma-butyrolactone	127 \pm 7	76 \pm 11	N/A	N/A
Lactone sum	350 \pm 8	327 \pm 17		
RARES				
N-acetyl tyramine	2040 \pm 11	0 \pm 0**	N/A	N/A
1H-Indole-3-ethanol, acetate (ester)	1377 \pm 8	0 \pm 0**	N/A	N/A
Rare sum	3417 \pm 4	0 \pm 0***		
TERPENES				
Linalool	12 \pm 2	28 \pm 13	Rose ^a	50
Alpha-terpineol	112 \pm 31	0 \pm 0	Floral, pine ^a	400
Citronellol	27 \pm 6	39 \pm 5	Sweet, floral ^b	18
Terpene sum	150 \pm 23	67 \pm 18		

Odor descriptor and odor thresholds reported in the literature are included.

*, **, *** indicate significance at $p < 0.05$, $p < 0.01$, $p < 0.001$ respectively.

^aFariña et al. (2015).

^bBoido (2002).

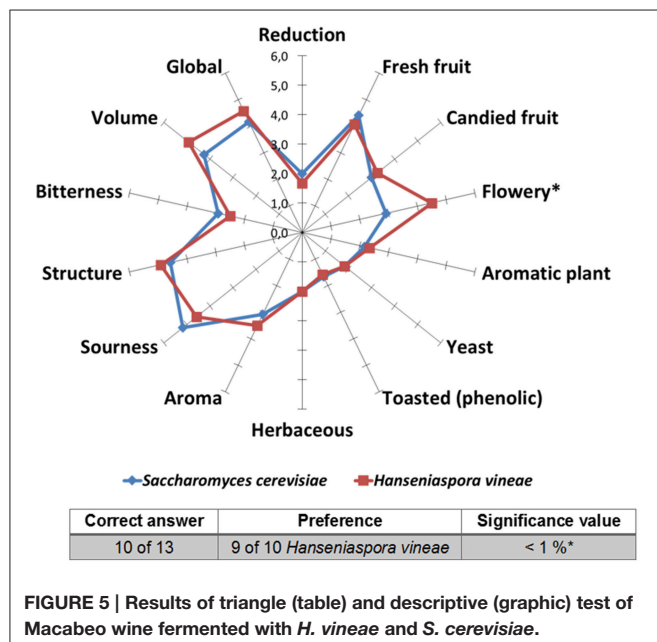
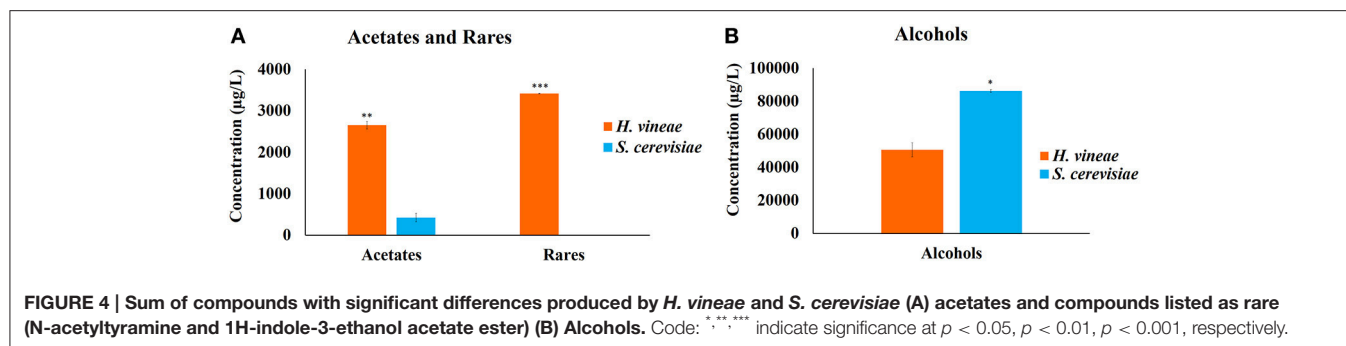


wine yeast with respect to *H. vineae*. However, rapid fermentation might not be a desired characteristic in the production of quality wines, due to flavor lost or high energetic demand for refrigeration (Medina et al., 2013).

The initial yeast diversity of the must was only analyzed after plate culturing. Before the inoculation, Merlot must presented a higher yeast diversity than Macabeo must, as evidenced by up to eleven different yeast species that were identified in the red variety, whereas the Macabeo contained only three species (*C.*

zemplanina, *H. uvarum*, and *T. delbrueckii*). As expected, only non-*Saccharomyces* yeasts were recovered by cultivation from both musts before the inoculation because *S. cerevisiae* is not present in relevant amounts in grapes and is mostly associated with cellar equipment (Pretorius, 2000; Torija et al., 2001; Beltran et al., 2002).

The PCR-DGGE analysis identified *S. cerevisiae* and *H. vineae* as the main yeasts in both the Macabeo and Merlot fermentations. *C. zemplanina* was found only in Merlot, and these



results corroborated those observed after the plate culture. Other minor yeast species were not detected by PCR-DGGE, especially if their population densities were below 10^3 – 10^4 CFU/ml or if their abundance was two orders of magnitude lower than that of the main species, as reported in previous studies (Mills et al., 2002; Prakitchaiwattana et al., 2004; Andorrà et al., 2008).

Even if must samples were not included in the HTS approach, this technique clearly detected higher levels of fungal diversity than the other techniques. Specifically, a total of 32 genera with a great diversity of OTUs were identified within each genus. The HTS technique was also able to detect yeast genera not related with fermentation, and some of these yeasts are associated with spoilage (like *Dekkera/Brettanomyces*). Although the proportion of these yeasts was very low, the changes in their proportion throughout the fermentation suggested that they were active and represented a potential risk for the spoilage of the final wine. Thus, the HTS technique confirmed the general trend obtained for the most abundant yeast populations by plate culturing and PCR-DGGE, but it also facilitated the detection and tracking of some minor yeast genera that may significantly impact the quality of the wine.

The culturing, PCR-DGGE and HTS analysis confirmed a decrease in the yeast genera diversity from the beginning to the end of fermentations, and these techniques also consistently indicated that the yeast diversity was higher in Merlot fermentations than in Macabeo fermentations. The low diversity exhibited by Macabeo must before inoculation may be a consequence of its treatment with a vacuum filter. The objective of this treatment was to clean the must and remove solid and colloidal particles, but it also reduced autochthonous yeasts and nutrients in the must. We used this protocol for two reasons: to clean the Macabeo must and to remove colloidal and solid particles and also it was affected by rain and exhibited some spoilage. Thus, we wanted to reduce the autochthonous yeast population because we planned to inoculate the must with *H. vineae*. We achieved these objectives. Furthermore, the Merlot was selectively handpicked in order to obtain the healthiest bunches of grapes. The results from plate culturing, PCR-DGGE and HTS indicate that *H. vineae* was able to overcome the autochthonous microbiota in the Macabeo must, constituting a high proportion of the yeast population until the middle of the fermentation and showing good fermentative capacity. However, *H. vineae* represented a very low proportion of the yeast population in Merlot must after the inoculation. However, after the inoculation (day 1), the percentages of the identified yeasts were different based on the method of estimation, being the population of *H. vineae* hardly recovered on plates. *S. cerevisiae* was the most abundant yeast recovered from plates, whereas it was present at much lower levels in all culture-independent methods (HTS and DGGE). This observation could be related to the well-reported interaction between *S. cerevisiae* and non-*Saccharomyces* yeasts during wine fermentation: non-*Saccharomyces* yeasts are quickly displaced by *S. cerevisiae*, which might kill or at least result in viable but not cultivable (VBNC) statuses, as indicated in several recent reports (Millet and Lonvaud-Funel, 2000; Pérez-Nevado et al., 2006; Andorrà et al., 2010, 2011; Wang et al., 2015). However, we should emphasize that these culture-independent techniques also detect DNA from dead cells, which could also be the case. At later fermentation time points, all methods again produced coincident results and identified *S. cerevisiae* as the main population. Interestingly, the dominant *S. cerevisiae* was not the inoculated strain, suggesting that a cellar-resident strain took over. Furthermore, Merlot grapes are among the latest in the harvest in this cellar, and, thus, the environmental contamination of the cellar is already

high. The *S. cerevisiae* population began to increase and became the dominant species according to HTS and produced the most intense band profile of DGGE, and this unique yeast was recovered at the end of the fermentation.

The final wine obtained by fermenting Macabeo must with *H. vineae* was preferred over the wine fermented with *S. cerevisiae* for its notable fruity and flowery aroma. This result corroborates those of studies that performed mixed fermentations with *H. vineae* and obtained high amounts of an acetate ester, phenethyl acetate, which is responsible of the fruity and flowery aroma of wine (Viana et al., 2009, 2011). The chemical analysis revealed that wines inoculated with *H. vineae* contained 50 times more phenethyl acetate than wines inoculated with *S. cerevisiae*, which explains the results of our sensory analysis and agrees with previous observations (Medina et al., 2013).

The production of N-acetyltyramine and 1H-indole-3ethanol acetate ester also differed. These compounds were abundant in wines inoculated with *H. vineae* and could not be detected in wines fermented with *S. cerevisiae*. These compounds could be derived from tyrosol, and this hypothesis is supported by the high concentrations of tyrosol in wines inoculated with *S. cerevisiae*. This difference could be explained by the production of unusual compounds from tyrosol in wines inoculated with *H. vineae*. However, aromatic descriptors associated with these compounds have not yet been reported.

CONCLUSION

Interest in the use of non-*Saccharomyces* yeasts in winemaking has been increasing. *H. vineae* is an apiculate non-*Saccharomyces* yeast that has demonstrated a good fermentative rate in Macabeo must and resulted in more flowery wines, which is likely related to the higher production of phenylethyl acetate. However, the need for inoculation with *S. cerevisiae* must be emphasized because

H. vineae is unable to finish the alcoholic fermentation. We did not use a *S. cerevisiae* strain in the inoculations with *H. vineae*, and the end of fermentation was consequently improperly controlled. Furthermore, the use of this yeast requires very healthy grape musts and is not recommended to use with grapes with a high and diverse yeast population or red musts, in which maceration with skins may be a significant source of yeast. In addition, the present study shows that the HTS technique detected not only the most abundant yeast populations obtained by plate culturing and PCR-DGGE but also some minor yeast genera that may significantly affect the quality of the wine.

AUTHOR CONTRIBUTIONS

JL: Performing the experiments, Design of experiments, Writing of the manuscript, Discussion of results, Performing Next Generation Sequencing, Analysis of results. VM: Performing the experiments, Design of experiments, Analysis of results, Discussion of results, Writing of the manuscript. MP: Performing Next Generation Sequencing, Analysis of results, Writing of the manuscript. FC: Design of experiments, Analysis of results, Discussion of results. GB: Design of experiments, Analysis of results, Discussion of results. AM: Design of experiments, Discussion of results, Analysis of results, Writing of the manuscript.

ACKNOWLEDGMENTS

MP is the recipient of a Beatriu de Pinós Postdoctoral Fellowship with the support of the Ministry of Universities and Research of the Department of Economy and Knowledge of the Generalitat of Catalonia and the program COFUND Marie Curie Actions of the 7th Framework Program for R+D of EU.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Non-conventional Yeast Species for Lowering Ethanol Content of Wines

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OPEN ACCESS

Edited by:

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Specialty section:

This article was submitted to
Food Microbiology,
a section of the journal
Frontiers in Microbiology

Received: 25 February 2016

Accepted: 18 April 2016

Published: 04 May 2016

Citation:

Ciani M, Morales P, Comitini F,
Tronchoni J, Canonico L, Curiel JA,
Oro L, Rodrigues AJ and Gonzalez R
(2016) Non-conventional Yeast
Species for Lowering Ethanol Content
of Wines. *Front. Microbiol.* 7:642.
doi: 10.3389/fmicb.2016.00642

Rising sugar content in grape must, and the concomitant increase in alcohol levels in wine, are some of the main challenges affecting the winemaking industry nowadays. Among the several alternative solutions currently under study, the use of non-conventional yeasts during fermentation holds good promise for contributing to relieve this problem. Non-*Saccharomyces* wine yeast species comprise a high number or species, so encompassing a wider physiological diversity than *Saccharomyces cerevisiae*. Indeed, the current oenological interest of these microorganisms was initially triggered by their potential positive contribution to the sensorial complexity of quality wines, through the production of aroma and other sensory-active compounds. This diversity also involves ethanol yield on sugar, one of the most invariant metabolic traits of *S. cerevisiae*. This review gathers recent research on non-*Saccharomyces* yeasts, aiming to produce wines with lower alcohol content than those from pure *Saccharomyces* starters. Critical aspects discussed include the selection of suitable yeast strains (considering there is a noticeable intra-species diversity for ethanol yield, as shown for other fermentation traits), identification of key environmental parameters influencing ethanol yields (including the use of controlled oxygenation conditions), and managing mixed fermentations, by either the sequential or simultaneous inoculation of *S. cerevisiae* and non-*Saccharomyces* starter cultures. The feasibility, at the industrial level, of using non-*Saccharomyces* yeasts for reducing alcohol levels in wine will require an improved understanding of the metabolism of these alternative yeast species, as well as of the interactions between different yeast starters during the fermentation of grape must.

Keywords: non-*Saccharomyces* yeasts, low alcohol wine, ethanol yield, yeast respiration, mixed starters

INDUSTRIAL AND SOCIAL INTEREST IN REDUCING ALCOHOL LEVELS IN WINE

The ethanol content in wine increased considerably over the past 20 years due to two main factors: the impact of climate change upon the global production of grapes, and the current quest for new wine styles, often requiring increased grape maturity (Jones et al., 2005; Grant, 2010; MacAvoy, 2010; Alston et al., 2011; Gonzalez et al., 2013). Late harvests are indeed required to meet present consumer's preferences toward well-structured, full body wines, and optimal phenolic maturity of grapes. This practice results in a noticeable increase in the sugar content of the berries (Mira de Orduña, 2010) with consequent higher alcohol levels in wine. On the other hand, global

climate change has deeply influenced the vine phenology and the grape composition, resulting in grapes with lower acidity, altered phenolic maturation and tannin content, and increasing sugar concentration (Jones et al., 2005). These changes further contribute to rising alcohol content in wines, in addition to modifying other wine sensory attributes as well as wine microbiology (Mira de Orduña, 2010). Alston et al. (2015) reported that the ethanol content in New World wines was higher than in European wines (13.65 vs. 13.01% v/v). The ethanol contents found in North American, Argentinean, Australian, and Chilean wines were 13.88, 13.79, 13.75, and 13.71% v/v, respectively. In Europe, Spain accounted the highest values (13.43% v/v). The high ethanol content in wine can lead to stuck and sluggish fermentations (Coulter et al., 2008) and to unbalanced wines that are unpleasant for consumers. Indeed, several studies reported that high ethanol concentration increase hotness and bitterness perceptions, while it decreases acidity sensations and masks the perception of some important aroma compounds such as higher alcohols, esters and monoterpenes (Escudero et al., 2007; Robinson et al., 2009; Fischer, 2010; Frost et al., 2015). This trend brings about some troubles for the wine industry, as well as social and public safety problems related to alcohol consumption (Grant, 2010; MacAvoy, 2010). In order to overcome these issues, the market focus is directed to wines with a moderate alcohol content. In addition, lowering ethanol content has an economic interest due to the high taxes imposed in some countries (Gil et al., 2013).

Pickering (2000) and Saliba et al. (2013) reported that wines with reduced ethanol content have been classified as dealcoholized or alcohol free (<0.5% v/v), low alcohol (0.5–1.2% v/v), reduced alcohol (1.2 to 5.5–6.5% v/v) and lower alcohol wine (5.5–10.5% v/v), even if these classifications, which are loosely based on labeling and legislative requirements, vary between different countries (Pickering, 2000). However, most winemakers are interested in developing practices aiming to reduce the alcohol concentration in wine by just 1–3% v/v, in order to compensate the impact of global warming and to obtain better-balanced wines (Meillon et al., 2010a,b; Gambuti et al., 2011). The winemaking industry is addressing this challenge by targeting almost all the different steps of the production cycle (Teissedre, 2013), starting from grapevine clonal selection, vineyard management, pre-fermentation and winemaking practices, microbiological approaches and post-fermentation and processing technologies (García-Martín et al., 2010; Gil et al., 2013; Poni, 2014; Varela et al., 2015).

In this regard, the viticultural practices to reduce ethanol content in wine act to manage grapes sugar content through different approaches such as reducing leaf area (defoliation or topping of shoots; Martínez de Toda et al., 2013; Poni, 2014), pre-harvest irrigation to cause a significant delay of ripening (Mendez-Costabel, 2007), application of growth regulators to postpone ripening (Symons et al., 2006) and manage harvest date (Bindon et al., 2013). At pre-fermentative stage the reduction of sugar concentration in must could be achieved by dilution of grape must with water (depending of country regulation) or using nanofiltration technologies (Harbertson et al., 2009; García-Martín et al., 2010). Another pre-fermentative strategy to remove

sugar from must could be the addition of glucose oxidase enzyme (Pickering, 2000). The ethanol reduction in wine could be also achieved at post-fermentation stage. In this regard, it is possible to mention the blending of low-high alcohol wines or physical removal of alcohol from wine with membrane-based system, vacuum distillation and supercritical CO₂ extraction (Gambuti et al., 2011; Kontoudakis et al., 2011; Schmidtke et al., 2012).

***S. cerevisiae* IS NOT THE BEST YEAST SPECIES FOR REDUCING ALCOHOL LEVELS IN WINE**

Development and application of yeast strains showing below normal alcohol production has been a recurrent objective for wine biotechnology for more than 20 years, starting even before increasing ethanol content in wines was widely perceived as a problem. Low alcohol production by yeasts might be related with two distinct metabolic features, alcohol tolerance, or ethanol yield on sugar. Traditional scientific literature on wine yeast often use the term fermentative power, to refer to the amount of alcohol produced by different yeast strains from natural or synthetic grape must (Lopes et al., 2006). Due to the assay conditions, this parameter is mainly related to alcohol tolerance, and tells little about the usefulness of yeast strains for alcohol level reduction. Indeed, oenological use of yeast strains having low fermentative power would result in either stuck fermentation or the starter being quickly replaced by native yeasts.

To attain a relevant alcohol level reduction in wine (fermented to dryness), the appropriate yeast metabolic trait to take into account is alcohol yield on sugar. Ethanol yield on sugar is formally expressed as grams of ethanol produced per gram of glucose or fructose consumed (g/g). The rule of thumb says consumption of 17 g/L of sugar will result in an increase of 1% v/v in alcohol content. Not surprisingly, being *Saccharomyces cerevisiae* the main yeast species responsible of alcoholic fermentation during winemaking, it has almost invariably been the species of choice for all research efforts aiming to reduce ethanol yields. However, evolution has shaped this species to quickly and efficiently produce ethanol from sugars under most environmental conditions, following the make-accumulate-consume life strategy (Piskur et al., 2006). Although, some natural variability can be found among wild isolates of this species, the distribution of ethanol yield values is rather narrow (around the values mentioned above).

Researchers have designed several alternative genetic engineering approaches in order to partially redirect *S. cerevisiae* normal carbon flux, starting with the overexpression of *GPD1* or *GPD2*, coding for isozymes of glycerol 3-phosphate dehydrogenase. The choice of *GPD* genes was additionally driven by glycerol contribution to sweetness, smoothness and wine body. Other strategies aiming to reducing alcohol yields also involve genetic manipulation of the central carbon and energy metabolism of *S. cerevisiae*. Target genes include for example *PDC2*, coding for pyruvate decarboxylase; *ADH1*, coding for alcohol dehydrogenase; or *TPI1*, coding for triose phosphate isomerase. An excellent review by Kutyna et al. (2010) gathers

additional genetic engineering strategies in order to reduce alcohol yield during wine fermentation. However, a recent experimental evaluation of genetic modifications to develop low ethanol yield wine yeast strains concluded that overexpression of *GPD1* was the most efficient strategy to lower alcohol yield (Varela et al., 2012). Also in agreement with early studies in this field (Remize et al., 1999; Cambon et al., 2006) they found overexpression of *GPD1* resulted in the overproduction of some metabolites negatively affecting wine quality. In order to avoid these drawbacks additional genetic modifications were required (Cambon et al., 2006; Ehsani et al., 2009). Metabolic pathways mentioned in this paragraph are summarized in **Figure 1**.

Limitations of the genetic engineering approach are twofold. First, commercial use of genetically engineered wine yeast strains does not seem to be feasible in the short term (Gonzalez et al., 2013). In order to circumvent this problem, some researchers are now using adaptive laboratory evolution (Cadière et al., 2011; Kutyna et al., 2012). Second, the increase in concentration required to reach a relevant impact on wine final alcohol content (2–3% v/v), would certainly compromise wine quality for most alternative metabolites. This holds true even for glycerol, one of the preferred targets for researchers in this field. Reduction of 2% v/v ethanol by diverting carbon flux toward glycerol production would result in more than 30 g/L extra glycerol (about five times the usual values). Almost any other chemical compound would also become unacceptable in wine at such elevated concentrations. Carbon dioxide is perhaps the only metabolite that would cause no trouble when overproduced by yeast during wine fermentation, in part because it is readily released to the atmosphere. The two main metabolic pathways for CO₂ production are respiration and fermentation. Concerning alcohol reduction, the advantage of respiration is that no ethanol is produced, since all six carbon atoms from each molecule of sugar end up as CO₂. Some researchers have suggested partial respiration of sugars from grape must as a way to decrease ethanol yield during winemaking (Gonzalez et al., 2013 and references therein). A possible way to reach this goal is shown in **Figure 2**.

There are, however, two restrictions to make yeast cells respire sugars under standard winemaking conditions, oxygen requirement and the Crabtree effect. Respiratory metabolism has a huge oxygen demand, but it is known to participate in many other chemical reactions that can be detrimental to wine quality. Proper management of dissolved oxygen during wine fermentation will be required in order to meet respiration requirements while preserving other wine compounds from excessive oxidation (see below). On the other side, *S. cerevisiae* is the archetype Crabtree-positive yeast species. This metabolic feature strongly favors fermentative over respiratory metabolism, despite oxygen availability (Pronk et al., 1996), and have played a key role in the adaptation of this species to sugar rich environments (Piskur et al., 2006). In *S. cerevisiae* “aerobic fermentation” involves usually above 98% of the sugars consumed in the presence of oxygen (de Deken, 1966). Only under conditions of very low sugar availability (which is not obviously the case for grape must), is respiration

the main energetic metabolic pathway in this species (Pronk et al., 1996). The possibility of reducing ethanol yields by promoting respiration of sugars by *S. cerevisiae* or other yeast species was initially suggested by Smith (1995), and the idea has been independently recovered and developed to different levels in recent years (Erten and Campbell, 2001; Contreras et al., 2015b; Morales et al., 2015; see below).

SUGAR METABOLISM OF NS YEASTS

Common ethanol yields on sugar after complete grape juice fermentation are 90–95% of theoretical, with the remaining 5–10% being explained by biomass biosynthesis, ethanol stripping, and alternative metabolic pathways (Konig et al., 2009). This mainly reflects anaerobic carbon flux distribution in *S. cerevisiae*. However, NS wine yeasts usually differ from *S. cerevisiae* in metabolic flux distribution during fermentation and, consequently, in ethanol production, biomass synthesis, and by-product formation (Ciani et al., 2000; Magyar and Toth, 2011; Milanovic et al., 2012; Tofalo et al., 2012). Under anaerobic conditions, the diversion of alcoholic fermentation and the abundant formation of secondary compounds may in part explain the low ethanol yield of some of these NS yeast species/strains. Indeed, some of these species are strongly characterized by species-specific patterns of fermentation by-products, which allows the differentiation of the majority of these yeast strains according to the species (Domizio et al., 2011).

The production of ethanol and the other main fermentation compounds are metabolically linked. In *S. cerevisiae* glycerol production is highly correlated with the production of acetic acid (Ciani and Rosini, 1995). Indeed, as mentioned above, genetic engineering of *S. cerevisiae* for glycerol overproduction often results a large production of acetic acid (Remize et al., 1999, 2000; Eglinton et al., 2002). The evaluation of the relation between fermentation by products and ethanol production among several NS wine yeasts revealed both direct and inverse correlations between acetic acid and ethanol production, for *Saccharomyces ludwigii* and *Kloeckera apiculata*, respectively (Ciani and Maccarelli, 1998). In contrast, *Torulaspora delbrueckii*, *Candida stellata*, and *Hanseniaspora uvarum* did not show any correlation between the two fermentation products. Ethanol is positively correlated with glycerol and ethyl acetate in *C. stellata* and *K. apiculata* respectively, while an inverse correlation between ethanol and succinic acid production was shown for *T. delbrueckii*.

The most striking metabolic trait of *S. cerevisiae* is perhaps the Crabtree effect. This feature makes *S. cerevisiae* preferentially consume sugars by fermentation in almost any growth condition, apart from carbon limited chemostat operated at low dilution rates. This trait has been often related to glucose triggered transcriptional repression of genes involved in respiratory functions (Barnett and Entian, 2005). However, current understanding of the Crabtree effect points to overflow metabolism at the level of the pyruvate node, as the main mechanism contributing to the observed distribution of carbon

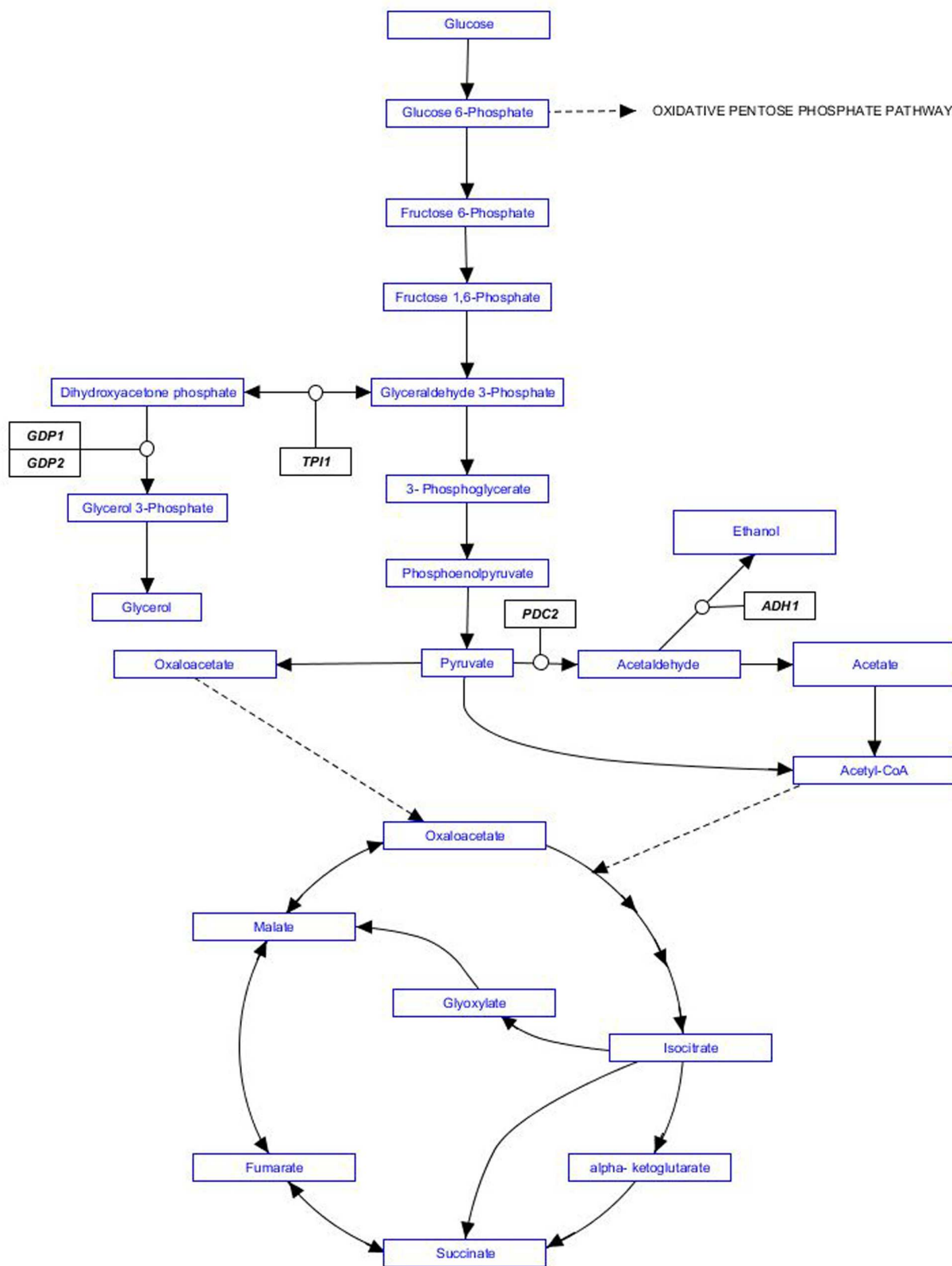
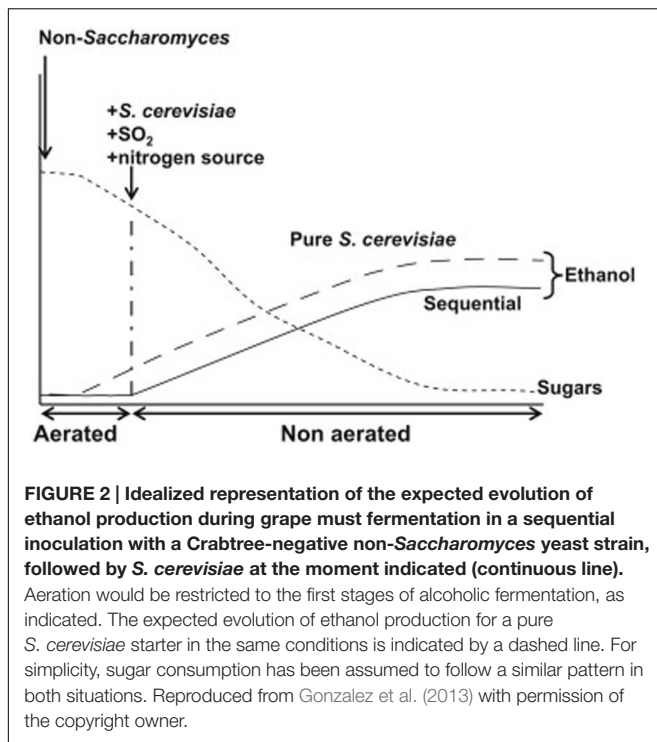


FIGURE 1 | Metabolic pathways involved in ethanol production by *Saccharomyces cerevisiae*. Genes targeted by genetic engineering strategies mentioned in the text are indicated in black boxes.



flux toward ethanol production (Holzer, 1961; Pronk et al., 1996). In addition, Aceituno et al. (2012) found cytoplasm-to-mitochondria NADH transport to be a limiting factor to get a fully aerobic metabolism in the presence of oxygen. Independent of the mechanism, the critical factor determining the respiro-fermentative balance in *S. cerevisiae* seems to be the rate of sugar consumption. Indeed, mutations slowing down the glycolytic rate result in a noticeable relief of the Crabtree effect (Otterstedt et al., 2004; Jansen et al., 2005).

Several classifications of yeast species, according to the way they regulate respiro-fermentative metabolism have been proposed (Gancedo and Serrano, 1989; Alexander and Jeffries, 1990). In general, they are categorized as either Crabtree-positive or Crabtree-negative, or as obligate respiratory. Assessment of the Crabtree status is generally based on studies under carbon limited chemostat conditions (Pronk et al., 1996). So, despite most yeast species found in the oenological environment have shown fermentative capacity (Kurtzman et al., 2011), most of them have never been evaluated for Crabtree status. Furthermore, according to recent studies (Quirós et al., 2014; Contreras et al., 2015b) the classification based on standard Crabtree assays has little prediction power on the behavior of yeasts under growth conditions more closely mimicking those found in wine fermentation. In addition, important differences can be found among yeast strains belonging to the same species.

Analysis of the respiro-fermentative behavior of yeast strains under controlled aeration conditions in high sugar containing media has usually confirmed *S. cerevisiae* as one of the most fermentation-prone yeast species. However, strains from some other species have shown even higher ethanol yield or RQ

values than control *S. cerevisiae* yeast strains under such assay conditions (Quirós et al., 2014; Contreras et al., 2015b). Interestingly, respiratory behavior of yeast strains seems to be strongly affected by other environmental factors, not only sugar abundance or oxygen availability (Rodrigues et al., 2016). The extent to which these environmental factors affect yeast respiro-fermentative metabolism, and secondary by-products like glycerol or acetic acid, is species or strain-specific. Further research is required in order to understand the metabolic diversity of NS yeast species and the relevance of this diversity for oenological applications, including reducing ethanol content of wines.

SELECTION OF NON-*Saccharomyces* WINE YEAST TO REDUCE THE ETHANOL CONTENT

During wine production, the non-*Saccharomyces* (NS) yeasts contribute to the fermentation process, either directly or through their effect on both growth kinetics and metabolic activity of *S. cerevisiae* (Ciani and Comitini, 2015). These NS yeasts are capable of anaerobic or aerobic growth and may persist during the fermentation, competing with *Saccharomyces* for nutrients, producing secondary compounds or modifying the *S. cerevisiae* metabolism (Milanovic et al., 2012; Sadoudi et al., 2012; Barbosa et al., 2015).

NS wine yeasts have been shown to modulate wine fermentation and to enhance sensorial complexity and aroma profile of wines (Fleet, 2008; Ciani et al., 2010). In addition, some of these NS species/strains are able to combat spoilage yeasts (Comitini et al., 2011; Oro et al., 2014; Alonso et al., 2015). Thus, over the last years, the role of NS yeasts in winemaking, previously neglected or demonized, has been re-evaluated, and their use has been proposed in controlled mixed fermentation with the aim to improve wine complexity, aroma profile and control of spoilage microorganisms (Rojas et al., 2001; Swiegers et al., 2005; Domizio et al., 2007; Renouf et al., 2007; Anfang et al., 2009; Comitini et al., 2011; Jolly et al., 2014). In this context, the metabolic traits of NS wine yeasts could also be profitably used to reduce the alcohol content in wine. This application would benefit from a better understanding of the metabolic pathways diverting carbon flux from ethanol production in NS yeasts, as well as the biological variability of these yeast species in terms of ethanol yield. One of these alternative pathways would be sugar respiration under suitable fermentation conditions, especially for Crabtree-negative yeast species, as discussed in other sections of this review. In summary, our current knowledge suggests several promising approaches for the use of NS wine yeast to limit ethanol production. However, taking into account the current interest on NS wine yeasts is mostly related to their impact on wine sensory quality (Lambrechts and Pretorius, 2000; Romano et al., 2003; Ciani et al., 2010; Belda et al., 2015, 2016; Wang et al., 2015; Hu et al., 2016; Masneuf-Pomarede et al., 2016; Medina et al., 2016), a positive contribution to wine aromatic complexity would certainly be a plus in yeast strain selection for this purpose.

Screening Based on Low Ethanol Yield under Anaerobic Fermentation Conditions

Over the recent years, there was a rising interest to investigate on the wine yeast variability in ethanol yield as a potential tool for the reduction of alcohol content in wine. Variability between different yeasts genera and species could be exploited at industrial level to produce wines better fitting consumer preferences. Reduction in ethanol yield is strictly dependent on the microbial strategies that divert sugar-carbon away from ethanol production.

As mentioned above, *S. cerevisiae*, shows high fermentation performance with high ethanol yield and fermentation efficiency, exhibiting a low intraspecies variability for these characters. In contrast, NS wine yeasts show, as a trend, lower ethanol production and lower ethanol resistance. Overall these features are considered to be a major factor of the dominance of *S. cerevisiae* over NS species during wine fermentation. Generally, the species belonging to *Hanseniaspora*, *Candida*, *Pichia*, *Kluyveromyces*, *Metschnikowia*, *Torulaspora*, and *Issatchenkia* genera, widely or occasionally found in grape juice, are not tolerant to ethanol concentrations above 5–7% v/v. Their decline and death as the fermentation progresses can be mostly explained by their low alcohol tolerance, even though recent studies indicate that the interactions with *S. cerevisiae* might be more complex (Arneborg et al., 2005; Branco et al., 2014; Ciani and Comitini, 2015). On the other hand, NS wine yeasts exhibit a broad spectrum of fermentation by-products, low fermentation purity (volatile acidity g/L ÷ ethanol % v/v) and, often, low ethanol yield (Muller-Thurgau, 1896; Ribéreau-Gayon and Peynaud, 1960; Romano et al., 1992). A systematic investigation on fermentation by-products formed by a wide collection of NS wine yeasts, belonging to five different species, was carried out by Ciani and Maccarelli (1998). In that work, “apiculate” yeast species showed a high production of acetaldehyde, ethyl acetate and acetoin; *C. stellata* exhibited high production of glycerol and succinic acid, while *T. delbrueckii* was shown to be a lower producer of secondary products of fermentation. In the various NS species tested, the ethanol production is differently related with the fermentation by-products. NS wine yeasts are generally low-ethanol producing yeasts. However, this feature does not necessarily mean that they exhibit also low ethanol yield.

In this context, only recent studies addressed the interspecies and/or intraspecies variability in ethanol yield among NS wine yeasts (Magyar and Toth, 2011; Contreras et al., 2014, 2015b; Gobbi et al., 2014). In a comparative evaluation of some oenological properties in several wine strains, Magyar and Toth (2011) found a very low ethanol yield for four *Candida zemplinina* strains. Gobbi et al. (2014), investigating on several NS wine yeast species, showed that strains belonging to the species of *H. uvarum*, *Zygosaccharomyces sapae*, *Zygosaccharomyces bailii*, and *Zygosaccharomyces bisporus* exhibited significant low ethanol yield and fermentation efficiency in comparison with *S. cerevisiae* under anaerobic conditions and using different grape juices. For *H. uvarum*, these data confirm the low ethanol yield previously described (Ciani et al., 2006), in contrast to species belonging to the

Zygosaccharomyces genus. Moreover, they found that ethanol yield, like other fermentation features, is a species-related trait. However, as indicated previously for other fermentation parameters (Ciani and Maccarelli, 1998; Comitini et al., 2011; Domizio et al., 2011), a pronounced intraspecies variability was also evident. In another recent work a screening on 50 different NS strains belonging to 24 different genera for their ethanol yield was carried out (Contreras et al., 2014). This led to the identification of four NS yeast strains (two strains of *Metschnikowia pulcherrima* and one strain each of *Schizosaccharomyces malidevorans* and *C. stellata*) that showed low ethanol yield. In a different study, under semi-aerobic condition, nine out of 48 NS strains showed ethanol yields lower than the *S. cerevisiae* control strain. Three of them (*T. delbrueckii* AWRI1152, *Pichia kudriavzevii* AWRI1220, and *Z. bailii* AWRI1578) gave promising results in the subsequent aerobic sequential trials, with *S. cerevisiae* AWRI1631 (Contreras et al., 2015b; see below for further discussion on this work).

Some *Saccharomyces* species, other than *S. cerevisiae*, have also shown potential for ethanol reduction. This is the case for *Saccharomyces uvarum*, a cryophilic species that has been described as a low ethanol and high glycerol producer (Giudici et al., 1995). Fermentation kinetics in must at 13°C is better for *S. uvarum* than for *S. cerevisiae*, but some strains get stuck at 8% v/v alcohol when run at 24°C (Kishimoto et al., 1994; Masneuf-Pomarede et al., 2010). In a sequential inoculation of *S. uvarum* (AWRI 2846) and *S. cerevisiae*, Contreras et al. (2015a) found an ethanol reduction of 0.8% v/v, and an increase of glycerol of 6.4 g/L. The decrease in ethanol production was not fully explained by the increase in glycerol, in terms of carbon mass balance.

Respiration Based Screening

As mentioned above, development of respiration based methods to reduce alcohol content in wine requires the use of NS yeast strains showing no or weak Crabtree effect. However, this metabolic feature, which is indeed rather common across the yeast phylogeny (de Deken, 1966), is not sufficient to warrant the utility of a given yeast species/strain for such purpose. Suitable yeast strains must be able to develop in grape must, a relatively harsh growth medium due to osmotic stress, low pH, and the presence of natural or added inhibitors of microbial growth. In addition sugar consumption kinetics should be relatively fast, in order to be compatible with industrial procedures; as well as being able to dominate fermentation processes, in competition with the microbiota naturally present in grape must. Finally, they must not generate secondary metabolic products that would result in wine spoilage, in either aerobic or anaerobic conditions.

Initial trials to follow the sugar respiration strategy analyzed the behavior of three to four yeast strains in synthetic or natural grape juice under aerobic or microaerobic conditions (Smith, 1995; Barwald and Fischer, 1996; Erten and Campbell, 2001). More recent studies use a higher number of yeast strains (around 60) and milder aeration regimes, than previous studies (Quirós et al., 2014; Contreras et al., 2015b). Quirós et al. (2014) chose respiratory quotient (RQ) as an indicator of the respiration capabilities of each yeast strain. RQ can be calculated as the

ratio of CO₂ produced to O₂ consumed. When hexoses are used as substrate RQ can range from 1 (full respiration) to ∞ (full fermentation). The relationship between RQ and the percentage of sugar consumed by respiration (%SR) can be expressed as follows: %SR = 100/(3RQ-2). They calculated RQ values, in synthetic medium containing 200 g/L sugar, pH 3.5, and high biomass content (OD₆₀₀ = 20), under strongly aerated conditions, and identified strains from several yeast species with RQ values close to 1 under these specific growth conditions. The advantage of RQ over direct calculation of ethanol yields is it is not affected by ethanol stripping. One alternative which is especially valid for mild aeration regimes is comparing ethanol yields with *S. cerevisiae*, in order to identify low yield candidates, and setting control experiments with pure nitrogen gas at the same flow rate, in order to compare ethanol yields between anaerobic and aerobic (or micro-aerobic) conditions (Contreras et al., 2015b; Morales et al., 2015). We must, however, stress that Contreras et al. (2015b) considered their aeration conditions to be not strong enough to trigger respiratory metabolism.

However, low respiratory quotient or low ethanol yields are not enough to ensure the usefulness of yeast strains for the purposes discussed in this review. Indeed, strains showing a strong preference for respiratory metabolism would be completely useless if the amount of sugar they metabolized were negligible (in a reasonable fermentation time). Hence, authors took into account sugar consumption after 3 or 4 days on synthetic grape must in order to identify interesting strains (Quirós et al., 2014; Contreras et al., 2015b).

The other main aspect to be taken into consideration for a proper yeast strain selection in this context is volatile acidity. There are already several reports showing an important increase in acetic acid yield for *S. cerevisiae* under aerated conditions, as compared to anaerobic growth (Giovannelli et al., 1996; Papini et al., 2012; Quirós et al., 2014; Contreras et al., 2015b; Rodrigues et al., 2016). Strains from other yeast species have also been found to produce high amounts of acetic acid under oxygenation (Quirós et al., 2014; Contreras et al., 2015b); and some of them also under standard fermentation conditions (Ciani and Picciotti, 1995; Viana et al., 2008).

MANAGING MIXED FERMENTATIONS

Apart from reducing ethanol yields, the main driver for the current development of NS commercial starters is related to the increasing consumer demand for wines showing improved sensorial properties and distinctive flavor (Pretorius and Hoj, 2005; Belda et al., 2015, 2016; Masneuf-Pomarede et al., 2016; Medina et al., 2016), in contrast to the limited complexity attributed to wines fermented with *S. cerevisiae* starter strains (Heard, 1999; Rojas et al., 2003; Romano et al., 2003; Ciani et al., 2006, 2010; Jolly et al., 2006). However, NS wine yeasts often show low fermentation power. For this reason *S. cerevisiae* starters have to be used to ensure consumption of all sugars from grape must, and to bring the fermentation process to completion. In addition, the interactions between *Saccharomyces* and NS yeasts can be exploited to modulate the content of ethanol in wine (Ciani

and Comitini, 2015; Wang et al., 2015). Temperature, sulphite content, sugar concentration, nitrogen composition, oxygen and pH, which influence glycerol and ethanol biosynthesis, must also be modulated and controlled.

Mixed starters can be used by either simultaneous or sequential inoculation. This later modality allows to take advantage of the metabolism of the first inoculated NS yeast without the influence of the *Saccharomyces* starter culture. In this way, the reduction in ethanol content will depend on the metabolic characteristics of the NS strain used, and on the actual opportunity it will have to stamp its metabolic footprint before *S. cerevisiae* takes over. Some important control parameters should be taken in account for this purpose: the inoculation concentration and the timing between the first and second inoculation, nutrient consumption and sulphite content. High inoculation level of NS yeast improves the competitiveness toward *S. cerevisiae* and other wild yeasts; while the interval between the first and the second inoculation affects the duration of this metabolic activity, which will quickly decline upon inoculation of *S. cerevisiae*. However, attention must also be paid to the consumption of nitrogen sources and vitamins from grape must by NS yeasts during the first stage of sequential inoculation fermentation (Kemsawasd et al., 2015). This consumption often requires to be compensated by suitable yeast nutrients in order to prevent stuck fermentations after inoculation of *S. cerevisiae* (Medina et al., 2012; Lage et al., 2014). Special attention is required in oxygenated fermentations, since a strong nutrient depletion is expected due to high biomass production by NS yeasts under these growth conditions. Concerning sulphite concentration, it must be adjusted during the first stage to the actual tolerance of the NS yeast strain used, since it will usually fall below the standard values for *S. cerevisiae* strains. Eventually, they might be raised to ordinary winemaking concentration after the second inoculation. Interestingly, controlled fermentation by sequential inoculation has been proposed as a way to reduce sulphite contents in the final wine.

The sequential inoculation strategy, using NS/*S. cerevisiae* has been employed in several studies. Many of them use *Starmerella bombicola* (formerly *C. stellata*) as the NS counterpart to *S. cerevisiae*. In these investigations a high production of glycerol and succinic acid and interactions involving some by-products (acetaldehyde, acetoin) with a consequent reduction of final ethanol amount were found (Ciani and Ferraro, 1996, 1998; Ferraro et al., 2000). The reduction in ethanol content in these assays varied from 0.64% v/v at pilot scale in natural grape juice to 1.60% v/v at laboratory scale using synthetic grape juice. Sequential fermentation trials using *Lachancea thermotolerans* (formerly *Kluyveromyces thermotolerans*) were carried out under industry condition using a high inoculation level (10⁷ cell/ml) with a delay of the second inoculum (*S. cerevisiae* strain) of 2 days resulting in an ethanol reduction of 0.7% v/v (Gobbi et al., 2013). A sequential inoculation of *M. pulcherrima* AWR1149 followed by a *S. cerevisiae* wine strain gave rise to a wine with an ethanol concentration lower than that achieved with *S. cerevisiae* (0.9 and 1.6% v/v in Chardonnay and Shiraz wines, respectively; Contreras et al., 2014). Di Maio et al. (2012) showed that *C. zemplinina* may be used in mixed fermentation with *S. cerevisiae* to reduce the

ethanol content in wine (0.32% v/v) and to increase the glycerol content. More recently, the use of sequential fermentation with immobilized non-*Saccharomyces* wine yeast, was proposed to reduce the ethanol content in wine using *S. bombicola*, *M. pulcherrima*, *H. uvarum*, and *Hanseniaspora osmophila* selected strains, in Verdicchio grape juice. Sequential fermentation of 72-h showed an ethanol reduction of 1.64% (v/v) for *S. bombicola*, 1.46% (v/v) for *M. Pulcherrima*, 1.21% (v/v) for *H. uvarum*, and 1.00% (v/v) for *H. osmophila*. The wines obtained did not exhibit any negative fermentation products, but rather an increase of some desirable compounds (Canonico et al., 2016). In **Table 1** are summarized the anaerobic sequential fermentations of some NS yeasts as compared to control *S. cerevisiae* proposed to reduce the ethanol content in wine.

As a general trend, using NS/*S. cerevisiae* pairs in mixed fermentation did not result in the overproduction of undesirable by-products, in contrast to some *S. cerevisiae* genetically engineered strains, which can dramatically accumulate acetic acid or other metabolites with a negative impact on wine sensorial quality (Michnick et al., 1997; Remize et al., 1999). Indeed, apart from the reduction of ethanol content in wine, positive interactions in fermentation by-products have been shown during sequential fermentation. In NS/*S. cerevisiae* mixed cultures, the interactions due to the wide inter-generic metabolic diversity should be higher. These interactions were investigated in *S. cerevisiae* and *S. bombicola* (Sipiczki et al., 2005) mixed fermentation. In this co-culture complementary consumption of glucose and fructose was observed (Ciani and Ferraro, 1998). Using sequential, continuous fermentation and immobilized yeast cells, preliminary evidence has highlighted the exchange of acetaldehyde between these two yeast species. The excess of acetaldehyde production by *S. bombicola*, due to the low activity of alcohol dehydrogenase (Ciani et al., 2000), was quickly metabolized by *S. cerevisiae*, which is a more active alcoholic fermentation species (Ciani and Ferraro, 1998). In this context, an acetaldehyde flux between *S. cerevisiae* and *Saccharomyces bayanus* has also been reported (Cherai et al., 2005). These interactions in acetaldehyde reduction,

were also detected in mixed fermentations using *S. cerevisiae*, *T. delbrueckii* (Ciani et al., 2006; Bely et al., 2008; Belda et al., 2015) and *L. thermotolerans* (Ciani et al., 2006). Another compound involved in interactions between two yeast species in mixed fermentation is acetoin; this is largely accumulated by *S. bombicola* in pure culture, and completely metabolized by *S. cerevisiae* in mixed fermentation (Ciani and Ferraro, 1998). More recently, the influence *Hanseniaspora guilliermondii* on genomic expression of *S. cerevisiae* in mixed culture wine fermentation was investigated (Barbosa et al., 2015).

On the other hand, oxygenated fermentation, as proposed above to stimulate yeast respiration, introduce a new challenge for managing mixed fermentations. Oxygen supply has a positive impact in several microbial and chemical processes during winemaking. It activates *S. cerevisiae* metabolism, in part because it is required for the biosynthesis of plasma membrane sterols, so aeration practices are often used in order to ensure good initial fermentation kinetics or to help recover sluggish fermentation (Alexandre and Charpentier, 1998; Valero et al., 2001; Fornairon-Bonnefond et al., 2002). Oxygen is also used in hyper-oxygenation treatments, in order to get rid of compounds highly sensitive to oxidation that would contribute to browning of white wines if oxidized in later stages of the winemaking process. In turn, macro- and micro-oxygenation of wines are used, alone or in combination with other oenological practices, in order to improve and stabilize wine color during the aging of red wines, or to avoid the “reduced” character sometimes associated to aging on yeast lees (Fornairon-Bonnefond et al., 2003).

Nevertheless, oxygen supply amounts required to ensure efficient yeast respiration are far beyond requirements for even the most demanding oxygenation practices, among those described above. There is a risk that the strong oxygenation levels required for yeast respiration would promote, as a side effect, the oxidation of key components for the sensory quality of wines, namely phenolics and aroma compounds. However, oxygen affinity of fermenting yeast cells has been determined to be about 1000 times higher than wine polyphenols (Salmon, 2006). Accordingly, the target to avoid oxidative damage to wine phenolics would be coupling air supply to oxygen consumption by yeast cells. Being able to keep dissolved oxygen values around 0% would be a good indicative of success for this objective. This goal was shown to be feasible by using controlled aeration conditions and an appropriate *M. pulcherrima* strain (Morales et al., 2015).

An additional major issue of strong aeration of wine during the fermentation step is acetic acid production. Several authors have described a boost in acetic acid production by *S. cerevisiae* when fermenting under aerobic or micro-aerobic conditions (Giovannelli et al., 1996; Papini et al., 2012; Quirós et al., 2014; Contreras et al., 2015b; Rodrigues et al., 2016). Other yeast species have also been shown to negatively impact volatile acidity under aerated growth conditions in synthetic grape must (Quirós et al., 2014). Rodrigues et al. (2016) analyzed volatile acidity across several growth conditions for four different yeast strains. They found a clear correlation between oxygen supply and acetic acid production. The good news is that some yeast species produce very little volatile acidity even under oxygenated

TABLE 1 | Reduction of ethanol content in anaerobic sequential fermentations of some NS yeasts as compared to control *Saccharomyces cerevisiae*.

Sequential fermentation	Grape juice	Ethanol reduction % (v/v)	Reference
<i>S. bombicola</i> / <i>S. cerevisiae</i>	White	0.64	Ferraro et al., 2000
<i>S. bombicola</i> / <i>S. cerevisiae</i>	Synthetic	1.60	Ciani and Ferraro, 1998
<i>S. bombicola</i> / <i>S. cerevisiae</i>	White	1.64	Canonico et al., 2016
<i>H. uvarum</i> / <i>S. cerevisiae</i>	White	1.21	Canonico et al., 2016
<i>H. osmophila</i> / <i>S. cerevisiae</i>	White	1.00	Canonico et al., 2016
<i>M. pulcherrima</i> / <i>S. cerevisiae</i>	White	0.90	Contreras et al., 2014
<i>M. pulcherrima</i> / <i>S. cerevisiae</i>	Red	1.60	Contreras et al., 2014
<i>M. pulcherrima</i> / <i>S. cerevisiae</i>	Red	0.90	Contreras et al., 2015a
<i>M. pulcherrima</i> / <i>S. cerevisiae</i>	White	1.46	Canonico et al., 2016
<i>L. thermotolerans</i> / <i>S. cerevisiae</i>	Red	0.70	Gobbi et al., 2013
<i>C. zemplinina</i> / <i>S. cerevisiae</i>	Red	0.32	Di Maio et al., 2012

conditions (Quirós et al., 2014; Rodrigues et al., 2016). It is possible to manage oxygen supply in fermentation trials driven by simultaneous inoculation of *S. cerevisiae* and a NS strain (Morales et al., 2015). However, the strict control of the process required under such growth conditions suggests that a better control of volatile acidity would be achieved by inoculating *S. cerevisiae* only after oxygen supply has been arrested (i.e., by sequential inoculation). In addition, proper control of yeast metabolism in aerated fermentations would benefit from the development of dedicated devices, able to monitor oxygen consumption and to adapt air supply to yeast requirements, so avoiding both excess oxidation and excess acetic acid production.

CONCLUSION

Research on yeast based strategies in order to reduce ethanol content of wines started about 20 years ago (Michnick et al., 1997). The growing evidence about global climate warming and its impact on sugar content of grapes at harvest has contributed to an ever increasing interest on this topic. The biotechnological strategies initially explored were based on genetic engineering of *S. cerevisiae*, a rational choice considering the preponderant role of this species in both spontaneous and inoculated fermentation. However, the use of these recombinant strategies soon faced hurdles coming from both yeast metabolism and the regulatory framework for genetically modified organisms. The fact that further genetic modification was required in order to overcome initial problems did not help much.

In this context, the intense research activity around NS wine yeasts, our increasing awareness about the metabolic diversity of yeasts, and the arrival to the market of NS starters, opened new opportunities to exploit yeast metabolism with the aim of reducing ethanol content of wines. Current knowledge indicate that, similar to other metabolic traits, ethanol yield on sugar is not only species-specific, but often strain-specific. Some NS yeast species can show ethanol yields similar or higher than *S. cerevisiae*, but many of them show reduced ethanol yields. It has also been shown that oxygenation during wine fermentation can help further reduce ethanol yields by these, often Crabtree negative, NS yeast species.

Since most NS wine yeasts are sensitive to ethanol concentrations above 6–8%, in order to keep the microbiological control of the fermentation process, and to avoid stuck or sluggish fermentation (and wine spoilage), the use of *S. cerevisiae* starters, in either sequential or simultaneous inoculation, will still be required. The introduction of mixed starter inoculation to routine winemaking practices also demands for a better control of the fermentation parameters, adapted to each specific combination of yeast starters. Some parameters to take into account are sulphite concentration, temperature, pH adjustments, inoculation levels

(and timing, for sequential inoculation), yeast nutrition, and eventually oxygenation levels and timing, among other parameters.

In order to perform knowledge based decisions in this field, further research will be required. Some of the topics that need to be addressed are common to other oenological applications of NS wine yeasts, while other are more specific for alcohol level reduction. Environmental factors influencing ethanol yield by different wine yeast species warrant a special attention, both for respiration-based and anaerobic fermentation strategies. As a general rule, research projects on NS wine yeasts should always pay attention to the production of unwanted metabolites, including acetic acid, which has been identified as a serious drawback, especially for respiration-based strategies or certain yeast strains. Reliable assessment of the impact of new yeast strains/species, and new oenological practices on quality related features of wines would require pilot scale experiments, use of natural grape must, and rigorous sensory analysis. One complex but very relevant aspect that is indeed already attracting attention by wine biotechnologists is physiological and ecological interactions between cells from the starter cultures, among them and with the natural microbiota. The few articles already published on this topic are just opening the window to a world of interactions, including competitions, metabolite exchanges, and production of narrow and wide spectrum antimicrobials. All these phenomena have a potential to impact alcohol level and overall quality of wines. Yeast inter and intraspecific diversity must always be taken into account both in the design of experiments and to draw general conclusions. Finally, the interaction of starter cultures with natural microbiota is a very relevant but complex topic, which might eventually benefit from the increasing availability of high throughput technologies, including metagenomic analysis.

AUTHOR CONTRIBUTIONS

MC, RG, and PM conceived the idea and outline of the review. All authors contributed to writing specific sections and approved the final version of the manuscript.

ACKNOWLEDGMENTS

Experimental work by researchers from ICVV is supported by the Spanish Government through MINECO and FEDER funds: MINECO AGL2012-32064 and AGL2015-63629-R grants, INIA RM2012-00007-00-00 grant, MINECO RTC-2014-2186-2 grant, MINECO training contract for AR, MINECO Formación Postdoctoral contract for JC. Experimental work by the authors from Polytechnic University of Marche is supported by Ricerca Scientifica di Ateneo RSA2015.

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- Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Biocontrol Ability and Action Mechanism of *Starmerella bacillaris* (Synonym *Candida zemplinina*) Isolated from Wine Musts against Gray Mold Disease Agent *Botrytis cinerea* on Grape and Their Effects on Alcoholic Fermentation

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OPEN ACCESS

Edited by:

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Specialty section:

This article was submitted to
Food Microbiology,
a section of the journal
Frontiers in Microbiology

Received: 24 March 2016

Accepted: 27 July 2016

Published: 15 August 2016

Citation:

Lemos Junior WJF, Bovo B, Nadai C,
Crosato G, Carlot M, Favaron F,
Giacomini A and Corich V (2016)
Biocontrol Ability and Action
Mechanism of *Starmerella bacillaris*
(Synonym *Candida zemplinina*)
Isolated from Wine Musts against
Gray Mold Disease Agent *Botrytis*
cinerea on Grape and Their Effects on
Alcoholic Fermentation.
Front. Microbiol. 7:1249.
doi: 10.3389/fmicb.2016.01249

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Gray mold is one of the most important diseases of grapevine in temperate climates. This plant pathogen affects plant growth and reduces wine quality. The use of yeasts as biocontrol agents to apply in the vineyard have been investigated in recent years as an alternative to agrochemicals. In this work, fermenting musts obtained from overripe grape berries, therefore more susceptible to infection by fungal pathogens such as *Botrytis cinerea*, were considered for the selection of yeasts carrying antifungal activity. Thirty-six isolates were identified as *Starmerella bacillaris*, a species recently proven to be of enological interest. Among them 14 different strains were studied and antifungal activity against *B. cinerea* was demonstrated, for the first time, to be present in *S. bacillaris* species. The production of volatile organic compounds (VOCs), tested *in vitro*, was found to be the main responsible of *S. bacillaris* antifungal effects. All the strains were able to reduce *B. cinerea* decay on wounded grape berries artificially inoculated with gray mold. The colonization level of wound was very high reaching, after 5 days, a concentration of 10⁶ cells per ml of grape juice obtained after berry crushing. At this cell concentration *S. bacillaris* strains were used to ferment synthetic and natural musts. The sequential yeast inoculation, performed by adding *S. cerevisiae* 48 h after *S. bacillaris*, was needed to complete sugar consumption and determined a significant increase in glycerol content and a reduction of ethanol and acetic acid concentrations. The high wound colonization ability, found in this work, together with the propensity to colonize grape berry and the interesting enological traits possessed by the selected *S. bacillaris* strains allow the use of this yeast as biocontrol agent on vine and grape berries with possible positive effects on must fermentation, although the presence of *S. cerevisiae* is needed to complete

the fermentation process. This work introduces new possibilities in wine yeast selection programs in order to identify innovative wine yeasts that are simultaneously antifungal agents in vineyards and alternative wine starters for grape must fermentation and open new perspective to a more integrated strategy for increasing wine quality.

Keywords: antifungal activity, SAU-PCR, grape must, VOCs, lytic enzymes, fermentation, glycerol

INTRODUCTION

Botrytis cinerea is one of the most important fungal plant pathogen that causes serious gray mold disease in more than 200 economically relevant plant species during pre-harvest (especially when plants are grown under protection), and post-harvest (Agrios, 2005). Grapes, vegetables, berries, and stone fruits cultivated worldwide are the most susceptible to this fungal disease (Rosslenbroich and Stuebler, 2000). The fungal agent infects leaves, stems, flowers and fruits of plants, either by direct penetration or through wounds caused by cultivation practices. This fungus kills host cells through the production of cell wall degrading enzymes, phytotoxic metabolites and reactive oxygen species accumulating after the induction of a plant-produced oxidative burst. Thanks to degrading enzymes, *B. cinerea* succeeds in the decomposition and consumption of different plant tissues (van Kan, 2006). Infestation is stimulated by high humidity, particularly if free moisture is present on the plant surface and low temperatures (Williamson et al., 2007). Generally the control of the disease is achieved by the use of synthetic fungicides (Elad and Evensen, 1995). From the middle of the 1990s, fungicides with excellent activity against *B. cinerea* came to the market and more recently the control of the disease was mainly achieved by integrating several cultural methods with the use of these fungicides (Rosslenbroich and Stuebler, 2000). Although, synthetic fungicides are effective, their continued or repeated application has disrupted biological control by natural enemies of the fungus and stimulated the development of resistant pathogen populations, leading to widespread outbreaks of the disease (Elad et al., 1992). The increasing concern over the adverse agronomical and environmental effects of synthetic fungicides brought to search new types of crop protection methods without or with reduced use of conventional fungicides. The salts of weak acids, such as sodium benzoate and potassium sorbate, can inhibit growth of several post-harvest fungal pathogens. These compounds present several benefits as they possess low toxicity toward mammals, a wide spectrum of activity and are relatively cheap. However, these compounds need to be used at concentrations that can determine potential organoleptic changes of the products. For example, calcium propionate completely inhibited mycelial growth of *B. cinerea* at a concentration of 5% (w/v) (Droby et al., 2002). Essential oils obtained from aromatic and medicinal plant species have been proposed as new classes of possibly disease control agents, since they are a rich source of bioactive chemicals. These chemicals are often active against a limited number of species, are biodegradable to nontoxic products and are potentially suitable for integrated use (da Cruz Cabral et al., 2013). Specific activity against *B. cinerea* was found in essential oils obtained from the

aerial parts of aromatic plants, which belong to the *Lamiaceae* family, such as origanum (*Origanum syriacum* L. var. *bevanii*), lavender (*Lavandula stoechas* L. var. *stoechas*), and rosemary (*Rosmarinus officinalis* L.; Soyly et al., 2010). Traditional medical plants from Africa and Asia were found to be a source of essential oils proposed for post-harvest control of gray mold (Tripathi et al., 2008). Among the alternatives to synthetic fungicides, the use of plant resistance inducers demonstrated the potential for large-scale application. The induced resistance can be defined as an increased expression of natural defense mechanisms of plants against different pathogens provoked by external factors of various type: elicitors of pathogenic origin (glucans, proteins, lipids, etc.); abiotic elicitors, including synthetic harmless chemical products (Edreva, 2004). Some molecules, that act as inducers, also present antimicrobial activity. That is the case of chitosan that decreases gray mold and other fungal diseases through the reduction of mycelial growth and spore germination and induction of morphological alterations in the fungal pathogen. Moreover, chitosan acts as a potent elicitor to enhance plant resistance (Amborabé et al., 2008; El Hadrami et al., 2010). An alternative strategy to reduce gray mold disease is based on the selection and application of biocontrol agents. Among the microorganisms used as biocontrol agents, yeasts have been targeted by many surveys as potential mold antagonists, focusing mainly on post-harvest diseases, since they are naturally occurring on fruits and vegetables, and have shown great ability to colonize wound sites (Bai et al., 2008). Some have been present on the market for a long time and showed specific activity against *B. cinerea*. *Candida oleophila*, the base of the commercial product “Aspire,” is recommended for the control of post-harvest decay in citrus and pome fruits. Its modes of action include nutrient competition, site exclusion, and direct mycoparasitism (Droby et al., 2002). The yeast *Cryptococcus albidus*, included in the commercial product “Yield Plus,” is an antagonist isolated from peach fruit and effective against the pathogen *B. cinerea* in apple (Fan and Tian, 2001). As regards other yeast species the investigations as biocontrol agents are still ongoing. Recently, the ascospore yeast *Metschnikowia fructicola* AL27 was tested on several apple varieties and found to be as competitive as the chemical fungicides used as control (Spadaro et al., 2013).

Focusing on viticulture, gray mold is one of the most important diseases of grapevine in temperate climates worldwide and can cause extensive economic losses through grape desiccation, rot and biochemical changes that reduce wine quality. Biological control of *B. cinerea* is a successful strategy that has been introduced as an alternative to synthetic fungicides in grapevine cultivation. Filamentous fungi from the genera *Trichoderma*, *Ulocladium*, and *Gliocladium*, bacteria from the

genera *Bacillus* and *Pseudomonas* and, lately, yeasts from the genera *Pichia* and *Candida* have been used as biocontrol agents (Jacometti et al., 2010). Recently, an integrated approach that combined low dosage of fungicides and antifungal yeasts has been tested in order to reduce chemicals concentration and enhance biocontrol efficacy. *Hanseniaspora uvarum* was tested under laboratory conditions in combined treatment with $\text{NH}_4\text{-Mo}$, showing inhibitory effects on spore germination and mycelial growth of *B. cinerea* *in vitro* and induced defense reactions in grape berries (Liu et al., 2010). Although several yeasts with antifungal property have been successfully identified, yeast selection to find out new biocontrol agents remains challenging, and species-to-species interaction studies are of great interest to understand native and introduced fungal population dynamics in both vineyard and cellar. Indeed, after grape harvest, antifungal yeasts become part of the must microbiota and, if well adapted to must condition, they could have a role during the fermentation process and therefore directly influence wine quality. At the moment, no information is available about the fate of selected yeasts proposed as biocontrol agents during must fermentation and winemaking, although they can be found on the grape surface at high level due to repeated treatments. Moreover, the possibility to select yeasts that are simultaneously antifungal agents in vineyards and wine starters for grape must fermentation is completely unexplored.

Non-*Saccharomyces* yeasts are a group of wine-related yeast species once defined spoilage microorganism. Generally they are well adapted to vineyard condition and are predominant in grape musts during the early stages of fermentation. Recently, there has been a re-evaluation of the role of these yeasts, as some of them were found to enhance the analytical composition and aroma profile of the wine (Ciani and Comitini, 2015).

Starmerella bacillaris (synonym *Candida zemplinina*) is a non-*Saccharomyces* yeast, commonly found on grapes and particularly associated with botrytized grapes and wines fermented from these grapes (Csoma and Sipiczki, 2008; Magyar and Tóth, 2011; Duarte et al., 2012; Masneuf-pomarede et al., 2015; Wang et al., 2015). Magyar and Tóth (2011) investigated the technological properties of *C. zemplinina* strains evidencing an extremely poor ethanol yield from sugar consumption, high glycerol and moderate volatile acids production. High glycerol production contributes to palate fullness ("body") of wine, whereas high acetic acid content confers an unpleasant vinegar aroma. Therefore, with the aim of improving wine quality, *S. bacillaris* was recently tested, together with *Saccharomyces cerevisiae*, in sequential and mixed yeast inoculations during grape must fermentation to balance the glucophilic character of the *Saccharomyces* species, to increase glycerol concentration in wine and, due to the low ethanol yield, to reduce ethanol content (Rantsiou et al., 2012; Bely et al., 2013; Wang et al., 2014).

With the aim to investigate the double role of *S. bacillaris* as both potential biocontrol agent and unconventional enological starter, 14 strains belonging to this species were studied in this work to evaluate their antifungal activity against *B. cinerea*, both *in vitro* and *in vivo*. Moreover, the technological properties of these non-*Saccharomyces* strains were evidenced at lab-scale,

both in single-strain fermentation and in sequential fermentation together with *Saccharomyces cerevisiae*.

MATERIALS AND METHODS

Isolation and Characterization of Yeast Isolates

The yeast strains used in this work were isolated from fermenting musts obtained from dried grape of Raboso piave variety. They were collected during two harvests in two wineries located in the winemaking area of Appellation of Origin Bagnoli (North-East of Italy) where the production of Friularo Bagnoli Passito wine is performed. A total of 360 yeast colonies were isolated on WL agar medium (Oxoid) plates. All yeasts were identified at species level by PCR-RFLP analysis of the ITS1-5.8S-ITS2 rDNA region and D1/D2 region sequence analyses as described by Bovo et al. (2011). A BLAST search on sequence results gave the most probable species identification. Thirty-six isolates identified as *S. bacillaris* were characterized at molecular level using SAU-PCR method, as described by Corich et al. (2005). SAU-PCR amplification patterns were analyzed using the software GelComparII V. 3.5 (AppliedMaths).

Extracellular Lytic Enzymes Activity

S. bacillaris strains were screened for the production of extracellular cellulase, xylanase, lipase, pectinase and proteinase using plate tests as described by Lorenzo et al. (2013). The presence of extracellular chitinolytic activity was tested on glycol chitin agar medium (yeast nitrogen base, 6.7 g/L, glycol chitin, 5 g/L, agar 16 g/L). After the growth of the yeast colonies a solution containing 500 mM Tris-HCl pH 8.9 with 0.01% w/v of Calcofluor white MR2 was poured on the plates. The plates were incubated for 10 min. Subsequently, the solution was discarded and replaced with water overnight. The presence of chitinolytic activity was evidenced by the observation of dark lytic plaques, where the colonies were present, on a light background under UV exposure. Extracellular β -glucosidase activity was evaluated using the esculin (esculetin 6-O-glucoside) agar hydrolysis test described by Njokweni et al. (2012) on Esculin agar (esculin 1 g/L, YNB 1.7 g/L, 0.5 g/L ferric citrate, agar 16 g/L) plates. Extracellular β -glucosidase activity were also tested by evaluating yeast growth on Cellobiose agar (cellobiose 5 g/L, YNB 6.7 g/L, agar 16 g/L) plates after incubation at 30°C for 72 h.

In vitro Antagonistic Activity

The antagonistic activity on agar plates and volatile organic compounds (VOCs) assay was performed as described by Parafati et al. (2015), and modified as follows. The *B. cinerea* strain used was BC0510.

Antagonistic Activity on Agar Plates

The yeast and mold strains to be tested were, respectively, growth on YPD for 24 h and on PDA for 5 days at 25°C. Each yeast strain was streaked orthogonally from the center of a plate, containing PDA (Potato Dextrose Agar) medium at two different pH (5.5 and 3.5). Simultaneously, for each plate 2 mycelial discs (6 mm

square plug) of *B. cinerea* were placed on agar plates 3 cm away from the yeast streak. A control plate was prepared inoculating only *B. cinerea*. At the end of the incubation period (5 days at 25°C) the radial growth reduction was calculated in relation to the growth of the control as follows: $\%I = (C - T/C) \times 100$, where $\%I$ represented the inhibition of the radial mycelial growth, C was the radial growth measurement in control and T was the radial growth of the pathogen in the presence of yeast strains. The assay was performed using four replicates for each yeast strain and pH.

Effects of Volatile Organic Compounds (VOCs)

A dual culture method was used to evaluate the efficacy of volatile compounds produced by yeasts against *B. cinerea*. Aliquots of 20 μ L of yeast suspensions (10^7 cells/mL) were seeded on plates with PDA at two pH values, 5.5 and 3.5, and incubated 4 days at 25°C. Aliquots (10 μ L) of the conidial suspension of *B. cinerea* (10^6 conidia/mL) were inoculated on PDA and dried at room temperature. The plates with *B. cinerea* conidia were individually covered face to face under the plates containing the yeast strains. The controls were prepared facing the plates containing *B. cinerea* suspension with unseeded PDA plates. Each plate pair was wrapped with two layers of Parafilm around the edges to prevent air leakage, and incubated at 25°C. The radial growth reduction of *B. cinerea* was calculated after 5 days of incubation as previously described.

In vivo Antagonistic Activity

In order to assess the efficiency of yeasts as biocontrol agents, the method described by Parafati et al. (2015), with slight modifications, was used. Table grape fruits derived from orchards located in Padova, Italy. Healthy and homogeneous grape berries were selected, washed and surface-disinfected. Artificial wounds were performed and inoculated with 10 μ L drop of 10^6 conidia/mL of *B. cinerea*. After air drying (2 h), a 10 μ L drop of 10^8 cells/mL of yeast were added to each wound. The same amount of 0.09% NaCl buffer (20 μ L) was used in the control. For each strain 10 grape berries were used. The grape berries were placed on plastic packaging trays. To create a humid environment, a wet paper was placed on cavity trays coated with a plastic bag. The trays were incubated at 25°C and 95% relative humidity (RH) for 5 days after inoculation to provide favorable conditions for the disease development. The disease severity (DS) was evaluated by using an empirical 1-to-4 rating scale evaluating both soft rot size and mycelium growth: + barely visible symptoms, ++ small, +++ intermediate, ++++ large (comparable to control).

Data concerning the disease reduction incidence (DRI) was calculated as follow $(DRI) = (C - T/C) \times 100$, where C was the average radial growth measurement in control (10 berries), and T was the radial growth of the pathogen in the presence of yeast strain in each berry.

The lesion diameter (LD) was evaluated by measuring the average diameter of the damaged area 5 days after *Botrytis* inoculation. Each yeast strain was tested on 10 berries.

Fermentation Trials in Synthetic and Natural Must

Inoculum Preparation

A loopful of a 3-days-old culture of each yeast strains from YPD agar plate (yeast extract 10 g/L, peptone 10 g/L, dextrose 20 g/L) was used to inoculate 10 mL of YPD broth in 50 mL tubes. A stationary phase culture with approximately 10^7 – 10^8 cells/mL, determined by OD measurements and confirmed by means of plate counts analysis (CFU/mL), was obtained after 24 h of incubation at 30°C. In single-strain fermentation the inoculum concentration was 2×10^6 cells/mL. In sequential fermentation the same inoculum size for both *S. bacillaris* strain and *S. cerevisiae* EC1118 (1 – 1.5×10^6 cells/mL) was used. EC1118 was added 48 h after the inoculum of *S. bacillaris*.

Fermentation Conditions

Fermentations were run in synthetic and natural musts. The synthetic must MS300 was prepared as described by Bely et al. (1990) with the addition of 100 g/L of glucose, 100 g/L of fructose and 6 g/L of malic acid, pH 3. Incrocio Manzoni grape must, containing 160 g/L of reducing sugars (pH 3.5) was used. In the fermentation trials 120 mL capacity bottles fitted with closures that enabled the carbon dioxide to escape and containing 100 mL of must were used. After yeast inoculation the bottles were incubated at 25°C. The fermentation process was followed by measuring twice a day the weight loss of each culture. When the weight loss was lower than 0.05 g per day the fermentations were considered concluded. All the fermentation trials were performed in triplicate.

HPLC Analysis

Ethanol, glycerol, fructose and glucose concentrations were quantified with HPLC (Shimadzu, Japan) equipped with a refractive index detector, set at 600 nm wavelength, while for the acetic acid quantification a UV detector was used.

The concentrations, expressed as g/L, were calculated by using calibration curves of the individual compounds. The chromatographic conditions were realized with the ROA-Organic Acid H+ column (Phenomenex, USA), which was run at 65°C with 5 mM H_2SO_4 as the mobile phase, with a flow rate of 0.5 mL/min.

Statistical Analysis

The statistical data analysis was performed with XLSTAT software, vers.7.5.2 (Addinsoft, Paris, France) using the principal component analysis (PCA) and the one-way analysis of variance (ANOVA) at 95% accuracy level. Fisher's test was used as comparison test when samples were significantly different after ANOVA ($p < 0.05$).

RESULTS

Isolation, Identification, and Evaluation of Extracellular Activity of Yeast Isolates

A total of 360 yeast colonies were isolated from fermenting musts for the production of Friularopassito wine. By means of PCR-RFLP analysis of the ITS1-5.8S-ITS2 rDNA region and D1/D2

TABLE 1 | Yeast strains used in this work.

Strain	Species	Origin
FRI719	<i>S. bacillaris</i>	Winery A
FRI728	<i>S. bacillaris</i>	Winery A
FRI729	<i>S. bacillaris</i>	Winery A
FRI751	<i>S. bacillaris</i>	Winery A
FRI754	<i>S. bacillaris</i>	Winery A
FRI779	<i>S. bacillaris</i>	Winery A
FRI7100	<i>S. bacillaris</i>	Winery A
PAS13	<i>S. bacillaris</i>	Winery B
PAS55	<i>S. bacillaris</i>	Winery B
PAS66	<i>S. bacillaris</i>	Winery B
PAS92	<i>S. bacillaris</i>	Winery B
PAS103	<i>S. bacillaris</i>	Winery B
PAS151	<i>S. bacillaris</i>	Winery B
PAS173	<i>S. bacillaris</i>	Winery B
EC1118	<i>S. cerevisiae</i>	Industrial strain

rDNA region sequencing (Kurtzman and Robnett, 1998) each isolate was identified at species level. A total of 36 isolates were identified as *Starmerella bacillaris*. The characterization at strain level, by means of Sau-PCR (Corich et al., 2005) and the cluster analysis of the amplification pattern (see Supplementary Material SM_1), allowed the selection of 14 different strains listed in **Table 1**. All the strains were tested for the production of extra-cellular enzymes using specific plate assays. Some of the activities are of industrial interest: beta-glucosidase, cellulase, lipase, and xylanase. Pectinase, protease, chitinase are involved in degrading mold cell wall. The results of the screening are reported in Supplementary Material SM_2. All the strains showed chitinase activity, although at low level. Only strains FRI719 and FRI751 produced proteolytic enzymes as they were able to grow on skin milk. None of the other activities tested was found in any strain.

In vitro Antagonistic Activity

Data from dual culture assays are reported in **Figure 1**. All the *S. bacillaris* strains were able to inhibit the growth of *B. cinerea* mycelium both at pH 5.5 and 3.5 when co-cultivated with *B. cinerea* (**Figure 1A**). The percentage of the inhibition of the radial mycelial growth ranged from 12 to 33. Strains FRI719, FRI779, PAS13, PAS66 showed higher antagonistic activity at pH 3.5 than at pH 5.5. On the contrary FRI100, PAS92 and PAS173 showed higher antagonistic activity at pH 5.5. In the other cases no significant differences were detected. In these conditions, for all the strains, the inhibition level found was limited, although comparable with that found in literature for some other yeast species (Parafati et al., 2015). This could be due to the different growth rate of *S. bacillaris* and *B. cinerea* on PDA medium where *S. bacillaris* can not found the optimal growth conditions. As the two microorganisms were inoculated simultaneously, *S. bacillaris* inhibited, only partially, the fungal growth.

The inhibition of *B. cinerea* mycelium growth due to the production of volatile compounds by *S. bacillaris* strains was tested, as well (**Figure 1B**). To overcome the different growth

rate between the two microorganisms, plates inoculated with *S. bacillaris* strains were incubated 4 days at 25°C before covering face to face each plate with that containing *B. cinerea*. Generally, results showed notably higher inhibition percentages than those found when *B. cinerea* and each yeast strains were co-cultivated on the same plate: the values ranged from 44 up to 79%. Comparing the results with those of the previous growth inhibition assay, only PAS13 and FRI100 confirmed their inhibition ability in relation to the pH of the growing medium.

In vivo Antagonistic Activity

A qualitative evaluation of the efficacy of the tested yeasts in reducing gray mold growth on grape berries is reported in **Table 2**. Although at different levels, all yeasts decreased the size of decay (soft-rot developed area) and the mycelium growth. Eight strains out of 14 showed remarkable effects on the developing of the *Botrytis* infection. In details (**Figure 2**), the DRI values ranged from 39 up to 85%. Strains FRI751, FRI754, PAS173 showed the highest gray mold decay as their values were significantly higher ($p < 0.05$) than those found for the other strains. The LD evaluation confirmed the remarkable ability of *S. bacillaris* strains to reduce the infection size, although only small differences were found between strains. When *S. bacillaris* strains were present in the wound the LD was always lower than 1 cm, while for the control the LD size was 1.8 cm. Strain PAS173 showed the highest level of LD reduction that was significantly different ($p < 0.05$) from those found for the other strains. At the end of the incubation time (5 days) each grape berry was squeezed and homogenized and *S. bacillaris* concentration was determined by plate counts (**Table 2**). The yeast concentration was very similar for all the strains ranging from 1.83×10^6 up to 2.35×10^6 CFU/ml.

Fermentation Activity in Synthetic Must

The fermentation activity of the 14 strains of *S. bacillaris* were evaluated in synthetic must MS300 at 20°C using an inoculum of 2×10^6 cells/ml. The CO₂ production was followed during all the fermentation process. To assess strain fermentation performances, the fermentation vigor, in terms of CO₂ production after 48 h of incubation, was considered in order to evaluate the adaptation ability to the must conditions. CO₂ production after 312 h was considered, as at these fermentation step the widest range of CO₂ production was found between strains. The industrial wine strain *S. cerevisiae* EC1118 was used as control. The fermentations of *S. bacillaris* strains were stopped after 624 h when the fermentation of *S. cerevisiae* EC1118 was completed. As expected, *S. bacillaris* strains showed a very low CO₂ production if compared to that of EC1118 (see Supplementary Material SM_3). Fermentation performances were very similar between strains as no significant differences were found after 312 and 624 h of incubation. Regarding fermentation vigor, strains FRI719, FRI728, and PAS92 showed a significant delay in the fermentation start (0.03, 0.01, 0.07 g/100 mL CO₂ after 48 h, respectively). Strain PAS173 showed the highest CO₂ production after 48 h (0.33 g/100 mL CO₂).

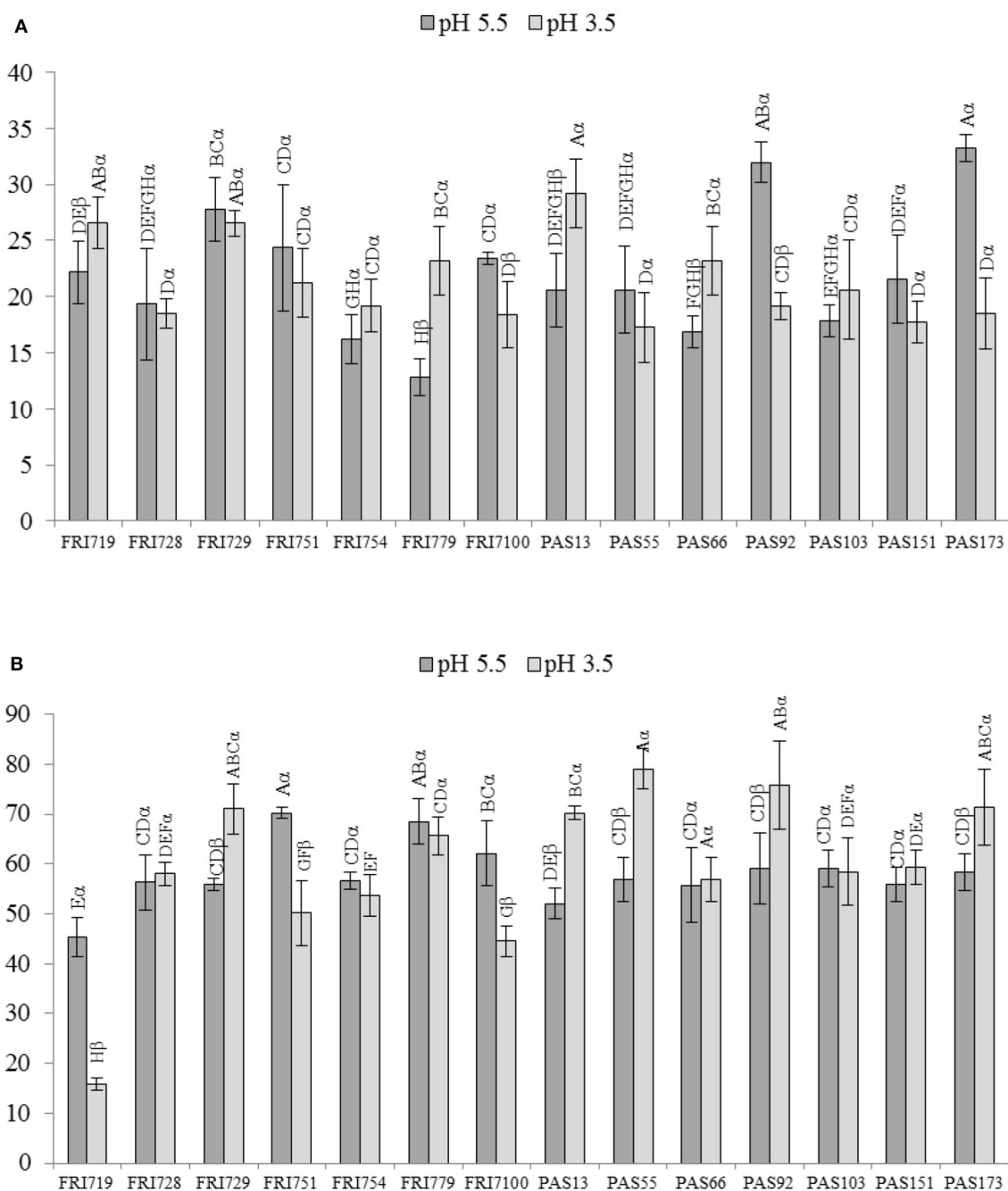


FIGURE 1 | *In vitro* antagonistic activity of *S. bacillaris* strains against *B. cinerea* on PDA plate at pH 5.5 and 3.5. Growth inhibition, measured as inhibition percentage of the radial mycelial growth, induced by yeast cells (A) and by volatile organic compounds (VOCs) (B). Vertical bars indicate the standard error of the mean. Statistical analysis: one-factor ANOVA ($p < 0.05$). At the same pH, mean values followed by the same Roman letter are not significantly different according to Fisher's test ($p \leq 0.05$). For each strain mean values obtained at pH 5.5 and 3.5, and followed by the same Greek letter are not significantly different according to Fisher's test ($p \leq 0.05$).

Concerning residual sugars, as expected, *S. bacillaris* consumed more fructose than glucose due to its fructophilic aptitude (Englezos et al., 2015). The sugars residues were very high (from 100.41 to 135.87 g/L), this was related to a limited ethanol production (from 4.12 to 6.19% v/v). Regarding

secondary metabolites, their production was strongly strain dependent. As expected glycerol production was very high (from 5.58 to 7.81 g/L), while acetic acid concentration was generally limited (from 0.28 to 0.45 g/L). In order to evaluate differences in fermentation performances among the strains all

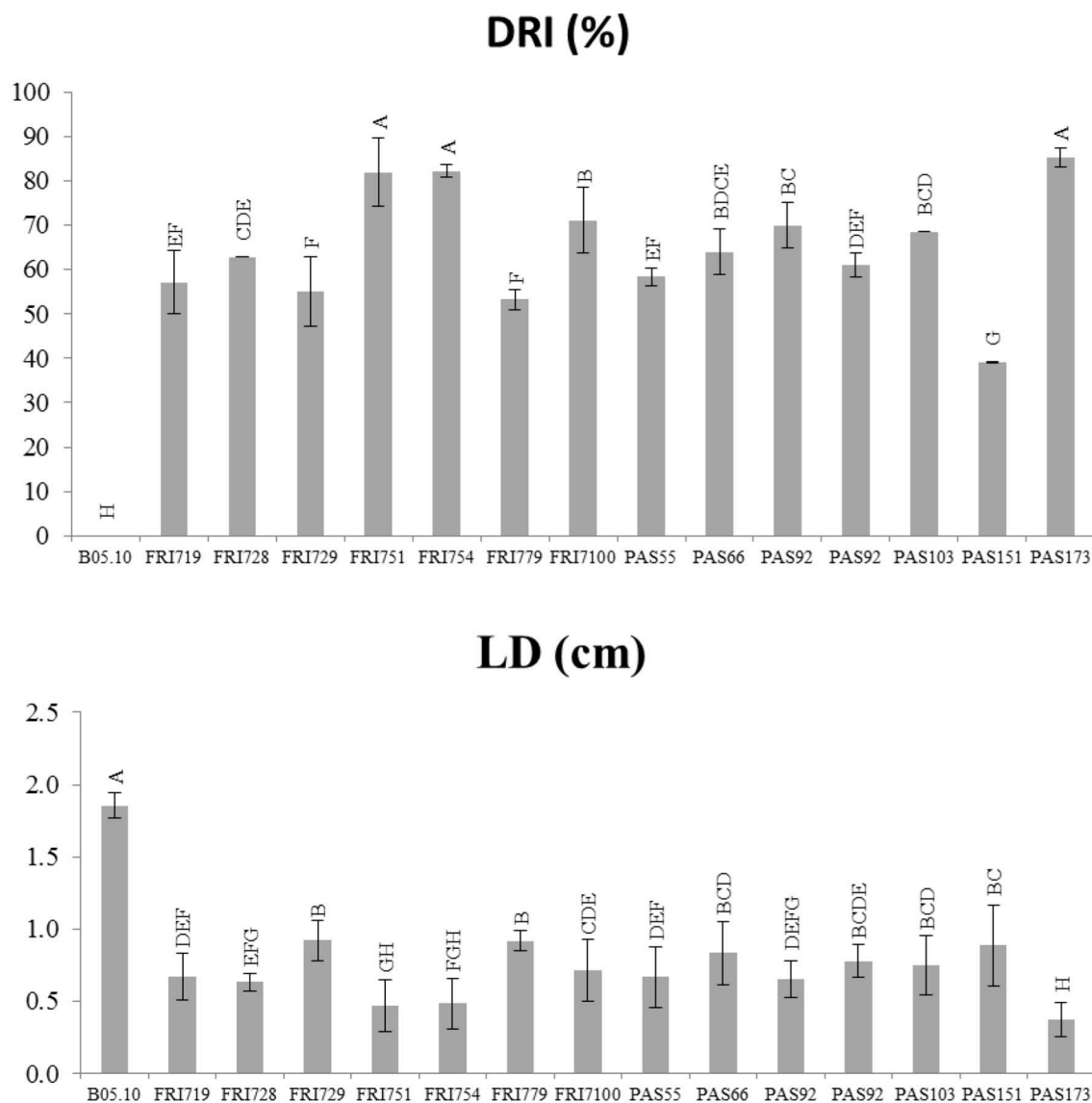


FIGURE 2 | In vivo antagonistic activity of *S. bacillaris* yeast strains in inhibiting gray mold decay on grape berries. Effect of yeasts is referred to disease reduction inhibition percentage (DRI%), and lesion diameter (LD) caused by *Botrytis cinerea* 5 days after incubation at 25°C. Vertical bars indicate the standard error of the mean. Mean values followed by the same letter are not significantly different according to Fisher's test ($p \leq 0.05$).

the collected data (CO_2 production after 48, 312, and 624 h, and the concentration of glucose and fructose residues, glycerol, acetic acid and ethanol) were analyzed by PCA (**Figure 3A**). Function (F1) accounted for 58.81% of the total variance and significantly correlated ($\alpha < 0.001$) with CO_2 production after 312, 624, and ($\alpha < 0.01$) 48 h, with fructose residue and ($\alpha < 0.01$) ethanol concentration. The second function (F2) explained 20.62% of the total variance and was correlated ($\alpha < 0.01$) with acetic acid concentration. No significant correlations were found with glucose residue and glycerol production. The analysis confirmed the high level of similarity between the fermentation performances of the different strains when they are tested as single starter, irrespectively of the strain origin.

FRI728 and PAS92 confirmed to be the strains with the worst fermentation aptitudes in terms of fermentation rate, and ethanol production. On the contrary PAS 173 showed the best fermentation performances.

All the strains were tested in co-fermentation with strain EC1118 in synthetic must MS300 at 20°C (see Supplementary Material SM_4). Each time sequential inoculations were performed adding *S. bacillaris* strain at first, followed, after 48 h, by *S. cerevisiae* EC1118. Both strains were added at the same concentration ($1\text{--}1.5 \times 10^6$ cells/ml). Strains PAS55, PAS66, PAS92, PAS103, PAS151, and PAS173 that showed high fermentation vigor (CO_2 production after 48 h), evidenced a significant lower CO_2 production after 312 h than that

TABLE 2 | Qualitative evaluation of the *Botrytis* infection severity on grape berries and yeast cell concentration in the grape juice obtained by berry squeeze after 5 days from yeast inoculation.

Strain	Soft rot	Mycelium	10 ⁶ CFU/mL
Control	++++	++++	–
FRI719	+	+	1.98 ± 0.07
FRI728	+	+	2.07 ± 0.09
FRI729	++	+	1.83 ± 0.08
FRI751	+	+	1.89 ± 0.10
FRI754	+	+	2.35 ± 0.01
FRI779	++	+	2.10 ± 0.07
FRI7100	+	+	2.20 ± 0.04
PAS13	++	+	2.25 ± 0.08
PAS55	++	++	2.05 ± 0.05
PAS66	++	++	2.08 ± 0.08
PAS103	++	++	2.38 ± 0.11
PAS151	+	++	2.04 ± 0.07
PAS92	+	+	2.07 ± 0.10
PAS173	+	+	2.34 ± 0.06

of the other strains. This means that after the addition of EC1118 a lower fermentation rate than that of EC1118 single-strain fermentation occurred. These strains, together with PAS13, showed the presence of sugar residues, although at low concentrations (from 1.92 to 7.9 g/L), confirming a lower fermentation rate than that of the other strains. The alcohol content was significantly higher in EC1118 single-strain fermentation than in sequential fermentations. Ethanol concentration in EC1118 single-strain fermentation was 13.16% (v/v), whereas sequential fermentations, where the reducing sugar were completely consumed, produced an average of 12.15% (v/v) ethanol, reducing 1% the alcohol content. These results confirmed the well-known ability of *S. bacillaris* species to reduce alcohol content in wine (Bely et al., 2013). Glycerol concentration was significantly lower in EC1118 single-strain fermentation (5.77 g/L) than in co-fermentations (average value 7.05 g/L). An average increase of 1.28 g/L was found. Strain glycerol production seems not to be related to sugar consumption, as strain PAS 13, that left 3.66 g/L of sugars, showed one of the highest levels of glycerol production. Only small differences were found in acetic acid production.

All the data obtained were analyzed by PCA (Figure 3B). Function (F1) accounted for 44.67% of the total variance and significantly correlated ($\alpha < 0.001$) CO₂ production after 312 h and ($\alpha < 0.05$) 624 h, with fructose residue and acetic acid production concentration. The second function (F2) explained 24.20% of the total variance and was correlated ($\alpha < 0.01$) with fermentation vigor and ethanol production. No significant correlations were found with glucose residue and glycerol production. In these conditions, strain origin seems to be the explanation of the strain clustering. “PAS” strains isolated from grape must B showed the worst fermentation performances with the presence of fructose residues, whereas “FRI” strains showed good fermentation performances, producing the highest level of acetic acid. EC1118 single strain fermentation clustered

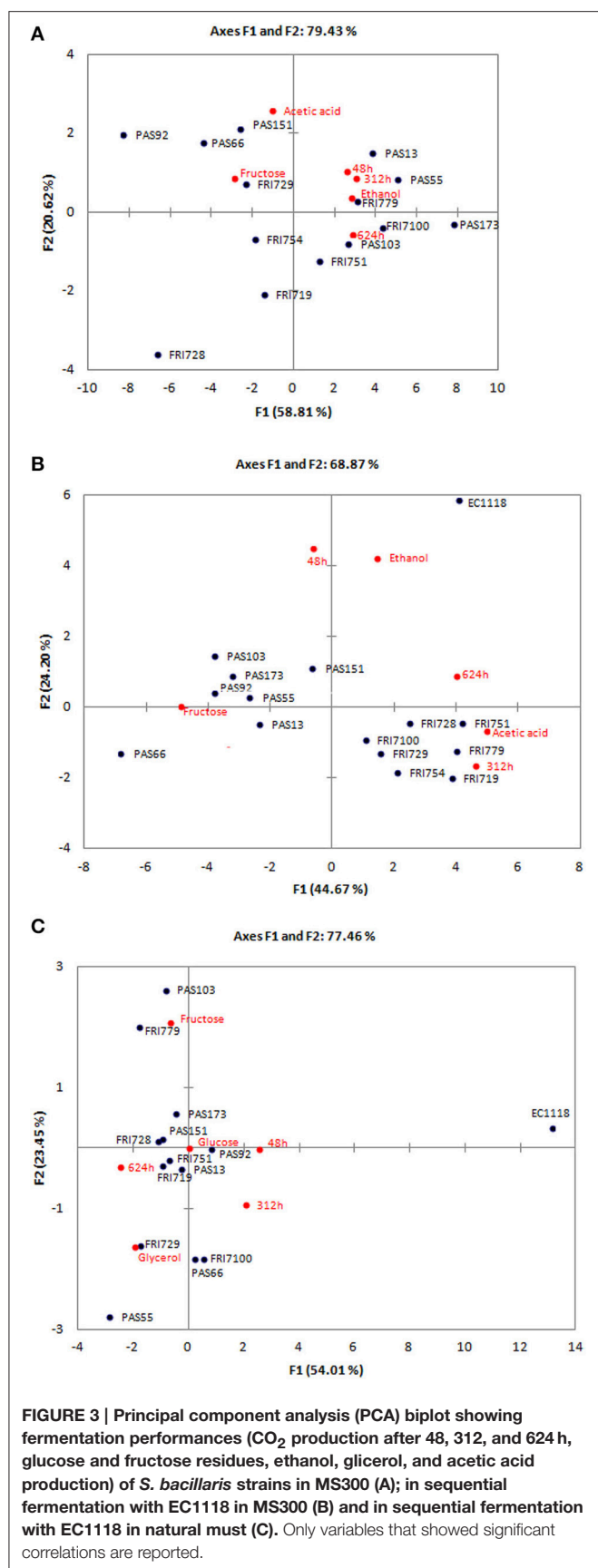
separately due to higher fermentation vigor and ethanol production than the sequential fermentations.

Fermentation Activity in Natural Must

Sequential fermentations of *S. bacillaris* and *S. cerevisiae* EC1118 were run in natural must in the same condition used for the synthetic must (see Supplementary Material SM_5). In this case the widest range of CO₂ production was found after 288 h. EC1118 completed the fermentation within 12 days, sequential fermentations in 16 days. Only when FRI754, FRI779 and PAS103 were tested a limited fructose residue was found (from 1.47 to 2.40 g/L). Glycerol was significantly higher in sequential fermentations than in EC1118 single strain fermentation. Glycerol concentration in EC1118 single strain fermentation was 4.86 g/L, whereas sequential fermentations produced an average of 5.84 g/L glycerol, with an average increase of 0.98 g/L. Ethanol concentration ranged between 11.19 and 11.61% (v/v). No significant differences were found between EC1118 fermentation and sequential fermentations for 5 out of 14 strains tested. This could be due to the lower sugar concentration present in the natural must than in the synthetic must of the previous trial. Indeed, glycerol production is directly proportional to the sugar content: the higher the sugar content, the higher the glycerol concentration, therefore the lower the ethanol concentration (Tilloy et al., 2014). Acetic acid concentrations were very limited and lower than those found during synthetic must fermentations (ranging from 0.28 to 0.36 g/L). All the data obtained were analyzed by PCA (Figure 3C). Function (F1) accounted for 54.01% of the total variance and significantly correlated ($\alpha < 0.001$) CO₂ production after 48, ($\alpha < 0.05$) 312, and 624 h, and ($\alpha < 0.05$) glycerol production. The second function (F2) explained 23.45% of the total variance and was correlated ($\alpha < 0.001$) glucose and ($\alpha < 0.05$) fructose residue. No significant correlations were found with acetic acid and ethanol content. In these conditions differences between sequential fermentations and EC1118 single-strain fermentation were more evident than in synthetic must in term of fermentation performances. In all the sequential fermentations a slower fermentation rate than that of EC1118 single-fermentation was found. The main differences among sequential fermentations were due to the presence of different level of sugar residues (ax F2).

DISCUSSION

With the aim of selecting wine yeasts carrying antifungal activity, fermenting musts obtained from late-harvest, overripe grape variety, naturally dried, were considered. The overripe grape berries show a very soft texture, due to the senescence or aging of fruit tissues. These physical features increase susceptibility to mechanical damage and infection by fungal pathogens (Genovese et al., 2007). Molds, such as *Botrytis cinerea* are abundant in this environment and yeasts must carry antifungal activity to compete. After yeast isolation and identification, 36 isolates were found to belong to the *S. bacillaris* species. This yeast possesses a fructophilic character and a poor ethanol yield from sugar consumed (Magyar and Tóth, 2011). Several ecological studies evidenced the presence of this species on grape berry



surface and during spontaneous fermentations of musts in several countries (Bokulich et al., 2013a,b; Milanović et al., 2013; Wang et al., 2015), suggesting that this species has a specific role in the fermentation process. *S. bacillaris* carries some very interesting enological traits, such as growth at high concentrations of sugars and low temperatures (Sipiczki, 2003; Tofalo et al., 2012), and production of low levels of acetic acid, acetaldehyde and significant amounts of glycerol from consumed sugars (Magyar and Tóth, 2011). Contrary to the most common non-*Saccharomyces* yeasts, it can survive until the end of the alcoholic fermentation due to its ability to tolerate high concentrations of ethanol present in the wine (Rantsiou et al., 2012; Englezos et al., 2015).

By means of SAU-PCR analysis at least 14 genetically defined groups were found.

One isolate for each group was selected to test antagonistic activity to *Botrytis cinerea* both *in vitro* and *in vivo*. The results of the antagonistic activity *in vitro* assay, obtained growing simultaneously the yeast strains together with the fungal mycelium, demonstrated that yeast isolates were able to limit the causal agent of gray mold disease and this seems not to be related to the acidic condition of the environment (PDA medium at pH 3.5). The values of the inhibition of the radial mycelium growth were comparable with those previously found for other antagonistic yeasts (Parafati et al., 2015). Due to the different growth rate of *S. bacillaris* and *B. cinerea* on PDA medium, where *S. bacillaris* can not found the optimal growth conditions, a 4 days pre-incubation of yeast strains was performed before testing the antifungal activity in the following *in vitro* assays.

Since several mechanisms have been reported to play a significant role in the biocontrol activity of antagonistic yeasts, in this study we evaluated the possible role of the main biocontrol modes of action, such as production of VOCs and cell wall-degrading enzymes, in controlling the *in vitro* growth of *B. cinerea*. Plate assays evaluating cell-wall degrading enzymes (pectinolytic, proteolytic and chitinolytic activities) indicated that pectinolytic activity was not present, chitinolytic activity was evident for all the strains although at low level, and only two strains FRI719 and FRI751 showed potential to produce proteolytic enzymes as they grew well on plates containing skin milk. The results regarding VOCs production were more promising. These compounds have been shown to have an antifungal effect and contribute to the biocontrol activity found in several yeast species, such as *Wickerhamomyces anomalus*, *Candida intermedia*, and *Sporidiobolus pararoseus* (Druvefors and Schnürer, 2005; Huang et al., 2011, 2012). In particular, more recently Hua et al. (2014) demonstrated that the biocontrol ability of *W. anomalus* can be attributed to the production of 2-phenylethanol, a secondary alcohol which affects spore germination, growth, toxin production, and gene expression in *Aspergillus flavus*.

It is well-known that volatile molecules, such as higher alcohols and esters are produced by non-*Saccharomyces* wine yeasts and their concentration is strain dependent (Rojas et al., 2001; Clemente-Jimenez et al., 2004; Jolly et al., 2006, 2014).

The inhibition percentage of the radial mycelial growth, during *in vitro* plate assay, was very high indicating a strong

antifungal activity and suggesting VOCs as main responsible for *S. bacillaris* antifungal effects. The inhibitory effect of the *S. bacillaris* strains was further proven on wounded grape berries artificially inoculated with *B. cinerea*. All the strains were able in reducing *B. cinerea* gray mold decay. In particular the lesion diameter reduction was comparable with that found previously for other antifungal yeasts (Parafati et al., 2015). Regarding the ability of the yeast strains to survive and multiply in artificial wounds made on grapes, results indicated that after 5 days from the inoculation, after squeezing the berries, the cell concentrations were very high (from 2 to 3×10^6 CFU/ml). This finding suggested that *S. bacillaris* strains can easily growth in the wound environment on grape berries and have a considerable colonizing potential. Due to the promising *S. bacillaris* antifungal activity and the well-proved enological property of this species, fermentation ability of the *S. bacillaris* strains isolated in this study were tested using an inoculum carrying a cell concentration similar to that found in the infected berries. This concentration is interesting from an enological point of view as natural yeast population size in grape must after pressing, usually ranges from 10^4 to 10^6 cells/ml (Fleet et al., 1984; Combina et al., 2005; Jolly et al., 2006). Moreover, in several studies where *S. bacillaris* was used in sequential fermentation together with *S. cerevisiae* the inoculum size was 10^6 cell/ml and at this concentration this yeast produced positive effect on wine (Andorrà et al., 2010; Rantsiou et al., 2012). *S. bacillaris* single-strain fermentation confirmed the fructophilic character, the high glycerol production and a fermentation rate slower than that of *S. cerevisiae* EC1118 (Magyar and Tóth, 2011; Englezos et al., 2015). When sequential fermentations were performed in synthetic must *S. bacillaris* strains significantly increased glycerol content and reduced ethanol concentration. In sequential fermentations of natural must the mixed starters consumed all the reducing sugars (only in few cases a minimal sugar residues remained in the wine) and *S. bacillaris* significantly increased the glycerol content, although the fermentation rate was slower than that of EC1118 single-strain fermentation. In all the fermentation trial *S. bacillaris* strains produced very low acetic acid concentrations. The level is lower than that found for other *S. bacillaris* strains isolated from another Italian winemaking region (Englezos et al., 2015). This finding is very interesting as one of the main concerns in the use of non-*Saccharomyces* strains in winemaking is their propensity to produce high level of volatile acidity (Jolly et al., 2006).

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In this paper we demonstrated for the first time that strains of *S. bacillaris* carry antifungal activity and this property can be used to control the growth of the fungal pathogen *B. cinerea* on grape. Moreover, the interesting enological properties possessed by these strains have been proven to enhance wine quality. The high wound colonization ability of *S. bacillaris* found in this work together with its propensity to colonize the grape berry surface (Wang et al., 2015) suggests that the use of this yeast as biocontrol agent on grape plant and berries could influence the following must fermentation, although the presence of *S. cerevisiae* is needed to complete the fermentation. Further studies will be needed to assess the efficacy of *S. bacillaris* as biocontrol agent directly in vineyard to couple the antifungal activity with the enological properties of these strains. In this sense our results provide a new insight in the management of non-*Saccharomyces* yeast for winemaking and open new prospects to a more integrated strategy for increasing wine quality.

AUTHOR CONTRIBUTIONS

Principal investigator: WL. Designed research: WL, BB, CV, AG. Performed antifungal activity: WL, FF, CN. Performed genetic characterization: WL, BB, GC. Performed fermentation trials and HPLC analyses: WL, CN, MC. Wrote the manuscript: VC. Edited the manuscript: VC, AG, WL, BB, CN.

FUNDING

The research financial support was provided by CAPES-Coordenação de Aperfeiçoamento de Pessoal de Nível Superior and MIUR Ex 60% (Ministero dell'Istruzione, dell'Università e della Ricerca) (Grant No. 60A08/3022/15).

ACKNOWLEDGMENTS

The authors wish to thank Valentino Pizzocchero for skilful assistance in HPLC analysis and the Consortium "Vini D.O.C. Bagnoli" to supply the Raboso piave musts used in this study.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fmicb.2016.01249>

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Corrigendum: Biocontrol Ability and Action Mechanism of *Starmerella bacillaris* (Synonym *Candida zemplinina*) Isolated from Wine Musts against Gray Mold Disease Agent *Botrytis cinerea* on Grape and Their Effects on Alcoholic Fermentation

OPEN ACCESS

Edited and reviewed by:

José Manuel Guzmán,
Spanish National Research Council,
Spain

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Specialty section:

This article was submitted to
Food Microbiology,
a section of the journal
Frontiers in Microbiology

Received: 29 August 2016

Accepted: 07 September 2016

Published: 27 September 2016

Citation:

Lemos Junior WJF, Bovo B, Nadai C,
Crosato G, Carlot M, Favaron F,
Giacomini A and Corich V (2016)
Corrigendum: Biocontrol Ability and
Action Mechanism of *Starmerella*
bacillaris (Synonym *Candida*
zemplinina) Isolated from Wine Musts
against Gray Mold Disease Agent
Botrytis cinerea on Grape and Their
Effects on Alcoholic Fermentation.
Front. Microbiol. 7:1499.
doi: 10.3389/fmicb.2016.01499

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Keywords: antifungal activity, SAU-PCR, grape must, VOCs, lytic enzymes, fermentation, glycerol

A corrigendum on

Biocontrol Ability and Action Mechanism of *Starmerella bacillaris* (Synonym *Candida zemplinina*) Isolated from Wine Musts against Gray Mold Disease Agent *Botrytis cinerea* on Grape and Their Effects on Alcoholic Fermentation

by Lemos, W. J. Jr., Bovo, B., Nadai, C., Crosato, G., Carlot, M., Favaron, F., et al. (2016). Front. Microbiol. 7:1249. doi: 10.3389/fmicb.2016.01249

Reason for Corrigendum:

Due to an incorrect earlier citation, the name of the first author should now read Wilson José Fernandes Lemos Junior.

The original article has been updated.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Alternative Glycerol Balance Strategies among *Saccharomyces* Species in Response to Winemaking Stress

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OPEN ACCESS

Edited by:

Alberto Mas,
University Rovira i Virgili, Spain

Reviewed by:

Maurizio Ciani,
Università Politecnica delle Marche,
Italy
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Specialty section:

This article was submitted to
Food Microbiology,
a section of the journal
Frontiers in Microbiology

Received: 10 February 2016

Accepted: 17 March 2016

Published: 31 March 2016

Citation:

Pérez-Torrado R, Oliveira BM,
Zemančíková J, Sychrová H and
Querol A (2016) Alternative Glycerol
Balance Strategies among
Saccharomyces Species in Response
to Winemaking Stress.
Front. Microbiol. 7:435.
doi: 10.3389/fmicb.2016.00435

Production and balance of glycerol is essential for the survival of yeast cells in certain stressful conditions as hyperosmotic or cold shock that occur during industrial processes as winemaking. These stress responses are well-known in *S. cerevisiae*, however, little is known in other phylogenetically close related *Saccharomyces* species associated with natural or fermentation environments such as *S. uvarum*, *S. paradoxus* or *S. kudriavzevii*. In this work we have investigated the expression of four genes (*GPD1*, *GPD2*, *STL1*, and *FPS1*) crucial in the glycerol pool balance in the four species with a biotechnological potential (*S. cerevisiae*; *S. paradoxus*; *S. uvarum*; and *S. kudriavzevii*), and the ability of strains to grow under osmotic and cold stresses. The results show different pattern and level of expression among the different species, especially for *STL1*. We also studied the function of *Stl1* glycerol symporter in the survival to osmotic changes and cell growth capacity in winemaking environments. These experiments also revealed a different functionality of the glycerol transporters among the different species studied. All these data point to different strategies to handle glycerol accumulation in response to winemaking stresses as hyperosmotic or cold-hyperosmotic stress in the different species, with variable emphasis in the production, influx, or efflux of glycerol.

Keywords: glycerol, yeast, *Saccharomyces*, stress, winemaking

INTRODUCTION

In the fermentation industry, especially winemaking, the resistance to osmotic stress and the ability to grow at low temperatures are required features for yeast strains (Pretorius et al., 2012). It is known that *S. cerevisiae* seeks to increase intracellular glycerol content when subjected to osmotic stress or cold in vinification or standard laboratory growth conditions (Panadero et al., 2006; Petelenz-Kurdiel et al., 2013; Oliveira et al., 2014). This intracellular accumulation is very important for osmotic equilibrium during the first phase of fermentation and to act as key cryoprotector agent for adaptation to cold environments allowing cellular viability with

Abbreviations: *GPD1*, glycerol-3-phosphate dehydrogenase 1 gene; *GPD2*, glycerol-3-phosphate dehydrogenase 2 gene; *STL1*, glycerol proton symporter gene; *FPS1*, glycerol channel gene; *ACT1*, actin gene; *RDN18-1*, 18S ribosomal RNA gene; qRT-PCR, quantitative real-time PCR; dithiothreitol, DTT.

implications in the fermentation yield (Remize et al., 2001; Tulha et al., 2010). A rapid and specific activation of the gene expression have been identified as an essential mechanism in the *S. cerevisiae* cells to respond to acute stresses, such as those associated with the different industrial fermentation processes (de Nadal et al., 2011). However, little is known about these stress responses in other *Saccharomyces* species associated with natural or fermentation environments such as *S. uvarum* (Naumov et al., 2002; Rementeria et al., 2003; Demuyter et al., 2004), *S. paradoxus*, isolated from Croatian vineyards (Redzepovic et al., 2002) or natural yeast hybrids between species of the genus *Saccharomyces* such as *S. cerevisiae* × *S. kudriavzevii* (Gonzalez et al., 2007) and *S. cerevisiae* × *S. uvarum* (Le Jeune et al., 2007; Pérez-Torrado et al., 2015) which may participate in the fermentative processes. *S. uvarum* and *S. kudriavzevii* present important physiological traits like the ability to grow at lower temperatures and produce more glycerol than *S. cerevisiae* (Gonzalez et al., 2007; Gamero et al., 2013; Oliveira et al., 2014). However, *S. paradoxus*, besides being a widely distributed yeast species mainly associated with natural environments and not very relevant in fermentations, is physiologically more similar to *S. cerevisiae* (Tronchoni et al., 2009).

It is well-known that *S. cerevisiae* and other yeast species are capable to modulate the glycerol synthesis and its intracellular content in accordance with environmental osmotic changes (Hohmann et al., 2007; Hubmann et al., 2011). They can also control an active glycerol import from the extracellular medium in symport with protons via Stt1 transporter (Tulha et al., 2010; Dušková et al., 2015a). Besides its important role in osmoregulation, the Stt1 function is directly related to cell survival and adaptation to cold stress in *S. cerevisiae* strains (Tulha et al., 2010). The yeast cells may also regulate their glycerol content by controlling its efflux via the Fps1 channel (Luyten et al., 1995). This channel can be quickly closed avoiding the glycerol efflux, and thus contributing to an efficient osmoregulation with direct implications on increasing the fermentation yield (Wei et al., 2013).

The understanding of the phylogenetic and physiological relationships between *S. cerevisiae* and other *Saccharomyces* species, as well as main ecological, environmental, and human factors that have driven the emergence of phenotypic changes among species of *Saccharomyces* genus, have been cleared in many works (Landry et al., 2006; Peris et al., 2014). Several studies have focused in understanding the cryophilic character of *S. uvarum* and specially *S. kudriavzevii* at the molecular level, including transcriptomic and metabolomic studies (Combina et al., 2012; López-Malo et al., 2013). Some aspects of *S. kudriavzevii* have been highlighted in relation to cold resistance and winemaking as membrane composition (Tronchoni et al., 2012), or translation efficiency (Tronchoni et al., 2014). However, little information about these species and the glycerol synthesis is available. In the case of *S. kudriavzevii*, the increased cold tolerance has been related to elevated glycerol synthesis as a consequence of increased expression and activity of Gpd1p in winemaking conditions (Oliveira et al., 2014). For this reason a better understanding of *Saccharomyces* species physiological and

TABLE 1 | Strains used in this study.

Strain	Species	Description
T73 ^a	<i>S. cerevisiae</i>	Wine strain, Alicante, Spain
FCry	<i>S. cerevisiae</i>	Wine strain, commercial (AEB), France
Chr 16.2	<i>S. paradoxus</i>	Wild strain, Oak bark, Hungary
108	<i>S. paradoxus</i>	Wild strain, Croatia
BMV58	<i>S. uvarum</i>	Wine, Spain
12600 ^a	<i>S. uvarum</i>	Sweet wine, Spain
CR85	<i>S. kudriavzevii</i>	Wild strain, Oak bark, Spain
IFO1802 ^b	<i>S. kudriavzevii</i>	Type strain, Soil, Japan
BY4741hog1Δstt1Δ	<i>S. cerevisiae</i>	Lab strain (Dušková et al., 2015b)
BY-hs-YEp352	<i>S. cerevisiae</i>	BY4741hog1Δstt1ΔYEp352 (This work)
BY-hs-pSTL1-T73	<i>S. cerevisiae</i>	BY4741hog1Δstt1ΔYEp352-STL1 _{T73} (This work)
BY-hs-pSTL1-BMV58	<i>S. cerevisiae</i>	BY4741hog1Δstt1ΔYEp352-STL1 _{BMV58} (This work)
BY-hs-pSTL1-IFO1802	<i>S. cerevisiae</i>	BY4741hog1Δstt1ΔYEp352-STL1 _{IFO1802} (This work)

Some strains are available from collections.

^aCECT; ^bNBRC.

molecular features with potential biotechnological interest is needed.

Hence, in this work we decided to investigate the expression of genes crucial to the balance of glycerol (*GPD1*, *GPD2*, *STL1*, and *FPS1*) in two yeast strains of each of the four species with a biotechnological potential (*S. cerevisiae*; *S. paradoxus*; *S. uvarum*; and *S. kudriavzevii*). We also studied the function of Stt1 glycerol symporter, in the survival to osmotic changes and cell growth capacity in winemaking environments.

METHODS

Yeast Strains and Growth Conditions

Yeast strains origin, availability are described in **Table 1**. Two different strains of each species were studied. For *S. cerevisiae*, T73 model wine strain (Querol et al., 1994; Lopes et al., 2010) and the commercial wine strain Fermol Cryophile FCry (AEB Group); selected as adapted to low temperature (Gamero et al., 2013) were chosen. The 108 and Chr 16.2 strains isolated from natural environment were used as representatives of *S. paradoxus*. For *S. uvarum*, the 12600 and BMV58 strains isolated from wine in Spain were studied. BMV58 was commercialized (Lallemand Inc) because of its high glycerol production and good fermentative properties (patent ES2330709 B1). For *S. kudriavzevii* species, IFO1802 (type strain), and the CR85 wild strain isolated in Spain (Dušková et al., 2015b) were used. The *S. cerevisiae* BY4741Δhog1Δstt1 (Pérez-Torrado et al., 2009) was used as a laboratory strain for the expression of *STL1* genes and comparison of the function of their products under hyperosmotic-stress conditions.

Yeast cells were maintained and grown in YPD medium (2% glucose, 2% Bacto peptone, and 1% Yeast extract) or SC-Ura medium (YNB 0.67%, glucose 2%, Drop-out –Ura 1.92 g/l (Formedium)) at 28°C for the *S. cerevisiae* and *S. paradoxus* species and 25°C for *S. kudriavzevii* and *S. uvarum* species.

The wine fermentations were performed in 250 ml bottles filled with 200 ml of MS300 synthetic must (100 g/L glucose, 100 g/L fructose, 6 g/L citric acid, 6 g/L malic acid, mineral salts, vitamins, anaerobic growth factors, 300 mg/L assimilable nitrogen) simulating standard grape juice (Bely et al., 2003) at 12°C with agitation (150 rpm) in triplicate. Overnight precultures were inoculated at 5.0×10^6 cells/ml density determined by measuring OD₆₀₀. To study the expression of genes related to glycerol balance under hyperosmotic stress, the cells from exponentially growing precultures were washed with water and transferred to YP (2% Bacto peptone and 1% yeast extract) with 2% glucose or 2% mannitol as a source of carbon, to the same medium supplemented with 1 M sorbitol (hyperosmotic stress), which is not assimilable for any of the species studied, or to H₂O (hypoosmotic stress). This experiment was performed in 21 flasks filled with 400 ml of media in triplicate at 25°C and 150 rpm.

The tolerance to hyperosmotic stress was evaluated by drop tests. Yeasts were grown overnight in YPD or SC-ura medium, then cultures were diluted to OD₆₀₀ = 0.2 and cells were allowed to grow in the same media until OD₆₀₀ = 1. Then, serial dilutions of cells were transferred to plates with YPD; YPD + 0.8 M NaCl; YPD + 1.25 M KCl, incubated at 12 and 25°C and evaluated each day. The growth of *Saccharomyces* species was also compared in plates with SC containing 2 M sorbitol or 2 M KCl and supplemented or not with 1 mM glycerol. To investigate the functional differences of *Stl1*, the growth of BY4741 $\Delta hog1 \Delta stl1$ cells transformed with appropriate plasmids was monitored on plates with SC-ura containing 0.7 M sorbitol, which or 0.3 M KCl and with or without 10 mM glycerol. Experiments were performed in triplicate, representative results are shown.

For the intracellular glycerol measurements, cells were grown in 250 ml flasks with SC-ura with 10% of glucose at 28°C with agitation (150 rpm) in triplicate until the glucose concentration achieved <2 g/l.

Plasmid Construction

Plasmids expressing the *S. cerevisiae* T73, *S. bayanus* BMV58, and *S. kudriavzevii* IFO1802 *STL1* genes under *NHA1* gene promoter were constructed by exchanging the *NHA1* coding sequence in pNHA1-985 (YEp352 derivative, Kinclová et al., 2001) by homologous recombination. All constructions were confirmed by diagnostic PCR and sequencing. The primers, listed in Table 2, were designed based on data from *Saccharomyces* Genome Database (Cherry et al., 2012) and used to amplify the DNA fragments (from genomic DNAs) with suitable flanking regions for homologous recombination and confirmation.

Analytical Determinations

The extracellular glycerol concentrations and residual sugars (glucose and fructose) were determined in must and medium samples by HPLC (Thermo Fisher Scientific, Waltham, MA)

equipped with a refraction index detector. The column employed was a HyperREZTM XP Carbohydrate H+ 8 μ m (Thermo Fisher Scientific) and the conditions used in the analysis were as follows: eluent, 1.5 mM H₂SO₄; flux, 0.6 ml/min; and oven temperature, 50°C. The samples were diluted, filtered through a 0.22- μ m nylon filter (Symta, Madrid, Spain) and injected in duplicate.

To determine intracellular glycerol content, 5 OD₆₀₀ units were harvested by filtration and quickly washed with 5 ml of water and transferred to a tube containing 1 ml of cold water. After no more than 20 s after sampling, the yeast suspension was boiled for 10 min, cooled on ice, and centrifuged at $15,300 \times g$ for 10 min (4°C). The supernatant was collected, filtered and directly analyzed by HPLC. A second sample (5 OD₆₀₀ units) was harvested by filtration in cellulose membrane, 25 mm pore size 0.45 μ m (MF-Milipore) previously dried in the microwave at 350W for 20 min. and weighed. To determine dry weight, the cells in the membrane were carefully washed with 1 ml of water and dried under the same conditions. The values obtained are expressed as μ g of glycerol per mg of yeast cells. Experiments were performed in triplicate.

Gene Expression Determination

For each culture, 10–20 ml sample was taken at different times. The cells were quickly harvested by centrifugation, washed and frozen in liquid N₂. Then, frozen cells were lysed and homogenized by FastPrep-24 (MP Biomedicals) in LETS buffer (10 mM Tris pH 7.4, 10 mM lithium-EDTA, 100 mM lithium chloride, 1% lithium lauryl sulfate) with acid-washed glass beads (0.4 mm diameter; Sigma-Aldrich) for 30 s six times alternating with ice incubation. Total RNA was extracted and purified using the phenol:chloroform method with minor modifications (Combina et al., 2012). Then the RNA was converted to cDNA and the expression of *GPD1*, *GPD2*, *STL1*, and *FPS1* genes was quantified by qRT-PCR (quantitative real-time PCR). The cDNA strand was constructed using 2 μ g of RNA mixed with 0.8 mM dNTPs, 80 pmol Oligo (dT) in 13 μ l. The mixture was heated to 65°C for 5 min and quenched on ice for 1 min. 5 mM dithiothreitol (DTT), 50 U of RNase inhibitor (Invitrogen), 1 \times First Strand Buffer (Invitrogen) and 200 U Superscript III (Invitrogen) were added to the 20 μ l mixture, which was incubated at 50°C for 60 min and the reaction was inactivated after 15 min at 70°C. qRT-PCR was performed with gene-specific primers (200 nM) designed for each specie (Table 2) from sequences consensus between the different strains in a 10 μ l reaction, using the Light Cycler FastStart DNA MasterPLUS SYBR green (Roche Applied Science, Germany) in a LightCycler® 2.0 System (Roche Applied Science, Germany). All samples were processed for melting curve analysis, amplification efficiency and DNA concentration determination. A mixture of all samples and serial dilutions (10^{-1} to 10^{-5}) was used as standard curve. Two different constitutive reference genes were used (*ACT1* and *RDN18-1*) to normalize the amount of mRNA and ensure accuracy, correct interpretation, and repeatability (Starovoytova et al., 2013). The results were normalized by using the normalization factor obtained from geNorm VBA applet (Vandesompele et al., 2002).

TABLE 2 | Primers used in this study.

Name	Sequence	Purpose	Species
GPD1-F	TGTGGTGCTTTGAAGAACG	qPCR and sequencing	<i>S.c.</i> , <i>S.u.</i> , <i>S.p.</i> , <i>S.k.</i>
GPD1-R	GTTTCTTCTCTAGATTCTGG	qPCR and sequencing	<i>S.c.</i> , <i>S.u.</i> , <i>S.p.</i> , <i>S.k.</i>
GPD2-F	GTTCCACAGACCTACTTCC	qPCR	<i>S.c.</i> , <i>S.u.</i> , <i>S.p.</i> , <i>S.k.</i>
GPD2-R	CCATCCCACACCTTCTACG	qPCR	<i>S.c.</i> , <i>S.u.</i> , <i>S.p.</i> , <i>S.k.</i>
FPS1-F	GTTTTGYGTTTTCCAAAGC	qPCR	<i>S.c.</i> , <i>S.u.</i> , <i>S.p.</i> , <i>S.k.</i>
FPS1-R	TGATAAGCCATRGARGCATT	qPCR	<i>S.c.</i> , <i>S.u.</i> , <i>S.p.</i> , <i>S.k.</i>
STL1-F	GCTTATTGGATTGATTTGGG	qPCR	<i>S.c.</i> , <i>S.u.</i> , <i>S.p.</i>
STL1-R	TGTTAACAGCATCGTGAAGC	qPCR	<i>S.c.</i> , <i>S.u.</i> , <i>S.p.</i>
STL1-F	ACAGCATCGTGAAGCATAGC	qPCR	<i>S. kudriavzevii</i>
STL1-R	TGGCTGATTCTCAAAGTCG	qPCR	<i>S. kudriavzevii</i>
ACT1-F	CATGTTCCCAGGTATTGCCG	qPCR	<i>S.c.</i> , <i>S.u.</i> , <i>S.p.</i> , <i>S.k.</i>
ACT1-R	GCCAAAGCGGTGATTTCT	qPCR	<i>S.c.</i> , <i>S.u.</i> , <i>S.p.</i> , <i>S.k.</i>
18S-F	TTGCGATAACGAACGAGACC	qPCR	<i>S.c.</i> , <i>S.u.</i> , <i>S.p.</i> , <i>S.k.</i>
18S-R	CATCGGCTTGAAACCGATAG	qPCR	<i>S.c.</i> , <i>S.u.</i> , <i>S.p.</i> , <i>S.k.</i>
P-NHA1	CAACTCTGTGTGATATAG	Verification	<i>S. cerevisiae</i>
ScSTL1 - R2	CAACCCTGTTCCAACACC	Verification	<i>S. cerevisiae</i>
ScSTL1 - F2	GGACAGTCCGGTTGGGGTTG	Verification	<i>S. cerevisiae</i>
SbSTL1 - F2	CTACCCTGAAACTGCTGG	Verification	<i>S. uvarum</i>
SbSTL1 - R2	GCCCAGTAGTCACGGAAGC	Verification	<i>S. uvarum</i>
SkSTL1 - F2	CCCTGAAACCGCTGGTAG	Verification	<i>S. kudriavzevii</i>
SkSTL1 - R2	GCCTTGGACATTTCCGAC	Verification	<i>S. kudriavzevii</i>
YEp352-R	GGGGATGTGCTGCAAGGCG	Verification	
YEp-SbSTL1-F	GTACATTATAAAAAAATCCTGAACCTAGCTAGATATTATGAAGGAATCAAAGTATCTAAG	Cloning YEp352	<i>S. uvarum</i>
YEp-SbSTL1-R	CACGACGTTGTAAACGACGCGCCAGTGCCAGCTTGCATGTTACTTTTCAGAGCTGTTTTCAT	Cloning YEp352	<i>S. uvarum</i>
YEp-ScSTL1-F	GTACATTATAAAAAAATCCTGAACCTAGCTAGATATTATGAAGGATTTAAATTATCG	Cloning YEp352	<i>S. cerevisiae</i>
YEp-ScSTL1-R	CACGACGTTGTAAACGACGCGCCAGTGCCAGCTTGCATGTCAACCTCAAATTTGCTT	Cloning YEp352	<i>S. cerevisiae</i>
YEp-SkSTL1-F	GTACATTATAAAAAAATCCTGAACCTAGCTAGATATTATGAGGAAATCAAAGTATC	Cloning YEp352	<i>S. kudriavzevii</i>
YEp-SkSTL1-R	CACGACGTTGTAAACGACGCGCCAGTGCCAGCTTGCATGCTAGTTTTCGGAATTTGGTTTC	Cloning YEp352	<i>S. kudriavzevii</i>

RESULTS

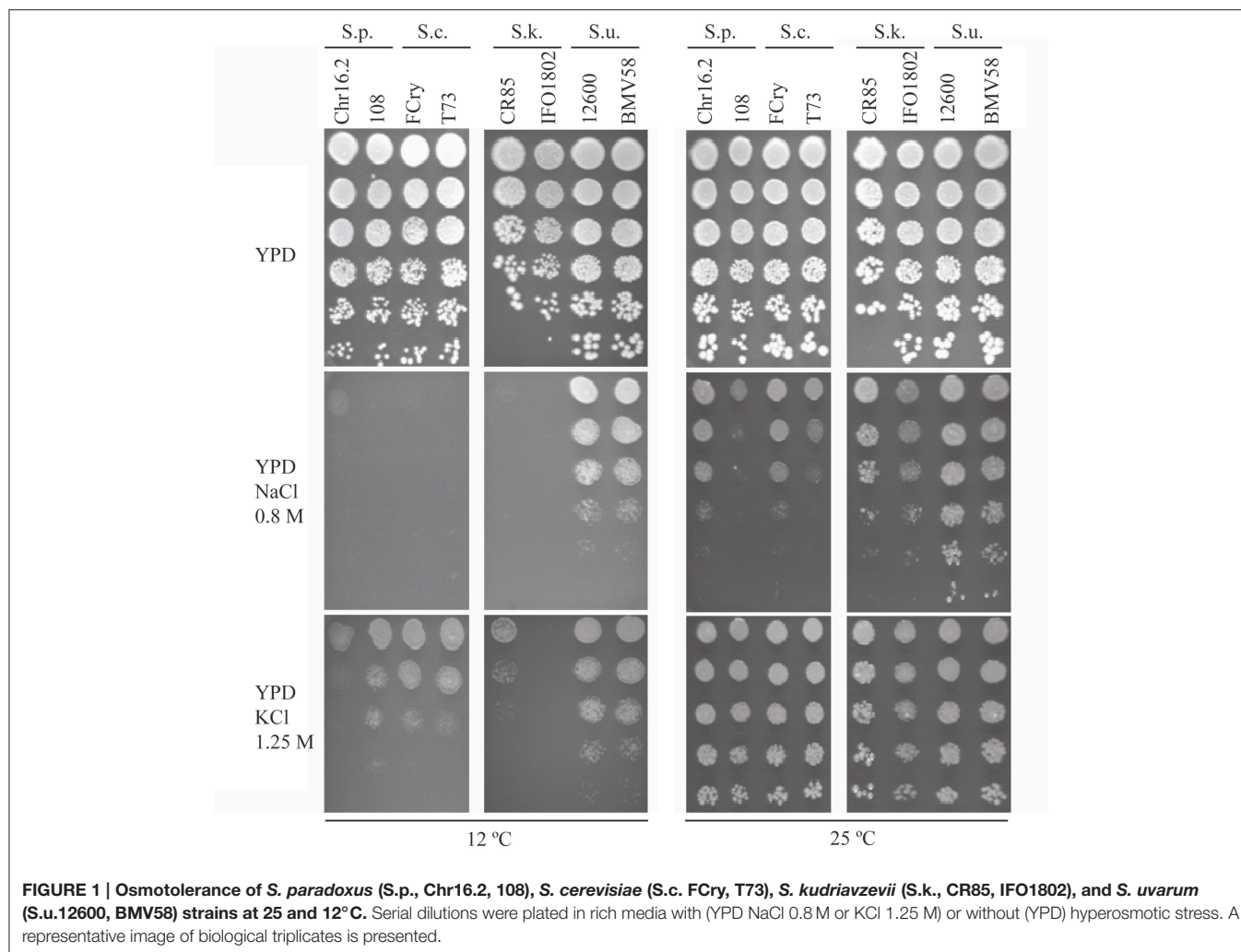
Saccharomyces Species Differ in Tolerance to hyperosmotic and Cold Stresses

The behavior of *S. cerevisiae* and other *Saccharomyces* species interesting for industrial applications was evaluated in response to wine fermentation relevant stresses. We selected hyperosmotic (NaCl 0.8 M and KCl 1.25 M) and a combination of hyperosmotic and cold stresses (12°C), two frequent suboptimal conditions during winemaking. We performed a drop test with two strains of each species (*S. paradoxus*, *S. cerevisiae*, *S. kudriavzevii*, and *S. uvarum*) on complete media and compared the growth in the above mentioned conditions (**Figure 1**). The results revealed that the used stresses have a very different effect on yeast growth depending not only on the species but even on the strain. The stress with KCl 1.25 M is the condition that has less effect on the yeast growth capacity, and the NaCl 0.8 M plus 12°C the most severe stress. The conditions NaCl 0.8 M hyperosmotic stress and KCl 1.25 M at 12°C hyperosmotic-cold stress generated intermediate growth capacity levels. The results showed clearly that the strains can cope better with a higher osmotic stress (KCl 1.25 M) than with the sodium toxicity (NaCl 0.8 M). In hyperosmotic stress mediated by NaCl 0.8 M we observed that

S. uvarum strains are the ones presenting the highest tolerance to hyperosmotic and a similar observation can be made in the most severe condition (NaCl 0.8 M plus 12°C). The other species showed similar behavior although *S. kudriavzevii* strains showed low growth levels in cold stress condition, especially IFO1802 strain. *S. cerevisiae* and *S. paradoxus* strains showed similar growth levels but strain 108 in NaCl 0.8 M at 25°C and strain Chr16.2 in KCl 1.25 M at 12°C presented lower growth levels than *S. cerevisiae* strains.

Glycerol Levels during Wine Fermentation

Since hyperosmotic and also cold stress responses are unequivocally related to glycerol accumulation we wanted to determine glycerol levels during hyperosmotic-cold stress in wine fermentations. Thus we performed wine fermentations in synthetic must with the studied *Saccharomyces* species and strains, and we measured intra- and extracellular amount of glycerol during the first hours and days of the fermentation. In the results presented in **Figure 2** we observed two steps regarding glycerol accumulation in *S. cerevisiae* strains. In the first step, glycerol starts to accumulate inside the cell (**Figure 2B**) immediately after inoculating into the cold-hyperosmotic condition, reaching a maximal value after 24 h. Also, minimal



glycerol levels are accumulated in extracellular media in the beginning of our experiment (**Figure 2A**). In the next 2 days, intracellular glycerol is reduced and tends to recover its original levels whereas extracellular glycerol increases with the time. In the case of *S. paradoxus* and *S. kudriavzevii*, maximal intracellular glycerol accumulation, which are approximately half of those in *S. cerevisiae* strains, occurs in the first hours and levels are maintained during 48 h. Analyzing the intracellular glycerol level (**Figure 2B**), it is interesting to note that, comparing with the other species, *S. cerevisiae* strains accumulated the higher levels of glycerol between 4 and 48 h of incubation. The *S. uvarum* strains showed the lowest values of intracellular glycerol with a maximal level after 1 h in the case of BMV58 and after 48 h in the case of 12600. Regarding extracellular glycerol (**Figure 2A**), *S. paradoxus* presented similar levels and accumulation pattern as *S. cerevisiae* and *S. uvarum* and, in addition, *S. kudriavzevii* showed a similar pattern but higher accumulation levels (around five times more). Is interesting to emphasize that *S. uvarum* and *S. kudriavzevii* showed a higher extracellular glycerol accumulation rate compared to the other two species. Interestingly, no extracellular glycerol was observed

at time 0 in any species. It should be noted that strains do not show significant growth after 1 or 4 h and maximal yeast biomass was observed at the 24 or 48 h time point except for the IFO1802 that show very low growth level in grape must (**Supplementary Figure 1**), in concordance with data observed in **Figure 1** in osmotic and cold stress conditions.

Changes in mRNA Levels of Genes Related to Glycerol Balance during Wine Fermentation and Hyperosmotic stress of Different *Saccharomyces* Species

To gain insights on the regulation of glycerol pools balance we studied variation in mRNA levels of key genes related to glycerol biosynthesis (*GPD1* and *GPD2*), efflux (*FPS1*), and influx (*STL1*) in the same wine fermentation conditions described above and in the same strains and species. The results (**Figure 3**) clearly revealed different patterns and levels of gene expression among the species in all four genes studied. In the case of *GPD1*, all the strains showed a general pattern of induction after the first hour but with marked differences in the expression levels. *S.*

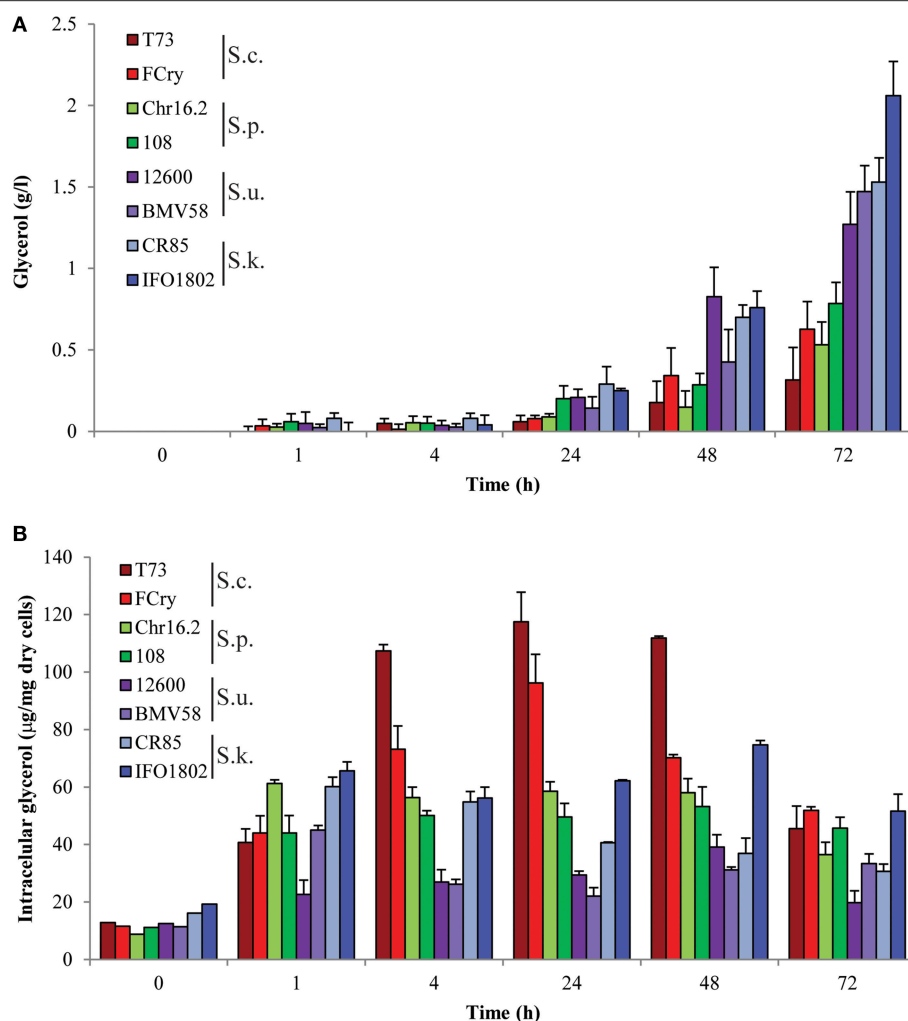


FIGURE 2 | Microvinification experiments in synthetic must at low temperature with *S. cerevisiae* T73 (dark red) and FCry (light red), *S. paradoxus* Chr16.2 (light green) and 108 (dark green), *S. uvarum* 12600 (dark purple) and BMV58 (light purple), and *S. kudriavzevii* CR85 (light blue) and IFO1802 (dark blue) strains. Precultured cells were inoculated in synthetic must at 12°C and samples were taken after 0, 1, 4, 24 and 48 h to determine extra (A) and intracellular (B) glycerol content for each strain. Three independent microvinification bottles were used for each strain and average \pm standard deviation is shown.

kudriavzevii strains showed the highest mRNA levels, specially IFO1802 strain that presented elevated expression of *GPD1* before stress and even more after 1 h of inoculation. For the *GPD2* gene, which is mainly involved in redox balance, some of the strains presented an induction with maximal levels after four (*S. uvarum* strains, FCry, 108, and CR85) or 48 (T73) hours whereas other strains (Chr16.2 and IFO1802) seem to not activate this gene showing low mRNA levels. The *FPS1* gene expression peaked after 1 h (108, CR85, *S. cerevisiae*, and *S. uvarum* strains) or 4 h (FCry), with the *S. cerevisiae* and *S. uvarum* strains showing the highest levels. The IFO1802, Chr16.2, and *S. kudriavzevii* strains did not showed significant increase of mRNA levels compared to the inoculum. Finally, the *SLT1* gene presented the most variable mRNA levels among the species showing highest values for the *S. uvarum* strains, especially BMV58, with a maximum after 1 h. Other species

showed a moderate amount of mRNA with maximum levels after 1 h (*S. kudriavzevii* strains) or 4 h (FCry). *S. paradoxus* strains showed very low *SLT1* mRNA levels along the experiment. Yeast growth phase does not has a dramatic impact in activation of gene expression in this conditions since the most important inductions were observed at 1 and 4 h, were grow was not observed. The comparison between glycerol content and gene expression results emphasize the importance of *GPD1* and *SLT1* in *S. kudriavzevii* and *S. uvarum* respectively, regarding their increased glycerol accumulation (Figure 2A). In the case of *S. cerevisiae* strains, increased *GPD2* levels, especially in FCry strain, could explain the high intracellular glycerol levels observed in Figure 2B. On the contrary, *FPS1* increased expression does not reflect extracellular glycerol levels in *S. cerevisiae* probably due to the tight regulation of this channel by posttranslational mechanisms.

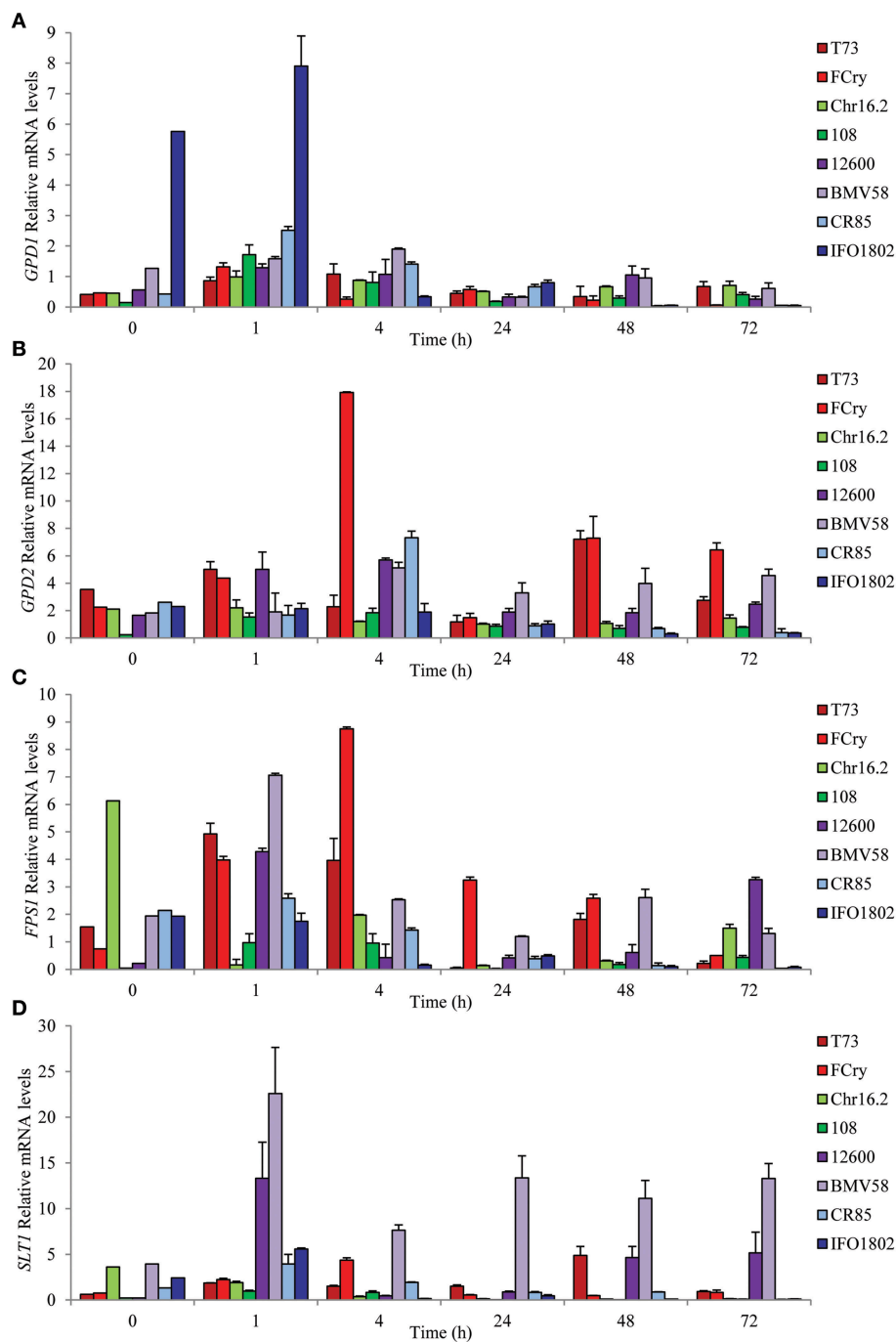


FIGURE 3 | Expression of glycerol balance related genes during first hours of low temperature microvinifications in synthetic must for *S. cerevisiae* T73 (dark red) and FCry (light red), *S. paradoxus* Chr16.2 (light green) and 108 (dark green), *S. uvarum* 12600 (dark purple) and BMV58 (light purple) and *S. kudriavzevii* CR85 (light blue) and IFO1802 (dark blue) strains. The genes related to glycerol biosynthesis, *GPD1* (A) and *GPD2* (B), and glycerol export, *FPS1* (C), and import, *STL1* (D), were studied. Samples were taken in the first part (0, 1, 4, 24, 48 and 72 h) of synthetic must microvinifications at 12°C. After RNA extraction, expression of the different genes was determined by qPCR and values were normalized with *ACT1* and *RDN18-1* constitutive genes. Three independent microvinification bottles were used for each strain and averages \pm standard deviation are shown.

To study the regulation of key genes related to intracellular glycerol balance under standard lab conditions (Figure 4) we used a representative strains of each species (T73, Chr16.2,

BMV58, and IFO1802) and measured mRNA levels of *GPD1*, *STL1* and *FPS1* after half, 1 and 2 h of transfer cells to a non-stress SC media (Figure 4A), hyperosmotic SC 1 M sorbitol

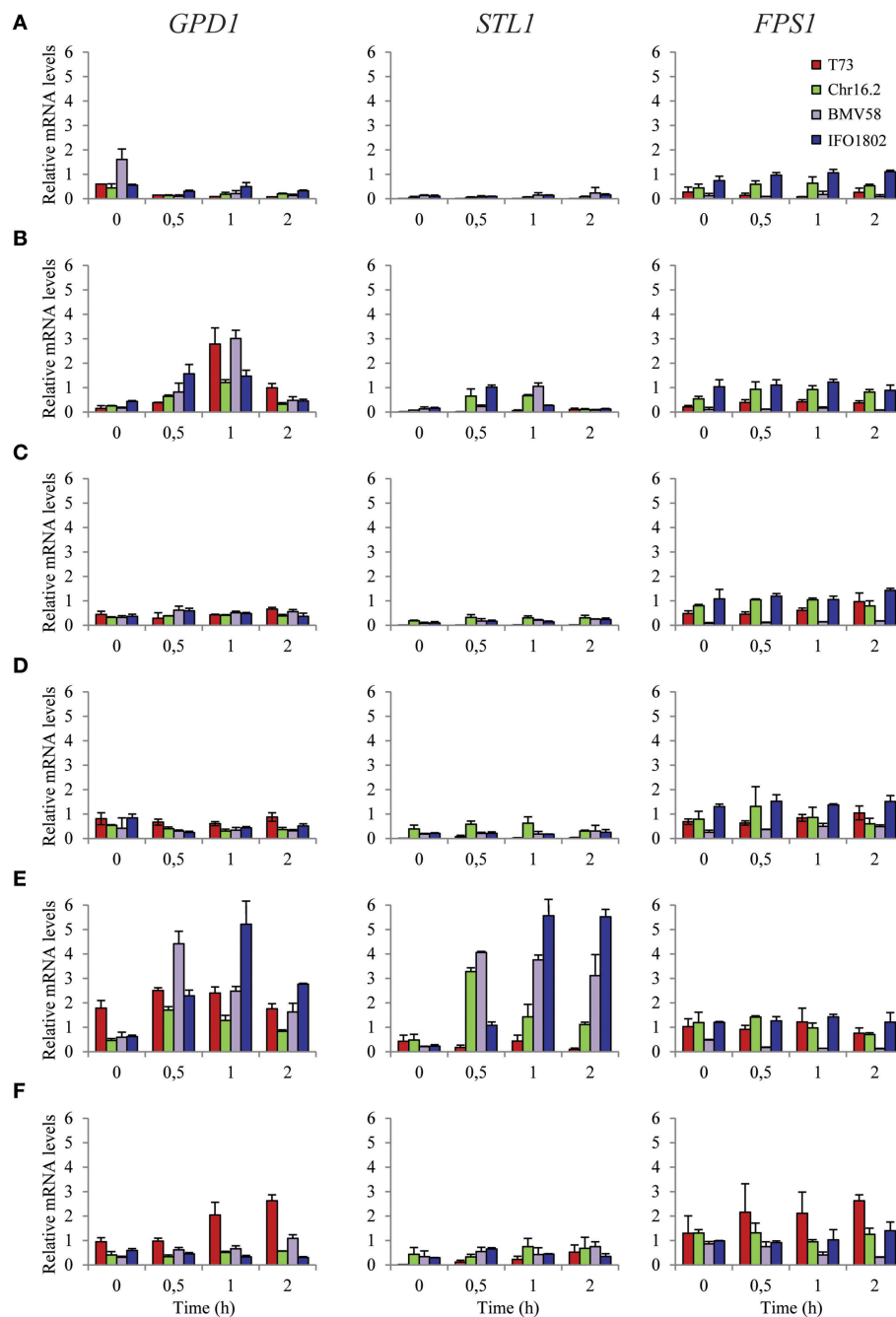


FIGURE 4 | Expression of glycerol balance related genes of *S. cerevisiae* T73 (red), *S. paradoxus* Chr16.2 (green), *S. uvarum* BMV58 (purple), and *S. kudriavzevii* IFO1802 (blue) strains in various conditions. The genes related to glycerol biosynthesis (*GPD1*) and glycerol transport (*FPS1* and *STL1*) were studied. Samples from non-stress SC media (A,D), hyperosmotic SC 1 M sorbitol (B,E) or hypoosmotic (water) media (C,F) cultures were taken after 0, 0.5, 1 and 2 h of the inoculation (from pre grown cultures in SC). The SC media were supplemented with 2% glucose (A–C) or 2% mannitol (D–F) as a carbon source. After RNA extraction, expression of the different genes was determined by qPCR and values were normalized with *ACT1* and *RDN18-1* constitutive genes. Three independent microvinification bottles were used for each strain and averages \pm standard deviation are shown.

(Figure 4B) or hypoosmotic (water) media (Figure 4C). In addition, another analog set of experiments were performed but using mannitol as a carbon source (Figures 4D–F), which is a non-fermentable carbon source that complicates the energy

supply for cellular processes. No yeast growth was observed during this experiment (results not shown). We can observe that all strains, especially T73 and BMV58, activate *GPD1* 0.5–1 h after hyperosmotic stress (Figure 4B) but is not activated in non-stress

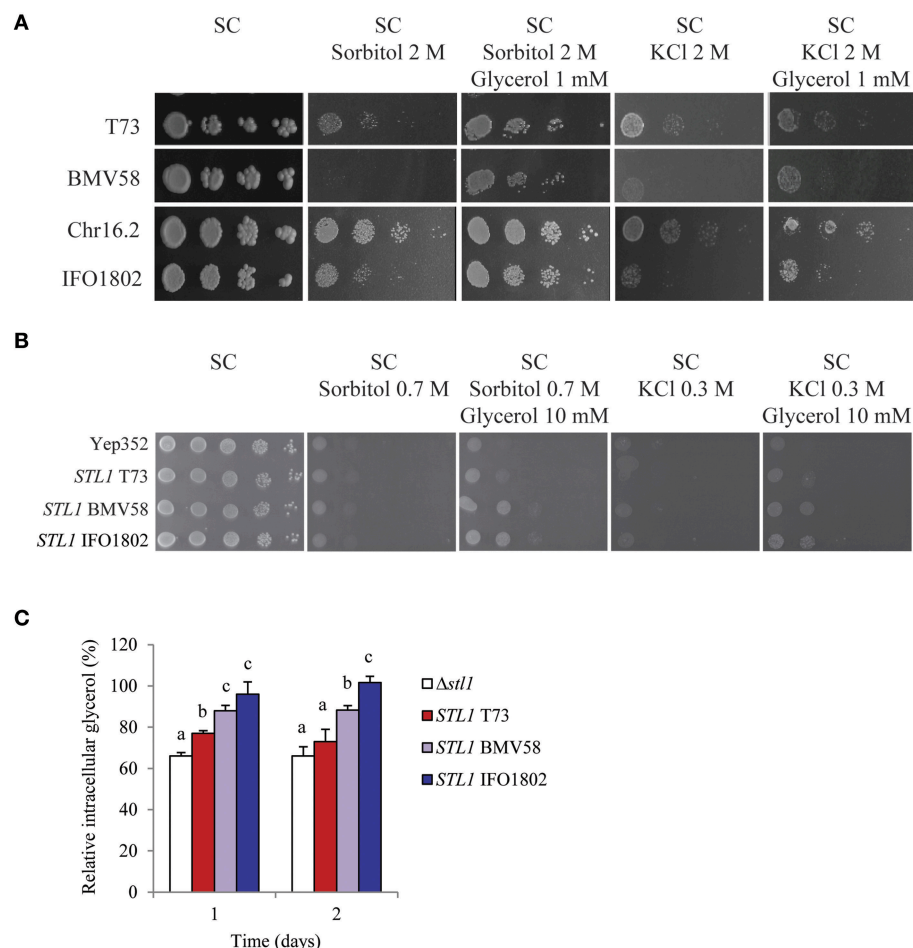


FIGURE 5 | Importance of glycerol import for osmotolerance of *S. cerevisiae* (T73), *S. uvarum* (BMV58), *S. paradoxus* (Chr16.2), and *S. kudriavzevii* (IFO1802) in drop test assays. (A) Serial dilutions of the different strains were plated in non-stress media (SC), in hyperosmotic stress media (SC with 2 M sorbitol or 2 M KCl) and in hyperosmotic stress media supplemented with glycerol (1 mM glycerol). **(B)** Growth of *S. cerevisiae* BY4741 $\Delta stl1 \Delta hog1$ strain expressing *STL1* alleles from *S. cerevisiae* (T73), *S. uvarum* (BMV58) or *S. kudriavzevii* (IFO1802) was monitored in drop tests on non-stress media (SC), in hyperosmotic stress media (SC with 0.7 M sorbitol or 0.3 M KCl), and in hyperosmotic stress media supplemented with 10 mM glycerol. A representative image of biological triplicates is presented. **(C)** In the same strains used in (B), intracellular glycerol accumulation was measured collecting samples after 0, 1, or 2 days of growth in SC with 10% glucose. Cells precultured in the same media were inoculated ($OD_{600} = 0.3$) and incubated at 25°C in 100 ml flasks. Data in time 0 for each strain was considered 100%. Three independent experiments were performed for each strain and averages \pm standard deviation are shown. ANOVA with fisher test ($p < 0.05$) was performed and significantly different values are labeled with different letters.

conditions (Figure 4A) or hypoosmotic stress (Figure 4C). A similar situation but with higher mRNA levels is observed in presence of mannitol instead of glucose where hyperosmotic stress (Figure 4E) activates *GPD1* gene, especially for BMV58 and IFO1802. In this case, hypoosmotic stress (Figure 4F) does activate the *GPD1* gene in the case of T73 and BMV58. The *STL1* gene reacts with a similar pattern as *GPD1* increasing mRNA levels in hyperosmotic stress (Figure 4B) but not upon hypoosmotic stress in the presence of glucose (Figure 4C). *STL1* shows also a similar pattern as *GPD1* in presence of mannitol, increasing expression levels after hyperosmotic stress (Figure 4E), though to higher levels compared to glucose (Figures 4B,E). Interestingly, *S. cerevisiae* T73 strain shows very low *STL1* levels in any conditions and no significant activation (Figure 4F). On the

contrary, the *FPS1* gene seems to be unresponsive to any condition in all the strains with except for the case of T73 growth in mannitol and hypoosmotic stress (Figure 4F). Similar levels are presented for all strains and conditions although BMV58 presented lower levels than the other strains. Altogether, it is the *STL1* gene whose expression shows the highest level of variation in different conditions and among the species and strains.

Stl1 Functional differences in *Saccharomyces* Species

Since *STL1* gene presented important differences in mRNA levels in strains from different *Saccharomyces* species we wanted to study the possible functional differences of this glycerol importer. For that we first compared the growth of a representative strain of

S. cerevisiae (T73), *S. uvarum* (BMV58), *S. paradoxus* (Chr16.2), and *S. kudriavzevii* (IFO1802) species in conditions where the activity of Stl1 is important (Figure 5A). A drop test with the four strains was performed in non-stress media (SC), in hyperosmotic stress media (SC with 2 M sorbitol or 2 M KCl) and in hyperosmotic stress media supplemented with a very low amount of glycerol (SC with 2 M sorbitol (or 2 M KCl) and 1 mM glycerol). In these conditions, if the cells are able to efficiently import glycerol to the cytosol they have a growth advantage when extracellular glycerol is present, i.e., before they synthesize the necessary amount to counterbalance the external osmotic pressure. The results show that cell growth is affected by hyperosmotic stress conditions proportionally to the osmotic pressure, i.e., more in the presence of 2 M KCl than in the presence of 2 M sorbitol. We can observe that BMV58 is the strain with the lowest and Chr16.2 the highest survival level in both hyperosmotic stress conditions. Interestingly, as shown in the Figure 5A, some strains, as IFO1802 and especially BMV58, benefit from the presence of glycerol in the medium more than others (e.g., T73 and Chr16.2). These results are indicative of different capacity to import glycerol in response to hyperosmotic stress among the studied strains. Is interesting to highlight that osmotolerances in minimal media can be different to complete media (see BMV58 in Figure 1 compared to Figure 5A). This reflects that strains disposition to cope to osmotic stress could be different since complete and minimal media induce very different gene expression programs (Gasch et al., 2000; Miura et al., 2008).

To confirm these Stl1 functional differences we cloned the different *STL1* alleles from T73, BMV58, and IFO1802 strains in an *S. cerevisiae* multicopy plasmid behind a weak and constitutive promoter, and expressed them in a laboratory osmosensitive *S. cerevisiae* strain (BY4741Δ*slt1*Δ*hog1*). As a control, this strain was also transformed with the empty YEp352. Then, the growth of strains was tested in non-stress media (SC), in hyperosmotic-stress media (SC with 0.7 M sorbitol or 0.3 M KCl) and in hyperosmotic-stress media supplemented with extracellular glycerol (SC 0.7 M sorbitol or 0.3 M KCl, and 10 mM glycerol). The results (Figure 5B) showed that the strains with the BMV58 and IFO1802 *SLT1* allele are clearly able to recover growth when they have extracellular glycerol in the presence of a hyperosmotic stress. However, the strain containing the T73 *STL1* allele presented only a minor growth recovery when it can use extracellular glycerol in the presence of a hyperosmotic stress.

We also evaluated the different Stl1 functionality by measuring the intracellular glycerol accumulation of the *S. cerevisiae* strains expressing different *STL1* genes after 1 and 2 days of growth in 10% glucose (Figure 5C) without any additional osmotic agents. The strain with IFO1802 Stl1 was able to recover the original intracellular glycerol levels by importing some of the diffused out glycerol. The strain with BMV58 Stl1 was able to recover more than 80% of the original intracellular glycerol levels. On the contrary, after 2 days the strain with the T73 Stl1 showed intracellular glycerol levels recovery no significantly different than a strain without Stl1. This results points in the same direction of the previous experiments and suggest a low functionality of T73 Stl1 compared with BMV58 and IFO1802.

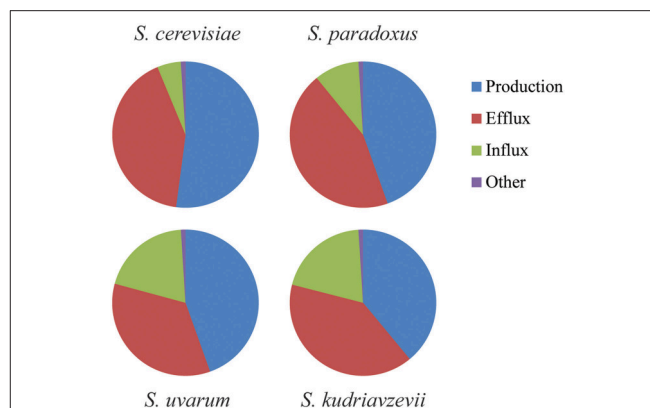


FIGURE 6 | Schematic representation of the weight of glycerol production, efflux, influx, or other actions regarding glycerol balance after hyperosmotic stress. This representation compares the dynamics of glycerol accumulation in response to hyperosmotic stress that has been quantitatively analyzed and modeled using physiologic, metabolic, enzymatic, and transcriptomic data of the key actors in *S. cerevisiae* (Petelenz-Kurdiel et al., 2013). Here we compared with the other species (*S. paradoxus*, *S. uvarum*, and *S. kudriavzevii*) using data provided in this work and others as Oliveira et al. (2014).

DISCUSSION

In this work we studied intracellular glycerol pool balance and regulation in response to stresses that occur upon inoculating wine-related yeast species in grape musts. We have analyzed strains belonging to four species that participate in winemaking directly (*S. cerevisiae*, *S. uvarum*, and *S. paradoxus*) or through hybrids (*S. kudriavzevii*). A first approach was to compare survival of these species during hyperosmotic and cold-hyperosmotic stress. Other studies have found that *S. cerevisiae*, *S. uvarum*, and *S. paradoxus* strains have similar tolerance to hyperosmotic stress whereas *S. kudriavzevii* strains show a decreased survival in 15% sorbitol at 30°C (Wimalasena et al., 2014). However, this result is doubtful since *S. kudriavzevii* strains are sensitive to this temperature (Arroyo-López et al., 2010). In our results using 25°C, an optimal temperature for *S. kudriavzevii*, this species shows similar or slightly higher tolerance to some hyperosmotic conditions compared to *S. cerevisiae* and *S. paradoxus*. In contrast, we observed an increased hyperosmotic stress tolerance in *S. uvarum* strains that is even more evident in hyperosmotic-cold stress conditions, where glycerol balance is determinant for cell survival. These results argue in favor to a more efficient handling of intracellular glycerol in *S. uvarum* strains in this condition. On the contrary, hyperosmotic tolerances in *Saccharomyces* species seems to be dependent on the media since *S. uvarum* strain BMV58 shows the lowest hyperosmotic tolerance in minimal media (Figure 5A) instead of the highest tolerance in complete media (Figure 1). All these data point to different strategies in the different species to handle glycerol accumulation in response to hyperosmotic or cold-hyperosmotic stresses.

In winemaking conditions, cells suffer hyperosmotic or cold hyperosmotic mild stresses that do not affect cell growth

capacity in any *Saccharomyces* species (results not shown). This hyperosmotic stress produced by the elevated amount of sugars may determine different lag phase adaptations. In fact, significant differences can be observed in extra and intracellular glycerol levels and also in gene expression of key genes involved in glycerol homeostasis. These data also suggest that the *Saccharomyces* species are using different strategies to face alterations in the osmotic pressure and cold temperatures. In fact this argument is not that surprising since *Saccharomyces* species are genetically quite distant showing coding region identities such as the one showed when comparing human and mouse (85%; Lapidot et al., 2001). The dynamics of glycerol accumulation in hyperosmotic stress has been quantitatively analyzed and modeled using physiologic, metabolic, enzymatic, and transcriptomic data in *S. cerevisiae* (Petelenz-Kurdiel et al., 2013). The strategy of this species consists in a transcriptional activation of *GPD1* to increase glycerol accumulation inside the cell by redirecting glycolytic flux. On the other hand, the glycerol efflux stops by the closing of Fps1 channel. These are the principal mechanisms to balance glycerol after a hyperosmotic shock. Glycerol influx and other elements contribute in a minor fraction (Figure 6). From the results of this work and others, we can hypothesize that non-*cerevisiae* *Saccharomyces* species have changed the weight of the different elements involved in glycerol balance. Based on *STL1* gene activation and Stl1 functionality assays we speculate that *S. uvarum* and *S. kudriavzevii* rely more in the glycerol import to compensate the osmotic pressure when extracellular glycerol is accumulated (Figure 6). This strategy is not exclusive of these species. In fact, it has been shown that the most osmotolerant yeasts species present a very efficient glycerol-import capacity (Lages et al., 1999).

A possible explanation of the different strategies applied by the *Saccharomyces* species to balance glycerol in osmotically non-optimal environments could be amount of intracellular glycerol that cells need to accumulate. We observed that, in our winemaking conditions, *S. cerevisiae* accumulates the highest amount of glycerol in the cells. This promotes the supposition that the other species can partially compensate the osmotic pressure by other means as cell volume changes for example. This will allow them to diversify the mechanisms available to compensate water efflux by using more frequently other elements that can be inefficient in specific situations, for example the glycerol import, which can be useless if there is no glycerol outside the cell. This variation could be

consequence of environmental adaptation to different niches. For example, cold stress adaptations could implement glycerol influx to better cope with low temperatures. Future research will shed more light in the effect of other conditions as redox unbalance and anaerobiosis in the glycerol pools in the different species.

In summary, the four species studied show different strategies to survive under osmotic or cold-osmotic stressful conditions (Figure 6). In all species, the balance of intracellular glycerol which depends on the production, efflux, influx and other minor elements is altered in order to increase its levels. However, whereas a species as *S. cerevisiae* relies more in changes in the production levels, others tend to depend more on the variation of the influx as *S. uvarum* or *S. kudriavzevii*.

AUTHOR CONTRIBUTIONS

RP conceived the study and participated in its design and coordination and draft the manuscript. GMO and JZ performed the experiments and analyzed the results. HS and AQ participated in the design and coordination of the study and in the draft of the manuscript. All authors read and approved the final manuscript.

ACKNOWLEDGMENTS

BO was supported by CAPES the Brazilian Federal Agency for the Support and Evaluation of Graduate Education (Brazilian Ministry of Education). This work has been supported by grants AGL2012-39937-C02-01 and AGL2015-67504-C3-1-R from the Spanish Government, FEDER, and Generalitat Valenciana PROMETEOII/2014/042 to AQ, GA CR 15-03708S from the Czech National Foundation to HS, and by the European Commission FP7: Marie Curie Initial Training Network CORNUCOPIA no. 264717 to AQ and HS.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fmicb.2016.00435>

Supplementary Figure 1 | Yeast biomass accumulation during low temperature microvinifications in synthetic must for *S. cerevisiae* T73 (dark red) and FCry (light red), *S. paradoxus* Chr16.2 (light green) and 108 (dark green), *S. uvarum* 12600 (dark purple) and BMV58 (light purple), and *S. kudriavzevii* CR85 (light blue) and IFO1802 (dark blue) strains.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Enhanced 3-Sulfanylhexasan-1-ol Production in Sequential Mixed Fermentation with *Torulaspora delbrueckii*/*Saccharomyces cerevisiae* Reveals a Situation of Synergistic Interaction between Two Industrial Strains

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OPEN ACCESS

Edited by:

Alberto Mas,
Rovira i Virgili University, Spain

Reviewed by:

Kalliopi Rantsiou,
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Specialty section:

This article was submitted to
Food Microbiology,
a section of the journal
Frontiers in Microbiology

Received: 03 November 2015

Accepted: 23 February 2016

Published: 15 March 2016

Citation:

Renault P, Coulon J, Moine V,
Thibon C and Bely M (2016)
Enhanced 3-Sulfanylhexasan-1-ol
Production in Sequential Mixed
Fermentation with *Torulaspora*
delbrueckii/*Saccharomyces cerevisiae*
Reveals a Situation of Synergistic
Interaction between Two Industrial
Strains. *Front. Microbiol.* 7:293.
doi: 10.3389/fmicb.2016.00293

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The aim of this work was to study the volatile thiol productions of two industrial strains of *Torulaspora delbrueckii* and *Saccharomyces cerevisiae* during alcoholic fermentation (AF) of Sauvignon Blanc must. In order to evaluate the influence of the inoculation procedure, sequential and simultaneous mixed cultures were carried out and compared to pure cultures of *T. delbrueckii* and *S. cerevisiae*. The results confirmed the inability of *T. delbrueckii* to release 4-methyl-4-sulfanylpentan-2-one (4MSP) and its low capacity to produce 3-sulfanylhexasyl acetate (3SHA), as already reported in previous studies. A synergistic interaction was observed between the two species, resulting in higher levels of 3SH (3-sulfanylhexasan-1-ol) and its acetate when *S. cerevisiae* was inoculated 24 h after *T. delbrueckii*, compared to the pure cultures. To elucidate the nature of the interactions between these two species, the yeast population kinetics were examined and monitored, as well as the production of 3SH, its acetate and their related non-odorous precursors: Glut-3SH (glutathionylated conjugate precursor) and Cys-3SH (cysteinylylated conjugate precursor). For the first time, it was suggested that, unlike *S. cerevisiae*, which is able to metabolize the two precursor forms, *T. delbrueckii* was only able to metabolize the glutathionylated precursor. Consequently, the presence of *T. delbrueckii* during mixed fermentation led to an increase in Glut-3SH degradation and Cys-3SH production. This overproduction was dependent on the *T. delbrueckii* biomass. In sequential culture, thus favoring *T. delbrueckii* development, the higher availability of Cys-3SH throughout AF resulted in more abundant 3SH and 3SHA production by *S. cerevisiae*.

Keywords: non-*Saccharomyces*, *Torulaspora delbrueckii*, wine, fermentation, mixed inoculation, volatile thiols, aroma precursors

INTRODUCTION

Volatile thiols are powerful aromatic compounds that contribute to the fruity notes of many white wines, especially Sauvignon Blanc. The three most important thiols in Sauvignon Blanc aroma are considered to be 3-sulfanylhexas-1-ol (3SH, formerly known as 3MH; Tominaga et al., 1998a), its acetate, 3-sulfanylhexas acetate (3SHA, formerly known as 3MHA; Tominaga et al., 1996), and 4-methyl-4-sulfanylpentan-2-one (4MSP, formerly known as 4MMP; Darriet et al., 1995; Tominaga et al., 1996, 1998a). Descriptors such as box tree and broom for 4MSP and grapefruit/passion fruit for 3SH match the occurrence of these compounds in box tree and yellow passion fruit, respectively (Tominaga and Dubourdieu, 1997, 2000). Due to their low perception thresholds (a few ng/L), they contribute significantly to the aroma profile of many wines (Roland et al., 2011 and cited references).

The release of volatile thiols by *Saccharomyces cerevisiae* yeast during alcoholic fermentation (AF), now relatively well described, results from the biotransformation of non-odorous precursors present in grapes (Tominaga, 1998; Marullo and Dubourdieu, 2010). 4MSP and 3SH are produced from cysteinylated (Cys-4MSP, Cys-3SH) and glutathionylated (Glut-4MSP, Glut-3SH) conjugates by yeast β -lyase cleavage (Darriet et al., 1995; Tominaga et al., 1998b; Peyrot des Gachons et al., 2002; Subileau et al., 2008a; Fedrizzi et al., 2009; Roland et al., 2011; Coetzee and du Toit, 2012). The biotransformation of these precursors by yeast involves their uptake through the membrane, followed by cleavage into their corresponding aromas (for a review, see Coetzee and du Toit, 2012). Concerning 3SH, in *S. cerevisiae*, the cysteinylated precursor form is taken up by amino acid transporters, such as Gap1p (Subileau et al., 2008b), while the glutathionylated form is assimilated through the Opt1p GSH transporter (Subileau et al., 2008a). Once transported into the cytoplasm, these precursors are transformed by α,β -elimination, catalyzed by β -lyases (Howell et al., 2005; Thibon et al., 2008; Holt et al., 2011; Roncoroni et al., 2011; Cordente et al., 2015). However, biotransformation rates by *S. cerevisiae* are low, with calculated yields ranging from <1% to about 5% (Murat et al., 2001; Dubourdieu et al., 2006; Grant-Preece et al., 2010; Kobayashi et al., 2010; Winter et al., 2011). 3SHA is produced after 3SH release by alcohol acetyltransferase, encoded by the ATF1 gene in *S. cerevisiae* (Swiegers et al., 2006). The final concentration of 3SHA depends on the activity balance between alcohol acetyltransferase (promoting esterification of the corresponding alcohol) and esterase (promoting its hydrolysis), encoded by the IAH1 gene (Coetzee and du Toit, 2012).

In recent years, several authors have highlighted the positive contribution of non-*Saccharomyces* yeasts to the analytical and sensory composition of wine, leading to the commercialisation of certain non-conventional yeasts. This is the case of the *Torulaspora delbrueckii* species, now available as an active dry yeast. Indeed, this species has been described as having a positive impact on the organoleptic quality of wines, due to its low production of compounds such as acetic acid, ethyl acetate, acetaldehyde, acetoin, hydrogen sulfide and volatile phenols, hence minimizing off-flavors (Cabrera et al., 1988; Herraiz et al.,

1990; Martinez et al., 1990; Ciani and Picciotti, 1995; Ciani and Maccarelli, 1998; Shinohara et al., 2000; Plata et al., 2003; Renault et al., 2009). A strong β -glucosidase activity, which enhances wine aroma by hydrolysing terpenyl-glycosides, was also described in several *T. delbrueckii* strains (King and Richard Dickinson, 2000; Hernandez-Orte et al., 2008; Comitini et al., 2011; Azzolini et al., 2012). Moreover, overall, *T. delbrueckii* alone produced lower quantities of esters than *S. cerevisiae* (Viana et al., 2008; Sadoudi et al., 2012) but a few minor esters (ethyl propanoate, ethyl isobutanoate, and ethyl dihydrocinnamate) were produced in larger concentrations, which had a positive organoleptic impact on the wine (Renault et al., 2015).

Despite a good ethanol production (up to 11% vol ethanol) compared to other non-*Saccharomyces* yeasts (Cabrera et al., 1988; Herraiz et al., 1990; Ciani and Picciotti, 1995; Ciani and Maccarelli, 1998; Renault et al., 2009; Velázquez et al., 2015), *T. delbrueckii* alone cannot complete AF under winemaking conditions. *T. delbrueckii*/*S. cerevisiae* multi starters have thus been proposed to modulate wine flavor and properties and to ensure complete AF. An increasing number of studies using these mixed cultures have, however, produced contradictory results concerning their impact on wine quality. In fact, the inoculation procedure, as well as the different strains used, drastically impact the population dynamics of both species, thus modifying aroma production (Renault et al., 2015).

Few researchers have investigated volatile thiol formation by the *T. delbrueckii* yeast metabolism in pure and mixed cultures. According to Zott et al. (2011), in synthetic medium, *T. delbrueckii* released significant concentrations of 3SH but lower than that of pure *S. cerevisiae* cultures. It also has a poor capacity to form 4MSP. These results were confirmed by Sadoudi et al. (2012) during AF of Sauvignon Blanc must. As a result, in a simultaneous mixed culture, at a 10:1 ratio (*T. delbrueckii*/*S. cerevisiae*), a decrease in 3SH and 3SHA production was observed, compared to a pure *S. cerevisiae* culture.

This study compared the volatile thiol profiles of Sauvignon Blanc wines fermented with pure *S. cerevisiae* and *T. delbrueckii*, as well as mixed cultures (simultaneous and sequential). To elucidate the nature of the interactions between these two yeast strains, 3SH, 3SHA, their related precursors, and 4MSP, as well as the population dynamics, were monitored throughout AF.

MATERIALS AND METHODS

Yeast Strains

In this study, two commercial strains from Laffort company (France) were used: *S. cerevisiae* Zymaflore® X5 and *T. delbrueckii* Zymaflore® Alpha^{TDn.sacch}. Yeasts were grown at 24°C on complete YPDA medium (1% yeast extract, 1% peptone, 2% dextrose) solidified with 2% agar, adjusted to pH 4.8.

Fermentation Medium

The medium used was a Sauvignon Blanc grape must from Bordeaux area, pH: 3.15, with a sugar concentration of 203 g/L and an available nitrogen concentration adjusted to 210 mg/L.

(i.e., amino acids: 114 mg/L and ammonia: 96 mg/L). The total and free sulfur dioxide concentrations were, respectively, 60 and 19 mg/L. Before yeast inoculation, the must was sterilized by filtration (0.45 μ m nitrate cellulose membrane, Millipore, Molsheim, France).

Fermentation Conditions

Fermentation kinetics were monitored by CO₂ release (Bely et al., 1990a,b). The amount of CO₂ release (g/L) was determined by automatic measurement of fermentor weight loss every 20 min. The CO₂ production rate (g/L/h) was obtained by polynomial smoothing of the last 11 CO₂ measurements. The large number of CO₂ acquisitions combined with precision weighing (0.01 g) gave three kinetic parameters with good accuracy: (1) lag phase (h) was the time between inoculation and the beginning of CO₂ release, (2) V_{max} (g/L/h) was the maximum CO₂ production rate, and (3) FD (h) was the time required to ferment all the sugars in the medium. Weight loss due to evaporation was under 2%.

Yeasts were pre-cultured in Erlenmeyer flasks filled with must at 24°C for 24 h (*S. cerevisiae*) or 48 h (*T. delbrueckii*). Fermentations were carried out at 24°C with agitation in 1.2 L fermenters locked to maintain anaerobiosis throughout AF (CO₂ was released through a sterile air outlet condenser). Four different trials were carried out: two pure cultures and two mixed cultures. Two types of mixed cultures were carried out: simultaneous mixed culture (called “simultaneous culture”) where *T. delbrueckii* and *S. cerevisiae* were inoculated at the same time and sequential mixed culture (called “sequential culture”) where *T. delbrueckii* was inoculated 24 h before *S. cerevisiae* yeast. Single and mixed cultures were inoculated with 1×10^7 viable cells/mL for *T. delbrueckii* and 2×10^6 viable cells/mL for *S. cerevisiae*. All experiments were performed in triplicate.

Population Kinetics

In mixed cultures, yeast growth was determined by plate counting on two different agar media. Samples were withdrawn throughout fermentation and diluted appropriately. Non-*Saccharomyces* cells were counted using a specific agar medium (NS): YPDA (1% yeast extract, 1% peptone, 2% dextrose, 2% agar; pH 4.8) supplemented with 1 μ g/mL cycloheximide to promote the growth of *T. delbrueckii* and inhibit that of *S. cerevisiae*. This low concentration allowed the growth of *T. delbrueckii* Zymaflore® Alpha^{TDn.sacch} but inhibited that of *S. cerevisiae* Zymaflore® X5 (data not shown). The number of *S. cerevisiae* was given as the difference between the total plate count using YPDA medium and the plate count using NS medium. Yeast growth in single cultures was determined using only the YPDA medium. At the end of AF, we controlled the species by PCR RFLP analysis of rDNA ITS region with digestion by Eco RI (Granchi et al., 1999). Plates were incubated at 24°C for 4 days before counting.

Wine Analysis

Ethanol concentration (% vol) was measured by infrared refractance (Spectra Analyser, Axflow, Plaisir, France) and sugar (g/L) was determined chemically by colorimetry (460 nm)

in continuous flux (Sanimat, Montauban, France). These analyses were performed by Sarco laboratory (Bordeaux, France).

Volatile Thiols Analysis

Volatile thiol quantification was performed by the wine analysis laboratory Sarco (Bordeaux, France). 4MSP, 3SH, and 3SHA were specifically extracted by reversible combination of the thiols with sodium-*p*-hydroxymercuribenzoate (*p*-HMB), from 50 mL wine previously preserved from oxidation by adding 50 mg/L of SO₂, as described by Tominaga and Dubourdieu (2006) and quantified by gas chromatography–mass spectrometry according to methods described by Tominaga et al. (1998a) and Tominaga and Dubourdieu (2000).

3SH Thiol Precursors Analysis

Cys-3SH and Glut-3SH were assayed according to the protocol described by Capone et al. (2010), modified as follows. An aliquot (25 μ L) of an aqueous solution containing *d*₃-Glut-3SH (final concentration 50 μ g/L) was added to 1 mL grape juice. The sample was diluted with 2 mL water and passed through a 6 mL, 500 mg LC-18 cartridge (Supelco), previously conditioned with 4 mL methanol, followed by 2 mL methanol-water (50/50) mix, and 3 mL water. After loading the juice, the sorbent was rinsed with 1 mL water, dried under air for 1 min, and eluted with 3 mL methanol solution (70%). The eluate was collected and dried in a Vacuum System with Vortex Motion (RapidVap, Labconco, US) at 10 mbar and 45°C. The extract was diluted in formic acid solution (700 μ L, 0.1%), filtered through a 0.45 μ m filter for LC–MS analysis. All LC–MS analyses were carried out on an Accela UHPLC (Thermo Fisher Scientific), connected in series to an Exactive (Thermo Fisher Scientific, Bremen, Germany) mass spectrometer, equipped with a heated ESI ion source. The column was a 100 \times 2.1 mm, i.d., 1.7 μ m, Synchronis aQ (Thermo Scientific). The solvents were: 0.1% aqueous formic acid (solvent A) and 0.1% formic acid in acetonitrile (solvent B), with a flow rate of 300 μ L/min. The gradient for solvent B was as follows: 0 min, 9%; 0.8 min, 9%; 5 min, 40%; 5.2 min, 90%. The column was equilibrated with 9% B for 1 min prior to injection. A 5 μ L injection volume was used for each sample. The ion source was operated in the positive ion mode at 3.5 kV. Source vaporizer temperature was set at 300°C, capillary temperature at 350°C, nitrogen sheath gas at 80, and the auxiliary and sweep gas at 5 (arbitrary units). A mass range of 100–500 was acquired in full scan MS mode. The resolution setting was 25 000 (m/ Δ m, fwhm at m/z400).

Statistical Analysis

In order to compare modalities, data were analyzed by single-factor variance (ANOVA, $p < 0.05$), following verification of variance homogeneity (Levene test, $p > 0.05$). Thereafter, a multiple comparison test (Duncan) was applied to classify the different culture protocols ($p < 0.05$). All statistics were analyzed using the R program.

RESULTS AND DISCUSSION

CO₂ Release and Population Kinetics in Pure and Mixed Cultures

Four different AF were conducted in Sauvignon Blanc grape must: two with pure cultures and two with mixed cultures (inoculated either simultaneously or sequentially). In all trials, the must was inoculated with 1×10^7 viable cells/mL for *T. delbrueckii* and 2×10^6 viable cells/mL for *S. cerevisiae*. The final ethanol concentrations (12% vol, corresponding to a final CO₂ release of 97 g/L) were reached in all fermentations except in the pure *T. delbrueckii* culture, which predictably stopped fermenting at 6.2%vol.

The overall fermentation kinetic profiles, i.e., the variation in CO₂ rate versus time, are shown in **Figure 1**. The rate curves varied markedly from one culture to another. Indeed, the trial involving inoculation with *T. delbrueckii* alone showed a short lag phase (17 h), but also a low fermentation rate, characterized by the lowest V_{max} (0.39 g/L/h). In contrast, even with a long lag phase (34 h), the *S. cerevisiae* culture had a high fermentation rate with the highest V_{max} (1 g/L/h) and the shortest fermentation duration (334 h). These results are in good agreement with previous investigations using a large number of strains (Renault et al., 2009), where *T. delbrueckii* was found to have a lower fermentation capacity than *S. cerevisiae*.

Mixed cultures exhibited intermediate fermentation kinetics (**Figure 1**). When both species were added at the same time, the fermentation curve showed similar profiles to that of pure *S. cerevisiae* culture, but with a lower V_{max} (0.84 g/L/h) and a shorter lag phase time (11 h), due to the larger amount of cells inoculated (1.2×10^7 viable cells/mL). On the contrary, when *T. delbrueckii* and *S. cerevisiae* were inoculated sequentially, the fermentation curve was close to that of *T. delbrueckii* alone, except that the V_{max} was higher (0.56 g/L/h). The fermentation

of the sequential culture took longer than that of the simultaneous or pure *S. cerevisiae* cultures.

The viable *S. cerevisiae* and *T. delbrueckii* populations in pure and mixed cultures were determined by plate counting (**Table 1**). The biomass kinetics are presented according to AF progress (expressed in % of CO₂ released; **Figure 2**). The maximum population (X_{max}) reached during AF by *T. delbrueckii* and *S. cerevisiae* was higher when they were inoculated alone (8.1×10^7 viable cells/mL for *T. delbrueckii* and 7.6×10^7 viable cells/mL for *S. cerevisiae*, in pure cultures, respectively) than in sequential and simultaneous cultures. Hence, both species influenced each other's development. It is noteworthy that the X_{max} of *T. delbrueckii* in the sequential culture was higher (6.1×10^7 viable cells/mL) than in the simultaneous culture (4.3×10^7 viable cells/mL). On the contrary, the X_{max} of *S. cerevisiae* was 4.4×10^7 and 2.4×10^7 viable cells/mL, in simultaneous and sequential cultures, respectively.

Indeed, in sequential culture, when the addition of *S. cerevisiae* (when 2.5 g/L CO₂ had been released) was delayed, *T. delbrueckii* was able to grow from 1×10^7 viable cells/mL to 4.1×10^7 viable cells/mL within the first 24 h, thus initiating AF (**Figure 2**). In that case, the *T. delbrueckii*/*S. cerevisiae* ratio after 24 h was largely in favor of *T. delbrueckii* (about 20:1) but *S. cerevisiae* developed sufficiently (from 2×10^6 to 2.4×10^7 viable cells/mL) to complete AF.

Consequently, in the sequential culture, the X_{max} of *T. delbrueckii* was maintained during the first 60% of AF and its viable population was higher than that of *S. cerevisiae* during the first 85% of AF (**Figure 2**). The dominance of *T. delbrueckii* throughout the AF, in sequential culture, is probably due to higher consumption of dissolved oxygen, nitrogen and vitamins than *S. cerevisiae* which was inoculated 24 h after.

The kinetics of the two yeast populations were very different following simultaneous inoculation, where the initial inoculation ratio of 5:1 (1×10^7 viable cells/mL *T. delbrueckii* and 2×10^6

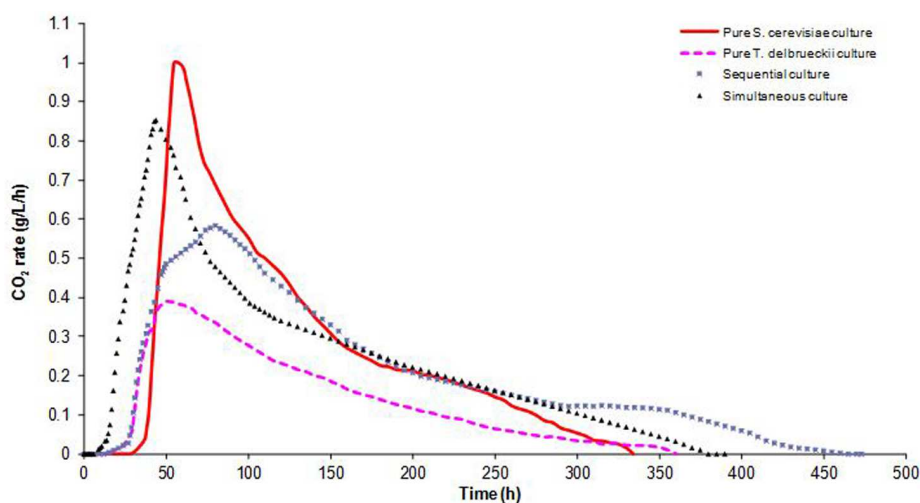
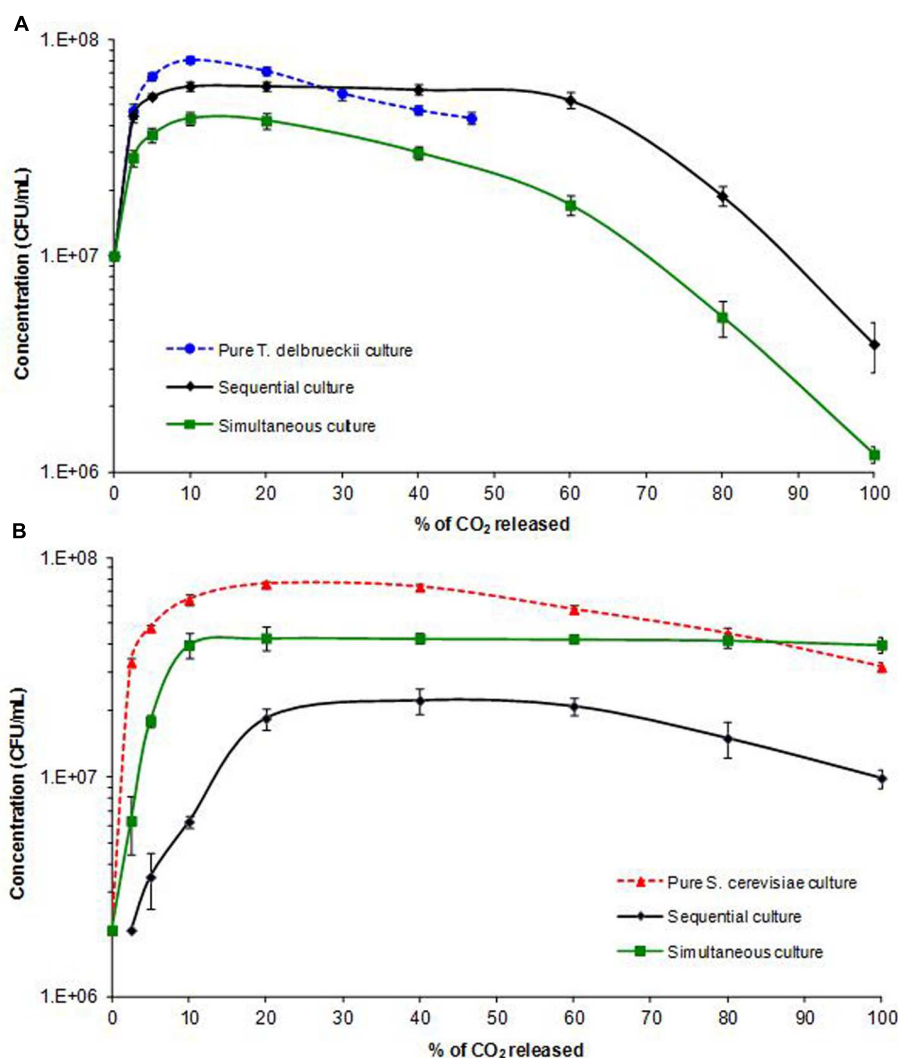


FIGURE 1 | CO₂ production rates (g/L/h) over time in pure and mixed *T. delbrueckii*/*S. cerevisiae* cultures. Average values of three experiments, standard deviation <5%.

TABLE 1 | Maximal cell population and final volatile thiol concentrations in pure and mixed *T. delbrueckii* and *S. cerevisiae* cultures.

	<i>Torulaspora delbrueckii</i> pure culture	Sequential mixed culture	Simultaneous mixed culture	<i>Saccharomyces cerevisiae</i> pure culture
Maximal population (viable cells/mL)				
<i>T. delbrueckii</i>	$8.1 \times 10^7 \pm 2.8 \times 10^{6c}$	$6.1 \times 10^7 \pm 7.1 \times 10^{6b}$	$4.3 \times 10^7 \pm 3.5 \times 10^{6a}$	/
<i>S. cerevisiae</i>	/	$2.4 \times 10^7 \pm 7.1 \times 10^{5a}$	$4.4 \times 10^7 \pm 2.3 \times 10^{6b}$	$7.6 \times 10^7 \pm 1.8 \times 10^{6c}$
Final volatile thiol concentrations (ng/L)				
3SH	623 ± 103^b	1312 ± 224^c	362 ± 97^a	303 ± 141^a
3SHA	14 ± 2^a	218 ± 34^c	79 ± 20^b	83 ± 9^b

Average values of three experiments \pm standard deviation. ^{a,b,c}Represents significantly different statistical groups ($p < 0.05$). 3SH, 3-sulfanylhhexan-1-ol; 3SHA, 3-sulfanylhhexyl acetate.

**FIGURE 2 |** Kinetics of *T. delbrueckii* (A) and *S. cerevisiae* (B) cell populations (CFU/mL) during alcoholic fermentation (AF) in pure and mixed cultures. Average value of three experiments.

viable cells/mL *S. cerevisiae*) was less favorable to *T. delbrueckii*, which was only dominant during the first 10% of AF. Indeed, the X_{max} of *T. delbrueckii* in simultaneous inoculation was lower than in sequential culture (4.3×10^7 and 6.1×10^7 viable

cells/mL, respectively). Furthermore, *S. cerevisiae* also reached its X_{max} during the early stage of AF and maintained this level of population until the end of AF, whereas the viable *T. delbrueckii* population decreased rapidly after 10% of AF. According to

several authors (Nissen and Arneborg, 2003; Nissen et al., 2003; Renault et al., 2013), the physical contact/proximity between *T. delbrueckii* and a large viable population of *S. cerevisiae* induced the rapid death of *T. delbrueckii*. Competition for oxygen may also explain the rapid death of *T. delbrueckii* cells (Hansen et al., 2001). Indeed, while *S. cerevisiae* yeast is able to grow rapidly under strictly anaerobic conditions, *T. delbrueckii* is affected by a lack of oxygen (Hanl et al., 2005).

To sum up, sequential culture facilitated the development of *T. delbrueckii*, resulting in a larger viable population than that of *S. cerevisiae* almost until the end of AF. Under these conditions, the kinetic parameters were close to those obtained in pure *T. delbrueckii* culture, except that AF was completed. In contrast, when both species were inoculated simultaneously, the maximal viable populations of both species were similar, but that of *S. cerevisiae* was larger than that of *T. delbrueckii* during 90% of the reaction, with AF showing similar profiles to those of pure *S. cerevisiae* cultures. Nevertheless, *T. delbrueckii* had a small impact on fermentation kinetics, as V_{max} was lower and AF was extended, in comparison to the pure *S. cerevisiae* culture.

Volatile Thiol Production

Torulaspora delbrueckii in pure culture did not produce 4MSP, unlike *S. cerevisiae* (33 ng/L at the end of AF). Very small amounts were detected in mixed cultures (<7 ng/L), suggesting the absence of any interaction between the species in producing this compound. These results confirmed the inability of *T. delbrueckii* to release 4MSP, as already reported in previous studies (Zott et al., 2011; Sadoudi et al., 2012).

As shown **Figure 3**, 3SH production was similar in both pure cultures during the first 20% of AF but diverged after this point, with differences in the final concentrations. Indeed, at the end of fermentation, the 3SH concentration in the pure *T. delbrueckii* culture was twofold higher than that in the pure *S. cerevisiae* culture (623 and 303 ng/L, respectively; **Table 1**). This result differed from previous findings using other *T. delbrueckii* strains (Zott et al., 2011; Sadoudi et al., 2012), suggesting that this production is strain-dependent.

Results were different for 3SHA for which *T. delbrueckii* produced very small amounts (14 ng/L), showing a progressive and linear production throughout AF (**Figure 3**). On the contrary, total 3SHA production by *S. cerevisiae* was higher (83 μ g/L), with 50% occurring during the growth phase. Furthermore, the 3SH/3SHA ratios were 3.6 and 44.5 in pure *S. cerevisiae* and *T. delbrueckii* cultures, respectively. These results confirmed those obtained by Sadoudi et al. (2012), showing that *T. delbrueckii* had a lower acetylation activity (i.e., a low conversion rate of 3SH to 3SHA) than *S. cerevisiae* (Coetzee and du Toit, 2012).

Furthermore, no significant difference in 3SH and 3SHA production was observed between the simultaneous and pure *S. cerevisiae* cultures at the end of AF (**Table 1**). However, it is interesting to note that, in the simultaneous inoculation protocol, the beginning of production was delayed (no production during the first 10% of AF; **Figure 3**).

Concerning the sequentially inoculated culture, 3SH production was similar to that of the pure *S. cerevisiae* culture

until 20% of AF, but diverged beyond that point, exhibiting a major increase during the last stage in AF, resulting in significantly different final concentrations. Indeed, at the end of AF, the 3SH concentration in the sequential culture was fourfold higher than in the pure *S. cerevisiae* culture (1312 and 303 ng/L, respectively; **Table 1**). 3SHA production in sequential culture was also different from the pure *S. cerevisiae* culture, remaining very weak until 20% of AF and then drastically increasing to reach a final concentration nearly threefold higher than in the pure *S. cerevisiae* culture (218 and 83 ng/L, respectively; **Figure 3**).

These results suggested that sequential inoculation of *S. cerevisiae* and *T. delbrueckii* in Sauvignon Blanc must result in synergistic interactions that affected 3SH and 3SHA production during AF.

Volatile Thiol Precursors

To investigate the possible synergistic interactions between the two species resulting in higher concentrations of 3SH and its acetate at the end of AF, their S-conjugate precursors (Cys-3SH and Glut-3SH) were monitored throughout fermentation (**Figure 4**). Cys-3SH and Glut-3SH were detected (0% of AF) at normal levels for a Sauvignon Blanc must: 20 μ g/L and 160 μ g/L, respectively.

In pure *S. cerevisiae* and *T. delbrueckii* cultures, only 60% of the initial Glut-3SH concentration was assimilated, leaving approximately 65 μ g/L of the precursor in the wine. However, in simultaneous and sequential cultures uptake was significantly higher. Indeed, up to 69 and 79% of Glut-3SH were assimilated, resulting in wines with 50 and 33 μ g/L precursor, respectively.

Figure 4 shows that, for all modalities, Glut-3SH was rapidly metabolized by the yeast in the earliest stage of AF, immediately after yeast addition. In the pure *S. cerevisiae* culture, the uptake stopped at 20% of AF. In all cultures involving *T. delbrueckii*, Glut-3SH kinetics were similar until 5% of AF, then the assimilation slowed down suddenly and stopped around 20% of AF in pure *T. delbrueckii* and simultaneous cultures. In the sequential culture, Glut-3SH assimilation continued slowly after 20% of AF, resulting in the lowest final Glut-3SH concentration in the medium. This enhanced precursor assimilation may explain the variations in total 3SH and 3SHA release observed at end of AF.

The kinetics of cysteine S-conjugate precursor (Cys-3SH) concentrations during AF is probably due to the fact that it is both produced and assimilated by the yeast (Cordente et al., 2015). Cys-3SH kinetics varied markedly from one culture to another (**Figure 4**). In the pure *S. cerevisiae* culture, Cys-3SH concentrations decreased after a short lag phase (<5% of AF) and completely disappeared after 20% of AF. In contrast, in all cultures involving *T. delbrueckii*, an increase in Cys-3SH concentrations was observed in the early stage of AF (**Figure 4**). In the pure *T. delbrueckii* culture, the concentration increased by 50% (21 μ g/L initial to 32 μ g/L at 5% of AF) during the very early stages of AF. In this phase, Cys-3SH production was concomitant to Glut-3SH depletion (**Figure 4**) and 3SH release (**Figure 3**), suggesting that *T. delbrueckii* was able to synthesize Cys-3SH and 3SH from Glut-3SH, but the

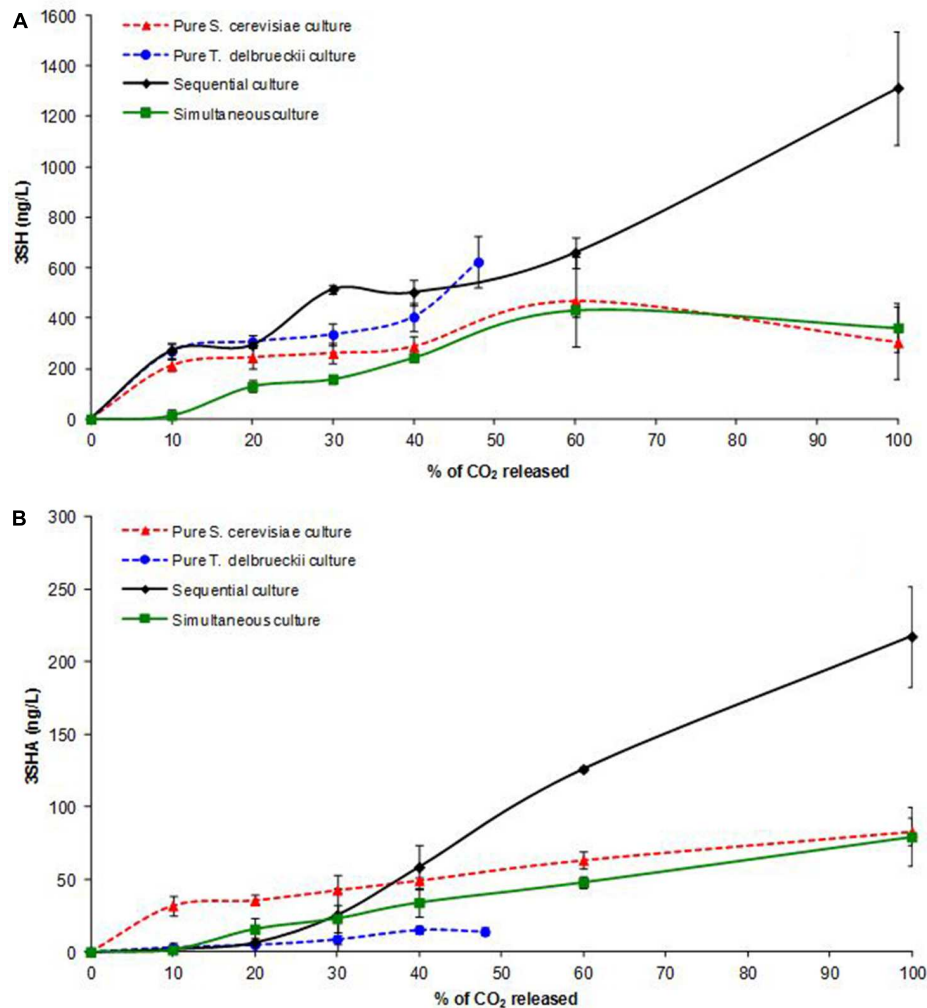


FIGURE 3 | Kinetics of 3SH (A) and 3SHA (B) concentrations (ng/L) in pure and mixed *T. delbrueckii*/*S. cerevisiae* cultures during AF. Average value of three experiments.

cysteinylation was hardly assimilated, if at all, by the yeast. In *S. cerevisiae*, its transport is provided by the Gap1p membrane protein (Subileau et al., 2008a). The *GAP1* gene has not been clearly identified in the genome sequence of *T. delbrueckii*, type strain CBS 1146T (CLIB230T). Indeed, the closest BLAST of Gap1p protein from *S. cerevisiae* S288c against CBS 1146T is a hypothetical protein (TDEL_OC00930) with only 74% identity along 96% of the sequence. It was, therefore, hypothesized that Gap1p permease was absent or dysfunctional in this species. Further experiments are required to validate this hypothesis, for example, an intracellular Cys-3SH assay.

When Glut-3SH stopped being converted into Cys-3SH, its concentration in the medium remained constant until the end of AF.

Interestingly, throughout AF in the two mixed cultures, the more *T. delbrueckii* developed, the higher the Cys-3SH concentration became. In the sequential culture, where

T. delbrueckii dominated *S. cerevisiae* for 85% of AF (Figure 2), the Cys-3SH accumulation phase in the must was much longer than in the simultaneous culture, resulting in a higher concentration at the end of this phase (34 $\mu\text{g/L}$ instead of 27 $\mu\text{g/L}$). After this accumulation phase, in both mixed cultures, an abrupt depletion reduced Cys-3SH to undetectable levels in the medium after around 40 and 60% of AF, in simultaneous and sequential cultures, respectively. It is worth noting that Cys-3SH uptake was apparently correlated with the development of *S. cerevisiae* (Figure 2). Precursor uptake began when *S. cerevisiae* reached its X_{max} , around 10 and 20% of AF (corresponding to 4.3×10^7 and 2.4×10^7 viable cells/mL in simultaneous and sequential cultures, respectively). Moreover, the Cys-3SH degradation rate (i.e., the slope of the line) of both mixed culture was apparently correlated to *S. cerevisiae* X_{max} level. The steep slope observed for the pure *S. cerevisiae* culture, with X_{max} around 7.5×10^7 viable cells/mL, supports this hypothesis.

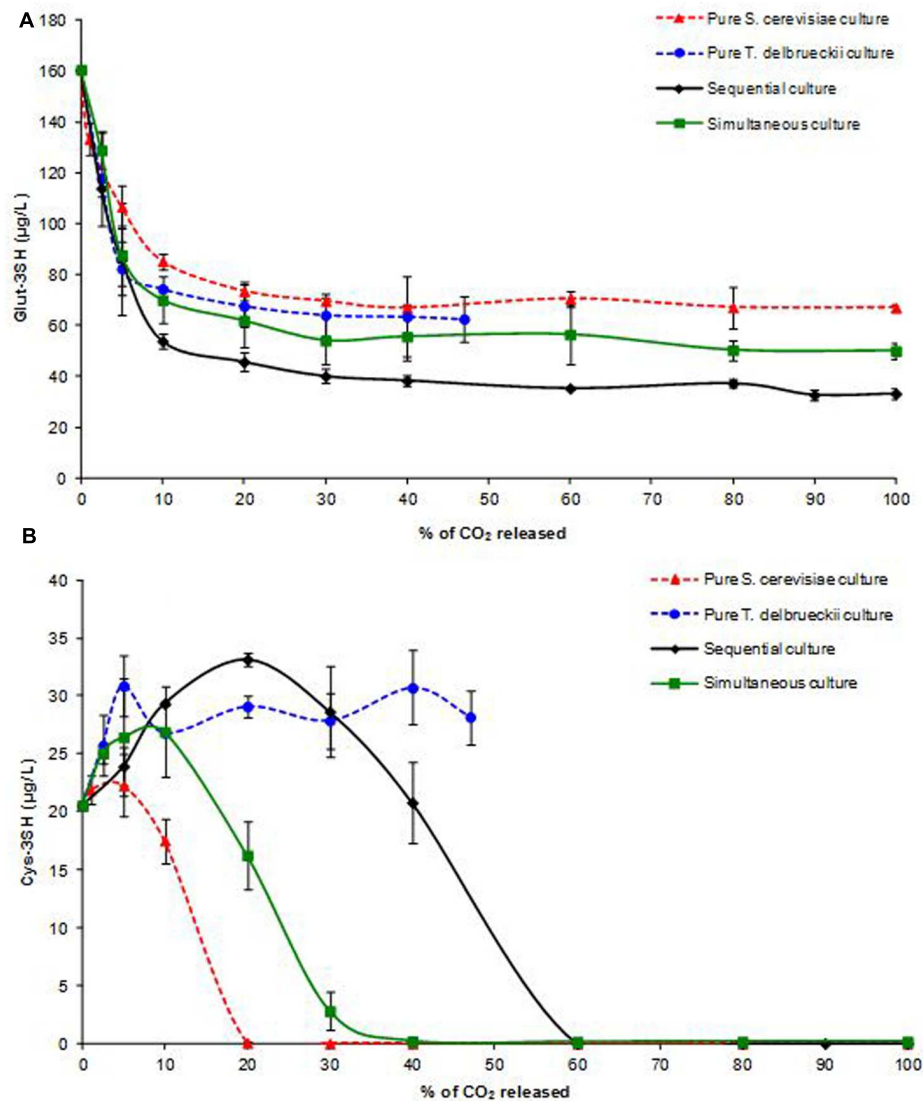


FIGURE 4 | Kinetics of Glut-3SH (A) and Cys-3SH (B) concentrations ($\mu\text{g/L}$) in pure and mixed *T. delbrueckii*/*S. cerevisiae* cultures during AF. Average value of three experiments.

CONCLUSION

This study, based on an analysis of the main thiols throughout AF of Sauvignon Blanc must fermented by *T. delbrueckii* and *S. cerevisiae* in pure or mixed cultures, provides interesting insights into the metabolic pathway of thiols in *T. delbrueckii* and reveals a synergistic interaction between the two species.

Under these experimental conditions, *T. delbrueckii* produced no 4MSP and only very small amounts of 3SHA, confirming previous findings. In contrast, high 3SH levels were found in wines fermented with pure *T. delbrueckii* and sequential *T. delbrueckii*/*S. cerevisiae* cultures, in comparison to wines resulting from AF with only *S. cerevisiae*. Monitoring 3SH and its precursors (Glut-3SH and Cys-3SH) throughout AF led us to conclude that *T. delbrueckii* only assimilates the

glutathionylated precursor, while both precursor forms are metabolized by *S. cerevisiae*. In pure *T. delbrueckii* cultures, Glut-3SH degradation produced significant amounts of 3SH and Cys-3SH in the wine. In mixed cultures, the more *T. delbrueckii* developed, the higher the Glut-3SH uptake and Cys-3SH release. In sequential cultures, which favored *T. delbrueckii* development compared to the simultaneous protocol, the results revealed an increase in the cysteinylated precursor followed by an increase in 3SH. Hence, once released by *T. delbrueckii*, the cysteinylated precursor was converted into 3SH by *S. cerevisiae* in the last stage of AF. The direct consequence was higher overall 3SH and 3SHA production than in pure *S. cerevisiae* cultures. Further work with different strains of *T. delbrueckii* and *S. cerevisiae* will help to confirm the synergistic interaction described between these two species.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: JC, VM, CT, MB, and PR. Performed the experiments: PR and CT.

ACKNOWLEDGMENTS

The authors thank Inno'vin for its precious help and Aquitaine Regional Council for its financial support.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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The Interaction between *Saccharomyces cerevisiae* and Non-*Saccharomyces* Yeast during Alcoholic Fermentation Is Species and Strain Specific

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OPEN ACCESS

Edited by:

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Specialty section:

This article was submitted to
Food Microbiology,
a section of the journal
Frontiers in Microbiology

Received: 22 February 2016

Accepted: 29 March 2016

Published: 13 April 2016

Citation:

Wang C, Mas A and Esteve-Zarzoso B
(2016) The Interaction between
Saccharomyces cerevisiae and
Non-*Saccharomyces* Yeast during
Alcoholic Fermentation Is Species and
Strain Specific.
Front. Microbiol. 7:502.
doi: 10.3389/fmicb.2016.00502

The present study analyzes the lack of culturability of different non-*Saccharomyces* strains due to interaction with *Saccharomyces cerevisiae* during alcoholic fermentation. Interaction was followed in mixed fermentations with 1:1 inoculation of *S. cerevisiae* and ten non-*Saccharomyces* strains. *Starmerella bacillaris*, and *Torulaspora delbrueckii* indicated longer coexistence in mixed fermentations compared with *Hanseniaspora uvarum* and *Metschnikowia pulcherrima*. Strain differences in culturability and nutrient consumption (glucose, alanine, ammonium, arginine, or glutamine) were found within each species in mixed fermentation with *S. cerevisiae*. The interaction was further analyzed using cell-free supernatant from *S. cerevisiae* and synthetic media mimicking both single fermentations with *S. cerevisiae* and using mixed fermentations with the corresponding non-*Saccharomyces* species. Cell-free *S. cerevisiae* supernatants induced faster culturability loss than synthetic media corresponding to the same fermentation stage. This demonstrated that some metabolites produced by *S. cerevisiae* played the main role in the decreased culturability of the other non-*Saccharomyces* yeasts. However, changes in the concentrations of main metabolites had also an effect. Culturability differences were observed among species and strains in culture assays and thus showed distinct tolerance to *S. cerevisiae* metabolites and fermentation environment. Viability kit and recovery analyses on non-culturable cells verified the existence of viable but not-culturable status. These findings are discussed in the context of interaction between non-*Saccharomyces* and *S. cerevisiae*.

Keywords: contact-dependent interaction, culturability loss, excreted compounds, viable but not-culturable (VBNC), wine

INTRODUCTION

Spontaneous wine fermentation is driven by a succession of different yeast species. A great variety of non-*Saccharomyces* yeast species originate from grape berries and survive during the early stages of fermentation, such as species from the genera *Candida*, *Hanseniaspora*, *Lachancea*, *Metschnikowia*, *Pichia*, and *Torulaspora* (Fleet, 2003). Some species such as *Starmerella bacillaris* and *Hanseniaspora uvarum* grow to a high density (10^5 – 10^7 cells/mL) and dominate other non-*Saccharomyces* species

(Wang et al., 2015b). There is a growing interest regarding the impact of non-*Saccharomyces* yeasts on the final wines, and some species are now used as fermentation starters (Jolly et al., 2014). However, fermentative *Saccharomyces cerevisiae* soon replaces non-*Saccharomyces* species to become the main or the only species present in the late stages of fermentation. Non-*Saccharomyces* strains are assumed to “die off” because these cells gradually lose their ability to form colonies on growth media, i.e., they lose the capacity to grow. The culturability loss of non-*Saccharomyces* strains has drawn widespread attention in recent years due to new findings that mention the role of excreted compounds in the interaction between *Saccharomyces* and non-*Saccharomyces* yeasts (Ciani and Comitini, 2015; Liu et al., 2015; Albergaria and Arneborg, 2016). Moreover, in a work by Branco et al. (2015), it was shown that viable but not-culturable (VBNC) status was related to interaction through excreted compounds. Therefore, as more non-conventional wine yeasts have been explored as wine starters in mixed fermentation with *S. cerevisiae* (Masneuf-Pomarede et al., 2016), studies on culturability loss of different non-*Saccharomyces* strains will help in understanding their final impact on wine quality.

The culturability loss of non-*Saccharomyces* strains at the late stages of alcoholic fermentation is a complicated phenomenon due to the multitude of factors involved. It is conventionally regarded to be related to their insufficient adaptability to environmental changes in fermentations, such as nitrogen limitation (Monteiro and Bisson, 1991), low oxygen availability (Holm Hansen et al., 2001) and inhibition of increased ethanol (Fleet, 2003), as well as extrinsic factors such as SO₂ (Ribéreau-Gayon et al., 2006). However, Nissen et al. (2003) proposed that *S. cerevisiae* S101 adopted a contact-dependent mechanism to induce the culturability loss of some non-*Saccharomyces* strains (*Lachancea thermotolerans* and *Torulaspora delbrueckii*). Subsequently, the contact-dependent mechanism was confirmed by studies using the same *S. cerevisiae* strain (Nissen et al., 2004; Renault et al., 2013; Kemsawasd et al., 2015a). However, it was found that *S. cerevisiae* CCMI 885 excreted toxic compounds, which inhibited the growth of *Hanseniaspora guilliermondii* and *H. uvarum*, demonstrating the interaction of these species through excreted antimicrobial compounds (Pérez-Nevado et al., 2006). Recent studies further elucidated that *S. cerevisiae* CCMI 885 produced antimicrobial peptides, which altered intracellular pH, membrane permeability and culturability of non-*Saccharomyces* strains (Albergaria et al., 2010; Branco et al., 2014, 2015). Interestingly, in the work of Wang et al. (2015c), not only the excreted products from *S. cerevisiae* NSa but also the synthetic media, induce a lack of culturability of *H. uvarum*. However, the synthetic must was weaker at inducing a lack of culturability of *H. uvarum* than *S. cerevisiae* supernatant, which included the same media plus the yeast metabolites. Thus, the role of environmental changes should be taken into consideration when studying the interaction between different yeasts.

Until now, studies on culturability loss of non-*Saccharomyces* yeasts have mainly focused on several potential wine starters: *H. guilliermondii*, *H. uvarum*, *Kluyveromyces marxianus*, *L. thermotolerans*, and *T. delbrueckii* (reviewed in Albergaria and Arneborg, 2016). However, few studies have focused on the

culturability differences among strains. According to Branco et al. (2014), different *D. bruxellensis* strains showed strain-specific sensitivity toward antimicrobial peptides excreted by *S. cerevisiae*. The differences between contact-dependent mechanisms and interactions through extracellular compounds were ascribed to the *S. cerevisiae* strains used (Kemsawasd et al., 2015a). Therefore, more yeast species and strains should be considered to gain a better understanding of the interaction between *S. cerevisiae* and non-*Saccharomyces* yeasts.

This study was aimed at (i) investigating the strain and species differences in culturability loss, (ii) analyzing the interaction mechanisms that exist in different strains, and (iii) determining the viable status of non-culturable cells. We investigated the interaction between *S. cerevisiae* NSa (the same strain used in our former work, Wang et al., 2015c) and 10 non-*Saccharomyces* strains from different sources belonging to *H. uvarum*, *S. bacillaris*, *M. pulcherrima*, and *T. delbrueckii* to analyze the interactions in mixed fermentation between *S. cerevisiae* and each individual strain. Through the use of three types of media (supernatants from *S. cerevisiae* fermentation, synthetic media mimicking *S. cerevisiae* fermentation and mixed fermentation), the performance of each non-*Saccharomyces* strain was compared and studied. Synthetic must was used to rule out other effects and to define the media to mimic the must at different stages of fermentation. Recovery analysis and viability assays were also conducted to evaluate the status of non-culturable cells.

MATERIALS AND METHODS

Yeasts Strains and Culture Conditions

Eleven yeast strains were used in this study, containing *H. uvarum* CECT13130, NSb and CECT1444^T, *S. bacillaris* NSc, NSd and CECT11046, *M. pulcherrima* Mp com and Mp 51, *T. delbrueckii* Td com and CECT13135, and *S. cerevisiae* NSa. These strains were obtained from different collections: CECT13130, NSa, NSb, NSc, NSd, Mp 51, CECT13135 and NSa were natural isolates from our collection (Wang et al., 2014). CECT1444 and CECT11046 were from Spanish Type Culture Collection. Mp com (Flavia) and Td com (Biodiva) were commercial strains from Lallemant Inc. (Canada).

The species identity of all strains was determined by 5.8S-ITS-RFLP analysis (Esteve-Zarzoso et al., 1999) and sequence analysis of the D1/D2 domain of 26S rDNA (Kurtzman and Robnett, 1998). Yeasts were grown overnight in YPD medium (1% yeast extract, 2% peptone, and 2% glucose, w/v, pH 6.2) at 28°C before use.

Alcoholic Fermentations, Sampling, and Setting Culturability

Synthetic must (100 g/L fructose, 100 g/L glucose, 290 mg N/L amino nitrogen, and 120 mg N/L ammonium nitrogen, pH 3.3) was prepared according to Andorrà et al. (2012). 350 mL of synthetic must was added to a 500 mL screw cap bottle, inoculated with 10⁶ cells/mL of each yeast strain and kept at 25°C in a shaker at a speed of 120 rpm. Fermentations were performed in the presence of air because the caps were not screwed tightly

on the bottles. Each of the mixed fermentations was inoculated with one non-*Saccharomyces* strain and *S. cerevisiae* NSa. As a comparison, a single *S. cerevisiae* fermentation was carried out with the NSa strain. Fermentations were conducted in duplicate; when an interaction was observed, the fermentations were repeated in another duplicate and thus four replicas were used to set the interaction analysis.

Samples were taken every day to follow sugar and nitrogen consumption, ethanol production and yeast population dynamics until the end of fermentation. Concentrations of ethanol, fructose and glucose were tested using an enzymatic kit from Roche Diagnostics (Germany). The level of individual amino acids and ammonium was analyzed by HPLC according to Andorrà et al. (2012). Yeast populations in all samples were quantified using a microscope and plating after appropriate dilution in sterile water. YPD agar medium was used to calculate the total number of yeast cells present, and lysine agar medium (Oxoid LTD., England) was used for quantification of non-*Saccharomyces* strains.

Three stages were set up for each species depending on the culturability of the non-*Saccharomyces* species in mixed fermentations (**Figure 1A**): 1. When culturable populations reached the highest level; 2. When culturable populations started to decrease; 3. When no colonies grew on plates, or at the end of fermentation for some strains if colonies were still seen on plates. The concentration of main chemical components (ethanol, fructose, glucose, individual amino acids, and ammonium), fermentation time and non-*Saccharomyces* strain culturability at these stages were listed to mimic the conditions of each fermentation stage where the interaction between non-*Saccharomyces* strains and *S. cerevisiae* was set (**Figure 1** and **Table 1**). Data from these stages at mixed and single fermentations were used for the interaction assays in next step.

Culture Assays Using Different Synthetic Media

To further understand the culturability of non-*Saccharomyces* strains in mixed fermentation, three types of synthetic media were prepared (**Figure 1**): supernatant from *S. cerevisiae* fermentation (S), synthetic medium mimicking *S. cerevisiae* fermentation (MS) and synthetic medium mimicking mixed fermentation (MM). S was collected from *S. cerevisiae* fermentation (**Figure 1B**), centrifuged and filtered using a 0.22 μm Whatman syringe filter (GE Healthcare Life Science, Germany). S was spread onto YPD agar plates to confirm the absence of *S. cerevisiae* cells. As a comparison, MS was prepared with metabolites (ethanol, fructose, glucose, individual amino acid, and ammonium) mimicking S, with the absence of *S. cerevisiae* excreted compounds (**Figure 1D**). By performing culture assays using S and MS, the effect of main fermentation metabolites (the same for S and MS) and other putative *S. cerevisiae* metabolites (only in S) could be observed. Considering the possible differences of the main metabolites produced by *S. cerevisiae* fermentation and mixed fermentation, MM was prepared with corresponding components mimicking the mixed

fermentation (**Figure 1C**). Moreover, no micronutrients or vitamins were added to MS and MM due to fast consumption at the beginning of alcoholic fermentation. All of the synthetic media were prepared for the three fermentation stages selected in 2.2 and were named with Arabic numbers to differentiate these stages (**Figure 1** and **Table 1**).

Single fermentations of each non-*Saccharomyces* strain were then performed to provide adapted cells as described in Wang et al. (2015c). These adapted cells were incubated in YPD to ensure viability and incubated in synthetic media to check their culturability on plates. Culture assays were conducted at 25°C in duplicate with a shaking speed of 120 rpm; when a culturability decrease was observed, the culture assays were repeated in another duplicate and thus four replicas were used to follow the culturability changes of non-*Saccharomyces* yeasts. Samples were taken at 24, 48, and 120 h to quantify yeast cells using a microscope and YPD plating after appropriate dilution in sterile water. Cells losing culturability in synthetic media were collected for the following recovery analysis and viability assay.

Recovery Analysis and Viability Assay of Non-culturable Cells from Synthetic Media

To test the viability of non-culturable cells from synthetic media, two approaches were used. Membrane integrity was analyzed by using the LIVE/DEAD® BactLight™ Bacterial Viability kit (Molecular Probes Inc., USA). In this assay, yeast cells were stained and observed using a fluorescence microscope equipped with filter system I3 and N2.1 (Leica DM 4000B) as in Hierro et al. (2006). The capacity to grow in rich liquid media was analyzed by incubating the cells in fresh YPD medium. Cells that could be recovered were considered to be viable but not culturable in synthetic media. Cells that could not be recovered after two consecutive 48 h incubations in fresh YPD medium were analyzed again by the LIVE/DEAD® BactLight™ Bacterial Viability kit.

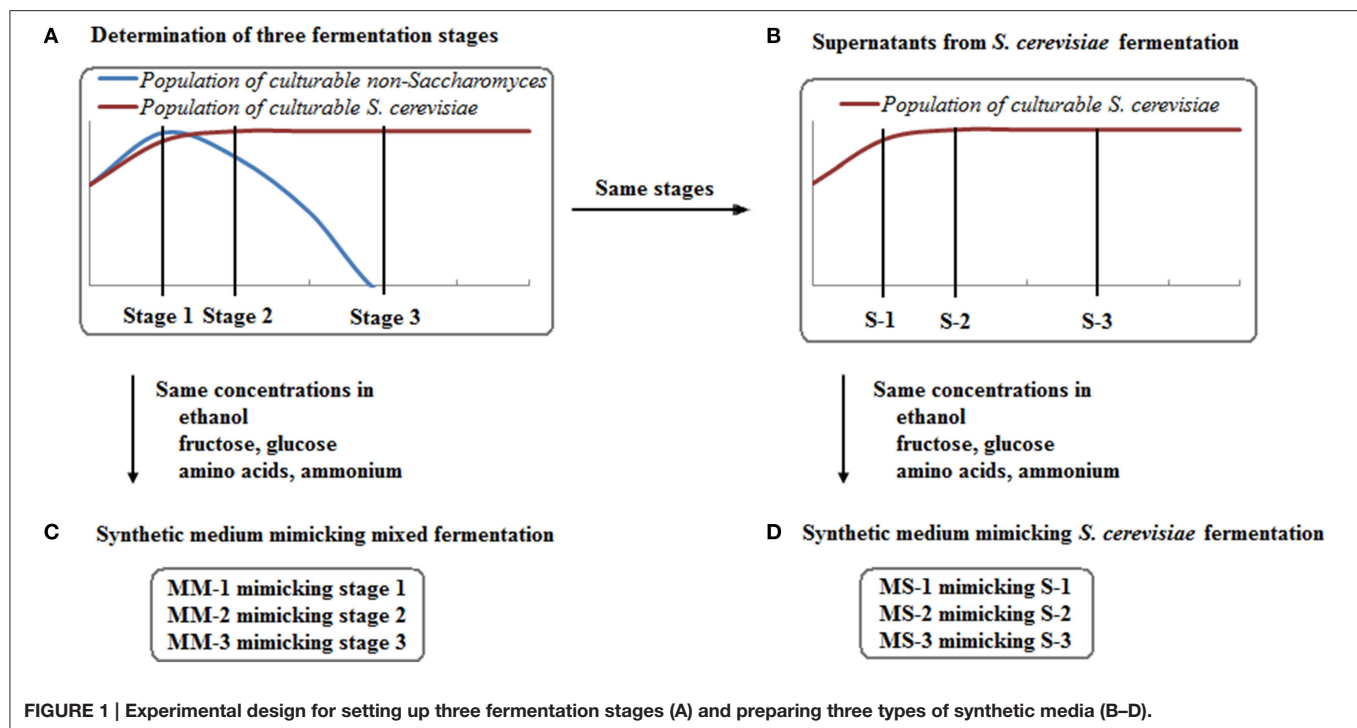
Statistical Analysis

One-way ANOVA by IBM SPSS Statistics 23 was used to calculate the value of significance for the variation analysis, and included a *post-hoc* Tukey test when needed. The consumption ratio (% of the total) of nutrients was used directly for the analysis of variation.

RESULTS

Culturable Population and Metabolic Characteristics of Non-saccharomyces Strains during Alcoholic Fermentation

Overall, both *S. cerevisiae* and non-*Saccharomyces* strains reached the maximum population number of 10^7 – 10^8 cells/mL 24 h after inoculation, and this size was maintained during mixed and single fermentations. Culturability of non-*Saccharomyces* strains decreased in all mixed fermentations. This decrease varied not only among different yeast species but also among some strains within the same species (**Figure 2**). Culturable *H. uvarum* increased to 10^7 – 10^8 cfu/mL at 24 h and began to decrease at



48 h. No colonies were formed on lysine plates for CECT1444 after 72 h and for CECT13130 and NSb after 96 h. Similar to *H. uvarum*, *M. pulcherrima* grew to 10^7 cfu/mL and quickly started to decrease. At 96 h, no colonies of Mp com were recovered on lysine plates, and for Mp 51, no colonies were recovered after 48 h. Culturable *S. bacillaris* maintained a population size of 10^7 – 10^8 cfu/mL until 96 h at which time the population started to decline. After 120 h, 10 to 100 cfu/mL of NSd and CECT11046 were recovered, however no colonies of NSc grew from lysine plates. Finally, *T. delbrueckii* strains reached approximately 10^7 cfu/mL and were maintained up to 48 h. After 48 h, CECT13135 started to decline, and no colonies were recovered after 144 h. The other *T. delbrueckii* strain, Td com, showed a slow decrease in culturable population, and at 144 h, 10^6 cfu/mL of Td com were still culturable. Based on the culturability of non-Saccharomyces strains in mixed fermentations, three stages were set up for each species. The fermentation times and main metabolites of these three fermentation stages are shown in Table 1. As a comparison, the same stage in fermentation of *S. cerevisiae* is also listed.

Despite the different culturability of non-Saccharomyces strains in mixed fermentations, no obvious variations in fermentation length were observed, and all fermentations finished after 120 or 144 h. Similar to *S. cerevisiae* fermentation, all mixed fermentations consumed glucose faster than fructose, and the final ethanol concentration reached 11–12% V (Table 1). However, analysis of the consumption of these main metabolites after 24 h revealed strain-dependent differences. As shown in Table 2, the strains that lost culturability faster were those that consumed some metabolites faster in the mixed fermentations: (i) Within *H. uvarum*, mixed fermentations inoculated with CECT1444 consumed glucose, ammonium and arginine faster

than the other two strains; (ii) For *M. pulcherrima*, mixed fermentations with Mp 51 metabolized fructose, glucose, alanine, ammonium, arginine and glutamine faster than mixed fermentations with Mp com; (iii) Arginine was consumed faster during mixed fermentations with *S. bacillaris* NSc than the other two strains; (iv) More arginine was consumed during mixed fermentations with *T. delbrueckii* CECT13135 than Td com.

The Influence of Excreted Compounds from *S. cerevisiae* and Media Composition on the Culturability of Non-saccharomyces Strains

To further elucidate the culturability of non-Saccharomyces strains and the interaction with *S. cerevisiae* during mixed fermentation, we performed culture assays using S (supernatant from *S. cerevisiae* fermentation), MS (synthetic media mimicking *S. cerevisiae* fermentation), and MM (synthetic media mimicking mixed fermentation) based on the three stages of fermentation (Table 1). Although the non-Saccharomyces strains maintained a population size of 10^7 – 10^8 cells/mL for 120 h, as determined by cell counting under a microscope, not all strains were culturable during the 5-day period. The culturability was dependent on the media used, as well as the yeast species and strain.

No effect on culturability was seen in any media in fermentation stage 1 (S-1, MS-1, MM-1), which corresponded to the fermentation stage where culturable populations of non-Saccharomyces strains were the highest (generally between 10^7 and 10^8 cfu/mL). However, in the media from fermentation stage 2 (only S-2), and in the media from fermentation stage 3 (all the media), a decrease in culturable populations was observed

TABLE 1 | Fermentation stages, population size and chemical characteristics of the media at different single and mixed fermentation stages.

Species	Fermentation time (h)	Culturable non-saccharomyces (cfu/mL)	Ethanol (v/v)	Fructose (g/L)	Glucose (g/L)	Total assimilable nitrogen (mg N/L)	Names of synthetic media
<i>H. uvarum</i>	24	$2.6 \pm 1.3 \times 10^7$	1.6 ± 0.2	74.6 ± 4.3	59.0 ± 4.1	66.8 ± 26.7	MM-1
	24	–	1.8 ± 0.0	76.7 ± 0.0	52.3 ± 0.0	5.0 ± 0.0	MS-1
	48	$2.0 \pm 1.1 \times 10^7$	3.1 ± 0.6	49.8 ± 2.1	25.1 ± 6.1	6.6 ± 3.3	MM-2
	48	–	4.2 ± 0.1	45.2 ± 1.6	13.7 ± 1.7	1.2 ± 0.0	MS-2
	96	nd	9.4 ± 0.5	12.7 ± 5.0	0.9 ± 0.7	nd	MM-3
	96	–	10.2 ± 0.4	4.1 ± 2.9	nd	nd	MS-3
<i>M. pulcherrima</i>	24	$8.1 \pm 3.3 \times 10^6$	2.2 ± 0.6	89.6 ± 4.1	53.1 ± 7.2	87.4 ± 54.1	MM-1
	24	–	1.8 ± 0.0	76.7 ± 0.0	52.3 ± 0.0	5.0 ± 0.0	MS-1
	48	$3.2 \pm 6.8 \times 10^6$	6.7 ± 0.9	56.1 ± 4.9	23.6 ± 5.7	2.5 ± 1.2	MM-2
	48	–	4.2 ± 0.1	45.2 ± 1.6	13.7 ± 1.7	1.2 ± 0.0	MS-2
	96	nd	10.2 ± 0.5	15.9 ± 6.0	0.7 ± 0.8	nd	MM-3
	96	–	10.2 ± 0.4	4.1 ± 2.9	nd	nd	MS-3
<i>S. bacillaris</i>	24	$4.2 \pm 3.7 \times 10^7$	1.5 ± 0.2	68.6 ± 3.0	50.7 ± 2.2	18.3 ± 4.7	MM-1
	24	–	1.8 ± 0.0	76.7 ± 0.0	52.3 ± 0.0	5.0 ± 0.0	MS-1
	96	$8.8 \pm 8.3 \times 10^6$	9.9 ± 0.5	5.1 ± 1.9	nd	nd	MM-2
	96	–	10.2 ± 0.4	4.1 ± 2.9	nd	nd	MS-2
	120	$4.7 \pm 8.2 \times 10^1$	11.6 ± 0.1	0.1 ± 0.1	nd	nd	MM-3
	120	–	11.5 ± 0.4	0.1 ± 0.1	nd	nd	MS-3
<i>T. delbrueckii</i>	24	$1.9 \pm 0.7 \times 10^7$	3.1 ± 0.1	90.8 ± 3.4	48.4 ± 0.9	20.1 ± 13.9	MM-1
	24	–	1.8 ± 0.0	76.7 ± 0.0	52.3 ± 0.0	5.0 ± 0.0	MS-1
	96	$1.1 \pm 1.9 \times 10^6$	10.4 ± 0.4	19.8 ± 5.1	1.1 ± 1.9	nd	MM-2
	96	–	10.2 ± 0.4	4.1 ± 2.9	nd	nd	MS-2
	144	$1.8 \pm 2.4 \times 10^6$	11.7 ± 0.6	1.5 ± 1.9	nd	nd	MM-3
	120	–	11.5 ± 0.4	0.1 ± 0.1	nd	nd	MS-3

This chemical composition was used to define the media mimicking the three selected fermentation stages. All values are the average of different strains within the same species. "MM" means synthetic media with main metabolites (ethanol, fructose, glucose and nitrogen) mimicking mixed fermentations, whereas "MS" is named after synthetic media with main metabolites mimicking *S. cerevisiae* fermentation. The Arabic numbers 1, 2, and 3 refer to the three stages selected in fermentations. "–" refers to the absence of a non-Saccharomyces population as derived from single *S. cerevisiae* fermentations, whereas "nd" means not detected. The total assimilable nitrogen is the sum of nitrogen from assimilable amino acids and ammonium. Only amino acid concentrations higher than 0.9 mg N/L are considered, and the concentrations are shown in Supplementary Table 1.

(Figures 3–6), although the extent of the decrease was species- and strain-dependent.

Within *H. uvarum* strains, the decrease of culturability was seen at 24 h in S-2 and S-3 and at 120 h in MS-3 and MM-3 (Figure 3). *H. uvarum* strains lost culturability in both S-2 and S-3; however, the decrease in culturability in S-2 occurred more slowly than in S-3. Further, among the three strains, CECT1444 showed a much slower decrease in culturability in S-2. The media (MS-3 and MM-3) also affected the culturability, but to a lesser extent, and they were evident only at 120 h. There was also a strain difference, such that NSb was more affected than the other two strains.

Regarding the *M. pulcherrima* strains, a slow decrease in culturability was observed in all media mimicking the fermentation stages (MS-2, MM-2, MS-3, and MM-3), whereas a sharp decrease was seen in the *S. cerevisiae* supernatants (S-2 and S-3). Mp com and Mp51 showed different culturability in both S-2 and MS-3 (Figure 4), with Mp com exhibiting higher sensitivity.

The culturability of three *S. bacillaris* strains was less affected by different synthetic media, as some colonies

were recovered (Figure 5). All of the strains showed a decrease in culturability during all studied periods (120 h), with no relevant differences between strains. Only in S-3 was a difference in sensitivity observed with strain NSc, which showed much lower culturability than the other two strains.

Similar to *S. bacillaris*, the effect of synthetic media on the culturability of the two *T. delbrueckii* strains was limited (Figure 6). However, when the cells were cultured in S-3, no colonies were recovered after 120 h. Instead, only a small decrease of culturability was observed in MS-3 and S-2.

Although the decrease of culturability varied among different species, S-3 consistently showed the most obvious effect compared with other synthetic media. For the two species more affected (*H. uvarum* and *M. pulcherrima*), a more obvious effect was shown in S-2 than in MS-3 or MM-3. Thus, it is likely that some substances secreted from *S. cerevisiae* played a principal role in the interaction between *S. cerevisiae* and non-Saccharomyces strains, and that changes in the media (ethanol, nitrogen and sugar) also mediated the interaction.

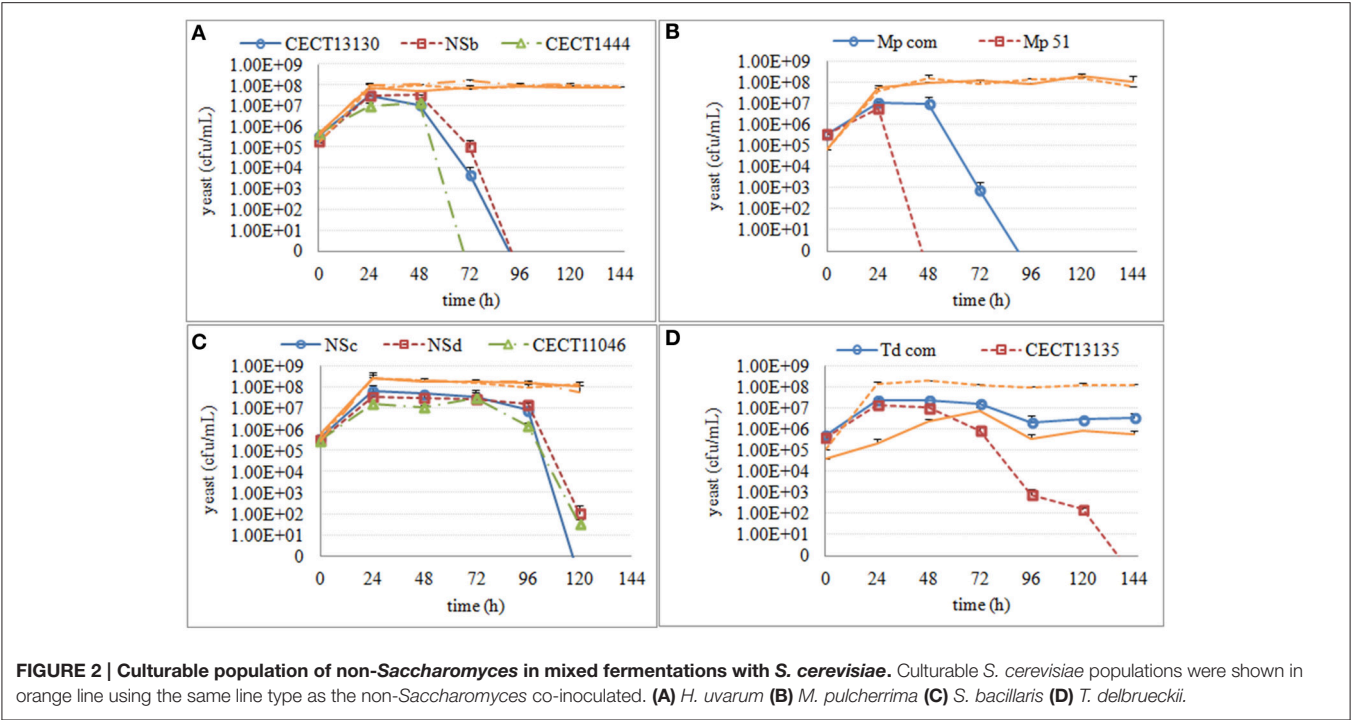


TABLE 2 | Consumption ratio of glucose, alanine, ammonium, arginine, and glutamine at 24 h of fermentation.

Non-Saccharomyces strains		% of the total					Time of culturability loss	
		Fructose	Glucose	Alanine	Ammonium	Arginine		Glutamine
sc		23.3	47.7	100.0	100.0	100.0	100.0	nd
sc+hu	CECT13130	27.2	37.6*	90.4*	68.1*	65.1*	100.0	96 h
	NSb	23.3	39.2*	93.1*	63.4*	66.7*	100.0	96 h
	CECT1444	25.7	46.2 [#]	91.6*	100.0 [#]	71.9* [#]	100.0	72 h
sc+mp	Mp com	6.9* [#]	40.7* [#]	13.4* [#]	69.1* [#]	53.7* [#]	88.4* [#]	96 h
	Mp 51	13.9*	53.2*	96.7	91.2*	67.8*	100.0	48 h
sc+sb	NSc	35.1*	51.6	100.0	100.0	91.4* [#]	100.0	120 h
	NSd	30.4*	48.5	96.9	100.0	82.7*	100.0	nd
	CECT11046	28.8*	47.7	98.3	100.0	84.1*	100.0	nd
sc+td	Td com	10.8*	51.6	99.4	100.0	75.9* [#]	100.0	nd
	CECT13135	7.7*	51.6	99.9	100.0	93.5*	100.0	144 h

sc means *S. cerevisiae* single fermentation, and mixed fermentations are presented as sc+hu (*S. cerevisiae* + *H. uvarum*), sc+mp (*S. cerevisiae* + *M. pulcherrima*), sc+sb (*S. cerevisiae* + *S. bacillaris*), sc+td (*S. cerevisiae* + *T. delbrueckii*). nd means not detected. *significance ≤ 0.05 with respect control (sc) by one-way ANOVA. #significantly different from the other strains of the same species as determined by a post-hoc Tukey test.

The Viability of the Non-culturable Cells

To improve the understanding of the lack of culturability of the non-*Saccharomyces* strains, all of the samples (34 cases in total) that did not grow on plates were tested using two different methods: membrane integrity using the LIVE/DEAD viability kit and recovery by suspension in liquid YPD with agitation. On one hand, these non-culturable cells were immediately analyzed using the LIVE/DEAD viability kit (Supplementary

Figure 1). The results showed that live fluorescent cells were only found three times: non-culturable cells of the Mp com strain at 24 h in S-2 yielded 0.13% of live fluorescent cells, at 48 h in MM-3 8.17% and the Mp 51 strain at 120 h in MM-2 2.84 %. All other non-culturable cells yielded dead fluorescent cells. On the other hand, all non-culturable cells were evaluated by recovery analysis. The non-culturable cells with live fluorescent

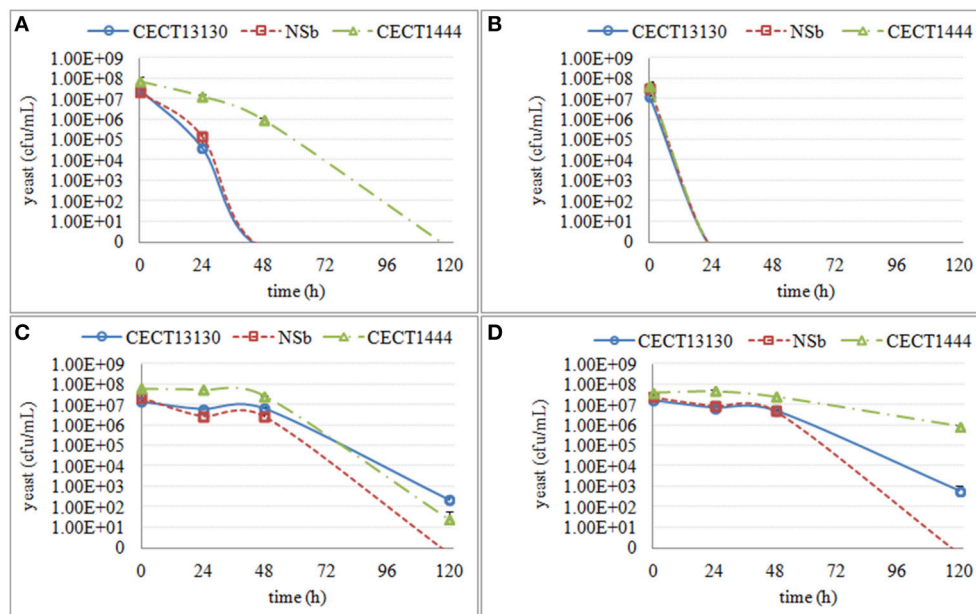


FIGURE 3 | The culturable population of three *H. uvarum* strains (CECT13130, NSb and CECT1444) grown in different synthetic media for 120 h. (A) the growth in supernatant from the second stage of *S. cerevisiae* fermentation S-2 (B) the growth in supernatant from the third stage of *S. cerevisiae* fermentation S-3 (C) the growth in synthetic media MS-3 (D) the growth in synthetic media MM-3.

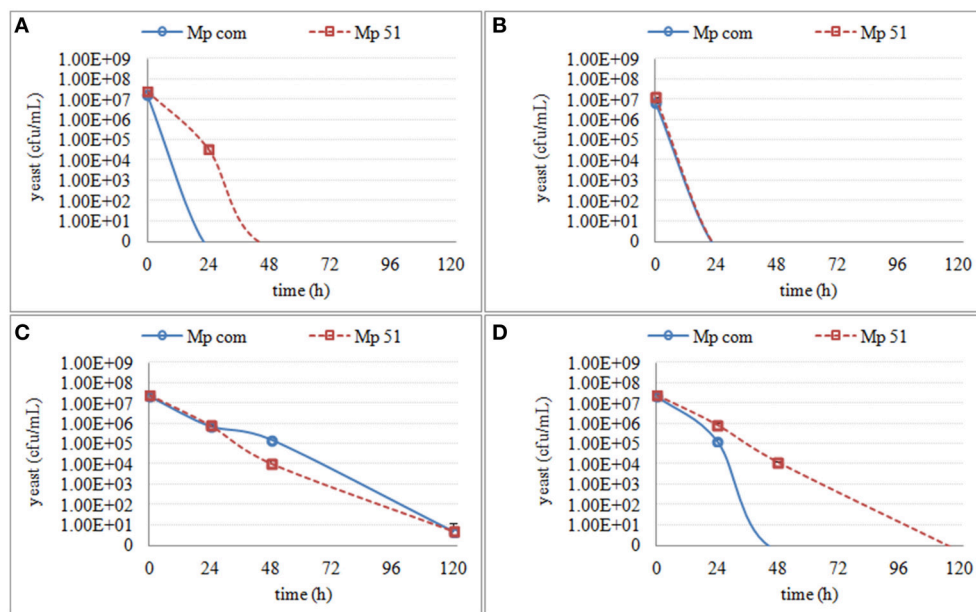


FIGURE 4 | The culturable population of two *M. pulcherrima* strains (Mp com and Mp 51) grown in different synthetic media for 120 h. (A) the growth in supernatant from the second stage of *S. cerevisiae* fermentation S-2 (B) the growth in supernatant from the third stage of *S. cerevisiae* fermentation S-3 (C) the growth in synthetic media MS-3 (D) the growth in synthetic media MM-3.

were recovered when incubated in liquid YPD medium, whereas some of the non-culturable cells with dead fluorescent could also be recovered. The latter cases were found seven times involving three non-*Saccharomyces* species. For example, after

120 h exposure to mimicking media MS-3 and MM-3, *H. uvarum* NSb, as well as *H. uvarum* CECT1444 after S-2 and the two *T. delbrueckii* strains after S-3, appeared dead in the fluorescence analysis but could be recovered. In the case of *H. uvarum*

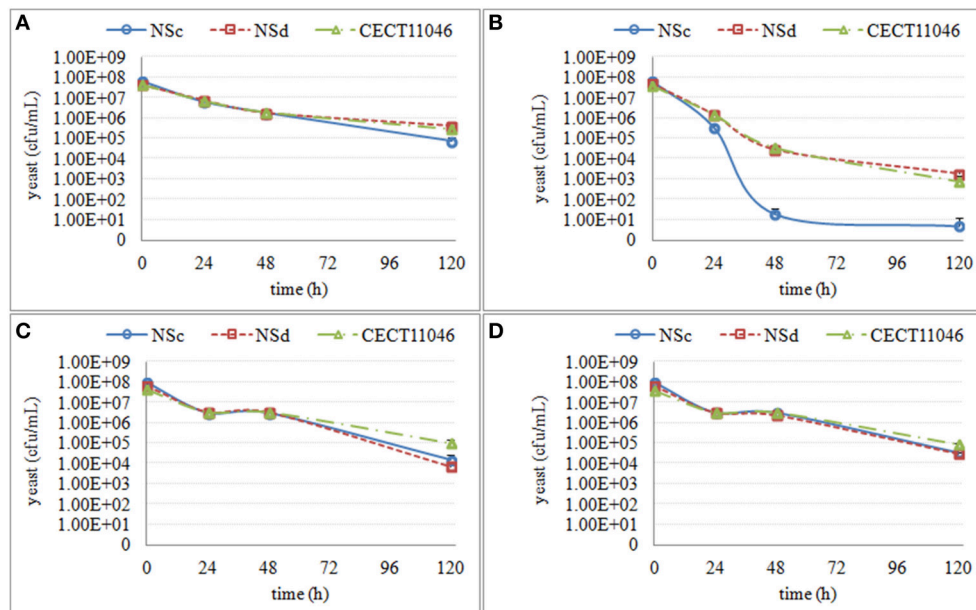


FIGURE 5 | The culturable population of three *S. bacillaris* strains (NSc, NSd and CECT11046) grown in different synthetic media for 120 h. (A) the growth in supernatant from the second stage of *S. cerevisiae* fermentation S-2 (B) the growth in supernatant from the third stage of *S. cerevisiae* fermentation S-3 (C) the growth in synthetic media MS-3 (D) the growth in synthetic media MM-3.

CECT13130, this observation was seen only in the early stages of exposure (48 h) to the media. Likewise, Mp com could be recovered after 120 h exposure to mimicking media MM-2 but could not be recovered from MM-3. Both cases indicate the existence of an intermediate, transient step before the cells are completely dead.

The LIVE/DEAD viability kit was again used to check the cells that could not be recovered by consecutive incubation in liquid YPD medium. All cells that could not be recovered yielded only dead fluorescent.

DISCUSSION

The culturability loss of non-*Saccharomyces* strains during late stages of alcoholic fermentation has been well documented (Fleet, 2003). However, despite recent advances, the cellular mechanism underlying culturability loss is still a matter of discussion (Ciani and Comitini, 2015; Liu et al., 2015; Albergaria and Arneborg, 2016). In a previous study (Wang et al., 2015c), we investigated how *S. cerevisiae* NSa interacted with *H. uvarum* NSb by the use of a compartmented dialysis system, cell-free supernatant and mimicking synthetic media. Due to the absence of a contact-dependent mechanism in *S. cerevisiae* NSa, in the present study we decided to focus on the effects of compounds secreted by *S. cerevisiae* NSa, and the changes in main metabolites (ethanol, glucose, fructose, amino nitrogen and ammonium nitrogen).

Our results indicated that cell-free supernatant from *S. cerevisiae* fermentation influenced cellular culturability much more than mimicking synthetic media at the same fermentation stage (same chemical composition for major

metabolites). Therefore, as mentioned in Wang et al. (2015c), some putative *S. cerevisiae* metabolites played a main role in the interaction between *S. cerevisiae* NSa and other non-*Saccharomyces* strains. A faster culturability loss was induced by *S. cerevisiae* supernatant at stage 3 than the initial two stages, which demonstrated the possible accumulation, or higher effect, of the *S. cerevisiae* secreted compounds as fermentation proceeded. Studies from Pérez-Nevaldo et al. (2006) and Williams et al. (2015) further related the accumulation to the amount of sugar consumed by *S. cerevisiae*. Likewise, antimicrobial peptides identified by Branco et al. (2014) were derived from a glycolytic enzyme, showing a probable link with sugar metabolism of *S. cerevisiae*. More research is still required to illustrate how sugar consumption regulates the secretion of antimicrobial peptides or other putative metabolites.

Moreover, according to our previous report (Wang et al., 2015c), as fermentation proceeds, the changes of the main metabolites also decreased the culturability of the cells, and the present results indeed showed that synthetic media at stage 3 caused a decrease in culturability. However, this effect occurs more slowly in the sensitive species and strains, and in our culture assays, complete culturability loss was mostly found after 48 or 120 h. This indicates that the changes in the main metabolites play a role in the interaction between *S. cerevisiae* NSa and other non-*Saccharomyces* strains, and vice versa. Because not all of the species were equally affected, it also showed the capacity of some non-*Saccharomyces* strains to withstand a harsh environment (ethanol higher than 10% vol, glucose lower than 1 g/L, fructose lower than 16 g/L and no available nitrogen).

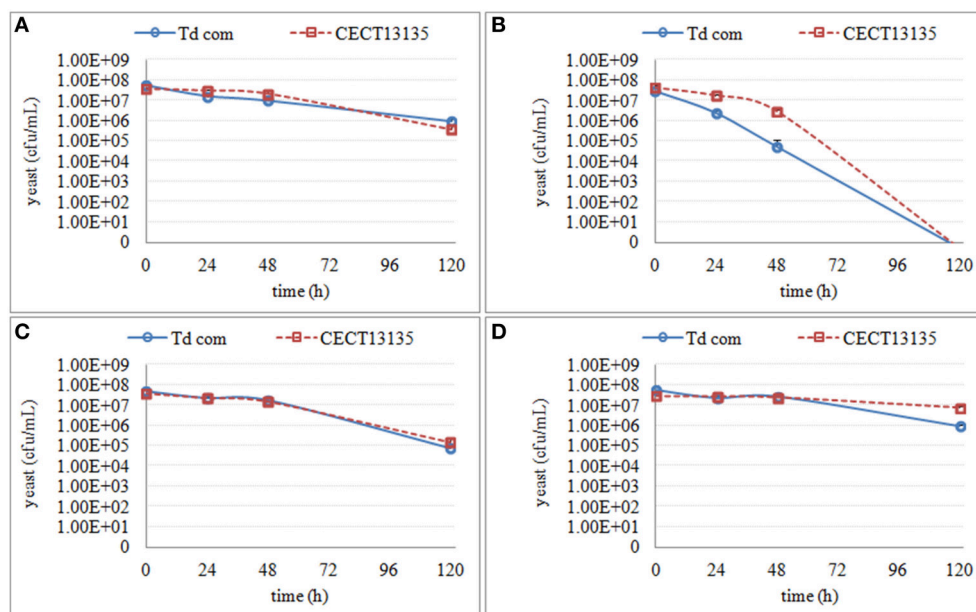


FIGURE 6 | The culturable population of two *T. delbrueckii* strains (Td com and CECT13135) grown in different synthetic media for 120 h. (A) the growth in supernatant from the second stage of *S. cerevisiae* fermentation S-2 (B) the growth in supernatant from the third stage of *S. cerevisiae* fermentation S-3 (C) the growth in synthetic media MS-3 (D) the growth in synthetic media MM-3.

The interaction between *S. cerevisiae* and non-*Saccharomyces* strains also relied on the participating yeast species. In our mixed fermentations, cells of *S. bacillaris* and *T. delbrueckii* could coexist longer with *S. cerevisiae* than *H. uvarum* and *M. pulcherrima*. Other studies proposed that oxygen availability, glucose uptake rate and nitrogen source might contribute to the longer co-existence (Holm Hansen et al., 2001; Nissen et al., 2004; Andorrà et al., 2012; Taillandier et al., 2014). We indeed found that mixed fermentation inoculated with *S. bacillaris* or *T. delbrueckii* present a consumption rate of glucose, alanine, ammonium and arginine more similar to single fermentations with *S. cerevisiae* than those mixed fermentations inoculated with *H. uvarum* and *M. pulcherrima*. However, the relation between species tolerance and consumption of some nutrients still needs further investigation.

As expected, strain differences within each species were observed in mixed fermentation, culture assays and recovery analyses. First, strains decrease their culturability to a different extent during mixed fermentation. Second, when incubated in the same synthetic media in culture assays, strains showed different culturability or tolerance to a harsh environment. Third, non-culturable cells from the same synthetic media showed different recovery abilities depending on the strain. The strain difference, to some extent, increased the complexity of interaction analysis between *S. cerevisiae* and non-*Saccharomyces* strains. In our case, the hypothesis of “strain tolerance to hard environment” cannot simply explain the strain differences in mixed fermentations. *H. uvarum* CECT1444 exhibited a slow culturability decrease in S-2, MM-2 and MM-3 as compared to the other two strains and thus was regarded as a strain

that is highly tolerant to harsh environments. However, despite being a highly tolerant strain, in mixed fermentation, the culturability decreased even earlier than the other two *H. uvarum* strains. When we analyzed the metabolites at 24 h of mixed fermentation inoculated with *H. uvarum* CECT1444, a faster consumption of glucose, ammonium and arginine was detected. Andorrà et al. (2012) and Kemsawasd et al. (2015b) reported the influence of nitrogen consumption on yeast growth and fermentation performance. Nevertheless, further research should be undertaken to elucidate this effect, which was also observed in strains Mp 51, *S. bacillaris* NSc and *T. delbrueckii* CECT13135 compared with other strains within the same species.

Another important finding was the appearance of non-culturable cells when incubated with synthetic media, yielding more than 90 % of cells with “dead” fluorescence by viability analysis but that could be recovered by incubation in YPD medium. However, when these cells were incubated longer in the synthetic media (24 h more), all showed dead fluorescence and could no longer be recovered in YPD medium. This phenomenon demonstrated the existence of VBNC status of at least *H. uvarum*, *M. pulcherrima*, and *T. delbrueckii* during alcoholic fermentation. As hypothesized by Branco et al. (2015), VBNC status could be understood as a transition status of yeast from culturable cells to dead cells, involving sub-lethally and severely injured cells. During this transition process, the ability to form colonies is the first lost vital activity and progressive changes in the permeability of cell membrane occur as found in this study, however the DNA or RNA remains stable (Andorrà et al., 2010; Wang et al., 2014, 2015a). Branco et al. (2015) measured how antimicrobial peptides secreted by *S. cerevisiae* affected cell viability and reported that

injured cells had a similar pH as the external pH, whereas cells without compromised membranes (impermeable to propidium iodide) maintained a higher pH. Further research is still required to determine how the interactions between *S. cerevisiae* and non-*Saccharomyces* impacts physiological status and metabolic capacity of cells in different status.

In conclusion, we investigated the interaction between one *S. cerevisiae* strain and ten non-*Saccharomyces* strains during alcoholic fermentation. We demonstrated that the decrease of culturability was mainly caused by metabolites secreted by *S. cerevisiae*, although the change of the composition in main metabolites in the media also played a role. We also found that culturability loss of non-*Saccharomyces* yeasts was not only species-dependent but also strain-dependent. The finding of VBNC status and strain differences in culturability is meaningful to the exploration of *Saccharomyces*-non-*Saccharomyces* interactions. The understanding of these interactions is relevant for the development of non-*Saccharomyces* strains as starters in wine production.

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AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: AM, BE. Performed the experiments: CW. Generated and analyzed the data: CW, AM, BE. Wrote the paper: CW, AM, BE.

ACKNOWLEDGMENTS

The present work was supported by the Wildwine EU Project (grant agreement 315065). CW is the recipient of a grant from China Scholarship Council (State Scholarship Fund No. 201206300028), and we thank the guidance from Rosa Pastor for HPLC analysis.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fmicb.2016.00502>

- culture fermentations of grape juice with *Saccharomyces cerevisiae*. *J. Appl. Microbiol.* 91, 541–547. doi: 10.1046/j.1365-2672.2001.01426.x
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- Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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The Genetics of Non-conventional Wine Yeasts: Current Knowledge and Future Challenges

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OPEN ACCESS

Edited by:

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Universitat Rovira i Virgili, Spain

Reviewed by:

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Científicas, Spain

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Specialty section:

This article was submitted to
Food Microbiology,
a section of the journal
Frontiers in Microbiology

Received: 06 November 2015

Accepted: 23 December 2015

Published: 11 January 2016

Citation:

Masneuf-Pomarede I, Bely M,
Marullo P and Albertin W (2016) The
Genetics of Non-conventional Wine
Yeasts: Current Knowledge and Future
Challenges. *Front. Microbiol.* 6:1563.
doi: 10.3389/fmicb.2015.01563

Saccharomyces cerevisiae is by far the most widely used yeast in oenology. However, during the last decade, several other yeasts species has been purposed for winemaking as they could positively impact wine quality. Some of these non-conventional yeasts (*Torulaspora delbrueckii*, *Metschnikowia pulcherrima*, *Pichia kluyveri*, *Lachancea thermotolerans*, etc.) are now proposed as starters culture for winemakers in mixed fermentation with *S. cerevisiae*, and several others are the subject of various studies (*Hanseniaspora uvarum*, *Starmerella bacillaris*, etc.). Along with their biotechnological use, the knowledge of these non-conventional yeasts greatly increased these last 10 years. The aim of this review is to describe the last updates and the current state-of-art of the genetics of non-conventional yeasts (including *S. uvarum*, *T. delbrueckii*, *S. bacillaris*, etc.). We describe how genomics and genetics tools provide new data into the population structure and biodiversity of non-conventional yeasts in winemaking environments. Future challenges will lie on the development of selection programs and/or genetic improvement of these non-conventional species. We discuss how genetics, genomics and the advances in next-generation sequencing will help the wine industry to develop the biotechnological use of non-conventional yeasts to improve the quality and differentiation of wines.

Keywords: non-conventional yeast, non-Saccharomyces, wine, enology, oenology, microsatellite

INTRODUCTION

In oenology, alcoholic fermentation is generally performed by *Saccharomyces cerevisiae* yeast, the “conventional” wine yeast. Currently, the winemakers have the choice between hundreds of *S. cerevisiae* starters that have been selected for various characteristics including their ability to complete alcoholic fermentation in oenological conditions, their low release of off-flavor compounds, their positive impact on wine aromas, etc., (Pretorius, 2000; Marullo and Dubourdieu, 2010). The growing demand for more diversified wines or for specific characteristics (low ethanol content, etc.) has led to the exploration of new species for winemaking. These non-conventional yeasts may contribute to the wine's flavor and taste by producing a broad range of secondary metabolites and extracellular enzymes (Hong and Park, 2013; Ciani et al., 2014; Wang et al., 2015). Some species could be interesting for alcohol level reduction in wine (Masneuf-Pomarede et al., 2010; Bely et al., 2013) or for greater fermentative ability in harsh conditions due to enhanced fructophily (Sutterlin, 2010; Magyar and Tóth, 2011). It has to be noted that, as only

some *Saccharomyces* species (i.e., *S. cerevisiae*, *S. uvarum*, and some interspecific hybrids) are able to consume all the sugar contained in grape must, non-*Saccharomyces* yeasts must be used in co- or sequential-fermentation with a *Saccharomyces* spp. able to secure AF completion (Jolly et al., 2006; Bely et al., 2013).

The wine industry currently proposes starters of a few non-conventional yeasts (*Torulaspora delbrueckii*, *Metschnikowia pulcherrima*, *Pichia kluyveri*, *Lachancea thermotolerans*, etc.), while several other species (*Hanseniaspora uvarum*, *Starmerella bacillaris*, etc.) are the subject of various studies to assess both positive contribution (Table 1) and negative impact (if any) on wine quality (Bely et al., 2013; Maturano et al., 2015). These non-conventional yeasts are widely distributed amongst the *Saccharomycetales* (Figure 1). In order to evaluate the oenological potential of a given species, several strains are usually compared for phenotypes of interest like fermentation ability (Renault et al., 2009) or glycerol production (Magyar and Tóth, 2011). However, in most cases, neither the relationships between the tested strains are described, nor the genetic structuration of the species is known. This lack of genetic knowledge is clearly detrimental, since we are not able to determine whether the phenotypic diversity described is representative of the species or not. The recent advances in next-generation sequencing (NGS) have triggered the development of genomic and genetic tools for some of these non-conventional yeasts, but the field is still in its infancy. The objective of this paper is thus to review the current state-of-art of the genetics of non-conventional wine yeasts and to discuss the future prospects and challenges from an oenological viewpoint.

BASIC GENETIC KNOWLEDGE OF WINE YEASTS

As a model organism, the genomic outline of *S. cerevisiae* is well-known: its genome size is around 12 Mb organized in 16 chromosomes, with a mitochondrial genome of 85 Kb (Table 1). The genome sequences of several hundreds of strains of various origins are available, and much more sequences are produced easily using NGS technology and subsequently assembled even by lab with moderate bioinformatics skills. The population genomics of *S. uvarum* has been improved recently with the sequencing of more than 50 strains of various origins (Almeida et al., 2014). The type strain CBS7001^T has a genome size of 11.5 Mb and 16 chromosomes (Cliften et al., 2003). By contrast, such basic knowledge (genome size, chromosome number, etc.) is available only for a small number of non-conventional wine species: *T. delbrueckii* has a genome of 9–11 Mb distributed on eight chromosomes; *L. thermotolerans* has a 10.4 Mb genome with eight chromosomes. Other wine yeast species usually have genome size ranging from 8 to 12 Mb, with chromosomes number unknown yet (*P. kluyveri*, *M. pulcherrima*, etc.). Moreover, there is still a lack of reference genome sequence for several non-conventional wine yeasts of interest like *S. bacillaris*, *P. fermentans*, etc., (Table 1). Disparities exist also for the mitochondrial genome, with full sequences available for some species like *L. thermotolerans* or *H. uvarum*, and partial

sequences for other species (*C. stellata*, *P. membranifaciens*, etc.). Thus, although the genomic data of non-conventional wine yeast greatly increased this last decade, there is still a lot of work to achieve in this field.

THE LIFE-CYCLE OF WINE YEASTS

The life cycle of *Saccharomyces* wine species is well-known: both *S. cerevisiae* and *S. uvarum* are diploid species that divide asexually by mitosis. They are able to enter meiosis and form asci containing generally four haploid spores (tetrads). While haploid cells can undergo mitosis, the haploid level is generally transient and crosses between haploid spores of opposite mating types are readily observed, leading to diploid zygote formation. Moreover, haploid cells are usually able to switch mating type at mitosis (homothallism). The physical proximity between mother and daughter haploid cells of opposite mating type usually results in high level of inbreeding (Ruderfer et al., 2006; Cubillos et al., 2009; Warringer et al., 2011). Variations in this breeding system were described for *S. cerevisiae* like near-dioecy or higher level of outcrossing, but seemed quite rare and associated with environmental specificities (Knop, 2006; Al Safadi et al., 2010; Murphy and Zeyl, 2010).

By comparison, the precise life-cycle of most non-*Saccharomyces* yeasts is unknown yet. Sporulation was observed for most non-conventional yeast, albeit forming non-tetrad asci in many cases (*T. delbrueckii*, *D. hansenii*, *H. vineae*, etc., Table 1). No evidence of sporulation ability was recorded to date for *Starmerella/Candida* species. Data regarding the occurrence of sexual reproduction is usually scarce for most non-*Saccharomyces* yeasts, so classical genetic manipulations are impossible to date. To circumvent this limitation, both intra and inter specific hybridizations by protoplast fusion can be achieved as demonstrated in the past (Ball, 1984; Pina et al., 1986).

The basic ploidy level is also usually unresolved (Table 1): *T. delbrueckii* has been considered as a haploid species for a long time, but the detection of several strains harboring several loci with two alleles (26.4% of strains showing heterozygosity), its ability to sporulate and the presence of mating type genes is more congruent with a diploid status (Albertin et al., 2014a). Conversely, for *S. bacillaris*, the proportion of heterozygous strains was almost null (0.01%). This, combined with its inability to sporulate, is more consistent with an hypothesis of an haploid status (Masneuf-Pomarede et al., 2015) but has still to be formally demonstrated. Finally, despite its fully sequenced genome, the ploidy status of *L. thermotolerans* is controversial: haploid or diploid depending on the authors (Souciet et al., 2009; Freel et al., 2014). In conclusion, the biological life-cycle of many non-*Saccharomyces* yeasts remains to be elucidated.

ECOLOGY OF WINE YEAST

Most wine yeasts can colonize several ecological niches, including wine-related environments like grape, must, winery equipment and premise (Table 1). Moreover, many of them can be isolated from other human-associated processes (brewery, bakery, dairy,

TABLE 1 | Comparison of wine yeast species.

Species/ synonym (anamorph)	Features of interest in winemaking	Genome size	Full nuclear genome sequence	Basic ploidy level	Sporulation/ zygote formation	Heterozygosity ^a	Ecological niches	Genetic subgroups	Genetic diversity from winemaking environments ^b
<i>Saccharomyces cerevisiae</i>	AF completion	Nucleus: 12.0 Mb, 16 chromosomes (Goffeau et al., 1996). Mitochondrion: 85 Kb (Foury et al., 1998).	Several hundred sequences: lab strain S288c (Goffeau et al., 1996), wine strains EC1118 (Novo et al., 2009) and AWRI1631 (Borneman et al., 2008), the 100-genomes strains (Strope et al., 2015), etc.	Diploid, occasional tetraploid associated with specific environments (Albertin et al., 2009; Al Safadi et al., 2010)	4 spores per ascus. Zygotes readily observed. (Kurtzman et al., 2011)	75.1–81.9% (308/410 clones, 136/166 clones) (Legras et al., 2007; Muller and McCusker, 2009)	Wild environments: fruit, plant, insect, soil. Anthropic environments: wine, other distilled and traditional fermented beverages, food fermentation, dairy product, bioethanol. Lab environments. Clinical environments. (Fay and Benavides, 2005; Legras et al., 2007; Kvittek et al., 2008; Diezmann and Dietrich, 2009; Schacherer et al., 2009; Wang et al., 2012)	Wild and domestic populations associated with wine, beer, bread, etc. (Fay and Benavides, 2005; Legras et al., 2007; Almeida et al., 2015), multiple domestication events (Schacherer et al., 2009).	0.39–0.65 (Albertin et al., 2014b); 0.00–1.00 (Schuller et al., 2012); 0.27–0.35 (Hall et al., 2011)
<i>Saccharomyces uvarum</i>	AF completion (Masneuf- Pomarede et al., 2010); reduced ethanol production (Bely et al., 2013); psychrophilism (Masneuf- Pomarede et al., 2010); Acetate ester production (Masneuf- Pomarede et al., 2010)	Nucleus: 11.5 Mb, 16 chromosomes (Almeida et al., 2014).	More than 50 genomes of which CBS7001 ^T (Clifton et al., 2003; Almeida et al., 2014)	Diploid	4 spores per ascus. Zygotes readily observed. (Kurtzman et al., 2011)	0% (0/40 strains) (Masneuf- Pomarede et al., 2007)	Wild environments: plant. Anthropic environments: wine, cider. (Almeida et al., 2014)	Wild and domestic populations associated with wine and cider. (Almeida et al., 2014)	0.00–0.62 (Masneuf- Pomarede et al., 2007)

(Continued)

TABLE 1 | Continued

Species/ synonym (anamorph)	Features of interest in winemaking	Genome size	Full nuclear genome sequence	Basic ploidy level	Sporulation/ zygote formation	Heterozygosity ^a	Ecological niches	Genetic subgroups	Genetic diversity from winemaking environments ^b
<i>Torulaspora delbrueckii</i> (<i>Candida colliculosa</i>)	Volatile acidity reduction (Bely et al., 2008); Aroma and complexity (Ciani and Maccarelli, 1998; Renault et al., 2009; Azzolini et al., 2012)	Nucleus: 9.2–11.5 Mb, 8 chromosomes (Gordon et al., 2011; Gomez-Angulo et al., 2015). Mitochondrion: 28–45 Kb (Wu et al., 2015).	2 genomes: CBS 1146 ^T and NRRL Y-50541 (Gordon et al., 2011; Gomez-Angulo et al., 2015)	Unclear, could be diploid (Albertin et al., 2014a)	One spore per ascus, occasional 2–3 spores/ascus (Kurtzman et al., 2011; Albertin et al., 2014a).	26.4% (29/110 strains) (Albertin et al., 2014a)	Wild environments: fruit, plant, insect, soil. Anthropic environments: wine, other distilled and traditional fermented beverages, food fermentations, dairy products. (Albertin et al., 2014a)	Wild and domestic populations associated with wine and other bioprocesses, geographical clustering for wild populations (Albertin et al., 2014a).	0.35–1.00 (Albertin et al., 2015)
<i>Hanseniaspora uvarum</i> (<i>Kloeckera apiculata</i>)	Aroma (Rojas et al., 2001)	Nucleus: 8.08–9.08 Mb, 8 to 9 chromosomes (Esteve-Zarzoso et al., 2001). Mitochondrion: 11Kb (Pranateftaki et al., 2006).	2 genomes: DSM 2768 and 34–9 (NCBI ¹)	Unclear, could be diploid (Albertin et al., 2016)	One, seldom two spores per ascus (Kreger-van Rij, 1977). Zygotes described ³ .	82.6% (95/115 strains) (Albertin et al., 2016)	Wild environments: fruit, plant, insect, bird, mollusc, shrimp, soil. Anthropic environments: wine, other distilled and traditional fermented beverages. (Grangateau et al., 2015; Albertin et al., 2016)	Geographical and temporal clustering (Albertin et al., 2016).	1.00 (but low number of strains per sample) (Albertin et al., 2016).
<i>Hanseniaspora guilliermondii</i> (<i>Kloeckera apis</i>)	Acetate ester production (Rojas et al., 2001; Moreira et al., 2008; Viana et al., 2008)	Nucleus: 8 to 9 chromosomes (Esteve-Zarzoso et al., 2001).	–	–	Four spores per ascus (Barnett et al., 2000). Zygotes described ³ .	–	Wild environments: fruit, soil. Anthropic environments: wine.	–	–
<i>Hanseniaspora vineae</i> (<i>Kloeckera africana</i>)	Acetate ester production (Viana et al., 2011)	Nucleus: 11.4 Mb, 5 chromosomes (Esteve-Zarzoso et al., 2001; Giorello et al., 2014).	1 genome: T02/19AF (Giorello et al., 2014)	–	One, seldom two spores per ascus (Kreger-van Rij, 1977).	–	Anthropic environments: wine.	–	–

(Continued)

TABLE 1 | Continued

Species/ synonym (anamorph)	Features of interest in winemaking	Genome size	Full nuclear genome sequence	Basic ploidy level	Sporulation/ zygote formation	Heterozygosity ^a	Ecological niches	Genetic subgroups	Genetic diversity from winemaking environments ^b
<i>Starmerella bacillaris</i> (<i>Candida zeylanoides</i>)	Fructophily (Magyar and Tóth, 2011; Tófaló et al., 2012; Englezos et al., 2015); reduced ethanol production (Di Maio et al., 2012; Bely et al., 2013; Giaramida et al., 2013); glycerol production (Di Maio et al., 2012; Giaramida et al., 2013; Zara et al., 2014); Aroma release (Andorrà et al., 2012); other characteristics (Mangani et al., 2011; Sadoudi et al., 2012; Tófaló et al., 2012; Domizio et al., 2014; Magyar et al., 2014)	Nucleus: 3 chromosomes (Spiczki, 2004). Mitochondrion: 23 Kb (Primatefaki et al., 2008).	–	Unclear, could be haploid (Masneuf-Pomarede et al., 2015)	No evidence of sporulation ability (Masneuf-Pomarede et al., 2015)	0.01% (1/163) (Masneuf-Pomarede et al., 2015)	Rare in wild environments. Anthropogenic environments: grape and wine. (Masneuf-Pomarede et al., 2015)	No evidence of domestication event, geographical clustering. (Masneuf-Pomarede et al., 2015)	0.90–0.97 (Masneuf-Pomarede et al., 2015)
<i>Candida stellata</i> / <i>Torulopsis stellata</i>	Glycerol production (Ciani and Maccarelli, 1998); Fructophily (Magyar and Tóth, 2011)	Nucleus: 3 chromosomes (Spiczki, 2004)	–	–	No evidence of sporulation ability	–	Anthropic environments: wine (Csoma and Spiczki, 2008)	–	–
<i>Lachancea thermotolerans</i> / <i>Kluyveromyces thermotolerans</i>	Glycerol overproduction (Comitini et al., 2011); Acetate ester production (Comitini et al., 2011); reduction of volatile acidity (Comitini et al., 2011)	Nucleus: 10.4 Mb, 8 chromosomes (Malpertuy et al., 2000). Mitochondrion: 21.9–25.1 Kb (Talla et al., 2005; Freel et al., 2014).	1 genome: CBS 6340 ^T (Malpertuy et al., 2000)	Controversial: haploid (Freel et al., 2014) or diploid (Souciet et al., 2009)	One to four spores per ascus (Barnett et al., 2000). Zygotes described ³ .	–	Wild environments: fruit, plant. Anthropogenic environments: wine and agave fermentations (Freel et al., 2014)	Geographical clustering (Freel et al., 2014)	–

(Continued)

TABLE 1 | Continued

Species/ synonym (anamorph)	Features of interest in winemaking	Genome size	Full nuclear genome sequence	Basic ploidy level	Sporulation/ zygote formation	Heterozygosity ^a	Ecological niches	Genetic subgroups	Genetic diversity from winemaking environments ^b
<i>Lachancea kluyveri</i>	NA	Nucleus: 11.3 Mb, 8 chromosomes (Souciet et al., 2009). Mitochondrion: 49–53.7 Kb piskur 1998; 51.5 (Jung et al., 2012)	1 genome: NCYC 543 ^T (Souciet et al., 2009)	Diploid, occasional triploid (Freel et al., 2014)	–	–	Wild environments: soil, insect, plant (Jung et al., 2012).	Geographical clustering (Jung et al., 2012)	–
<i>Debaryomyces hansenii/Pichia hansenii (Candida famata)</i>	Enzymatic activities (Yanai and Sato, 1999)	Nucleus: 11–46–12.18 Mb, 7 chromosomes (Dujon et al., 2004) Mitochondrion: 29.5 Kb (Dujon et al., 2004)	2 genomes: CBS 767 and MTCC 234 (Dujon et al., 2004; Kumar et al., 2012)	Haploid (Breuer and Harms, 2006)	One (occasionally two) spores per ascus (Barnett et al., 2000). Zygotes described (Breuer and Harms, 2006)	–	Wild environments:ocean. Anthropic environments: cheese, grape.	–	–
<i>Pichia kluyveri/Hanseluna kluyveri</i>	Aromas (Anfang et al., 2009)	Mitochondrion: 43.1 Kb (CBS 7907) ¹ .	–	Diploid (Starmer et al., 1992)	Four spores per ascus (Barnett et al., 2000). Zygotes described (Starmer et al., 1992)	–	Wild environments: fruit, insect. Anthropic environments: wine. (Starmer et al., 1992)	–	–
<i>Pichia kudriavzevii/ Issatchenkia orientalis (Candida krusei)</i>	Under assessment (Clemente- Jimenez et al., 2004; Wang and Liu, 2013; Steensels and Verstrepen, 2014)	Nucleus: 10.18–12.94 Mb (Chan et al., 2012).	3 genomes:SD108, M12, NBRC 1279 (Chan et al., 2012)	Diploid	One or two spores per ascus (Barnett et al., 2000). Zygotes described ³ .	–	Wild environments: plant. Anthropic environments: wine, other traditional fermented beverages, food fermentation, dairy product. (Chan et al., 2012)	–	–

(Continued)

TABLE 1 | Continued

Species/ synonym (anamorph)	Features of interest in winemaking	Genome size	Full nuclear genome sequence	Basic ploidy level	Sporulation/ zygote formation	Heterozygosity ^a	Ecological niches	Genetic subgroups	Genetic diversity from winemaking environments ^b
<i>Pichia</i> <i>membranifaciens</i> (<i>Candida valida</i>)	Esters production (Viana et al., 2008)	Nucleus: 11.58 Mb ² , between 2 and 8 chromosomes (Naumov and Naumova, 2009)	1 genome ²	–	One to four spores per ascus (Barnett et al., 2000).	–	Wild environments: plant. Anthropic environments: AF and food spoilage yeast.	–	–
<i>Pichia fermentans</i> (<i>Candida lambica</i>)	Aromas (Clemente- Jimenez et al., 2005)	Maybe 2 chromosomes (Miller et al., 1989).	–	–	Two to four spores per ascus (Barnett et al., 2000). Zygotes described ³ .	–	Wild environments: plant, water, soil. Anthropic environments: wine, brewery. Clinical environments.	–	–
<i>Pichia anomala</i> / <i>Hanseluna</i> <i>anomala</i> (<i>Candida</i> <i>pelliculosa</i>)	Aromas (Rojas et al., 2001; Domizio et al., 2011a,b); killer against Dekkera/Brettanomyces (Comitini et al., 2004)	Nucleus: 26.55 Mb, 6 chromosomes (Friel et al., 2005).	1 genome: NRRL Y-366 ¹	Diploid	One to four spores per ascus (Barnett et al., 2000). Zygotes described ³ .	–	Wild environments: soil, water, plant, animal. Anthropic environments: wine, fermentation contaminant, ensilage (Kurtzman et al., 2011)	–	–
<i>Metschnikowia</i> <i>pulcherrima</i> / <i>Torulopsis</i> <i>pulcherrima</i> (<i>Candida</i> <i>pulcherrima</i>)	Aromas and esters production (Clemente- Jimenez et al., 2004; Parapouli et al., 2010; Zott et al., 2011; Sadoudi et al., 2012)	–	–	Diploid	One to two spores (Barnett et al., 2000).	–	Wild environments: plant. Anthropic environments: wine	–	–
<i>Zygosaccharomyces</i> <i>ballii</i>	Fructophily (Sutterlin, 2010)	Nucleus: 10.27–21.14 Mb, 5 to 13 chromosomes (Mira et al., 2014)	2 genomes: CLIB 213 ¹ and ISA 1307 (NCBI ¹)	Haploid and diploid strains (Rodrigues et al., 2003)	One to four spores per ascus (Barnett et al., 2000).	–	Wild environments: fruit, tree. Anthropic environment: food spoilage	–	–

^aProportion of strains with heterozygous microsatellite loci^bGenetic diversity (0 means fully clonal population and 1 means fully diversified population)Web sites: NCBI¹, <http://www.ncbi.nlm.nih.gov/genome/>; JGI², <http://genome.jgi.doe.gov/>; UCDAV/IS³, <http://wineserver.ucdavis.edu/industry/enology/winemicro/wineyeast/diversity.html>.

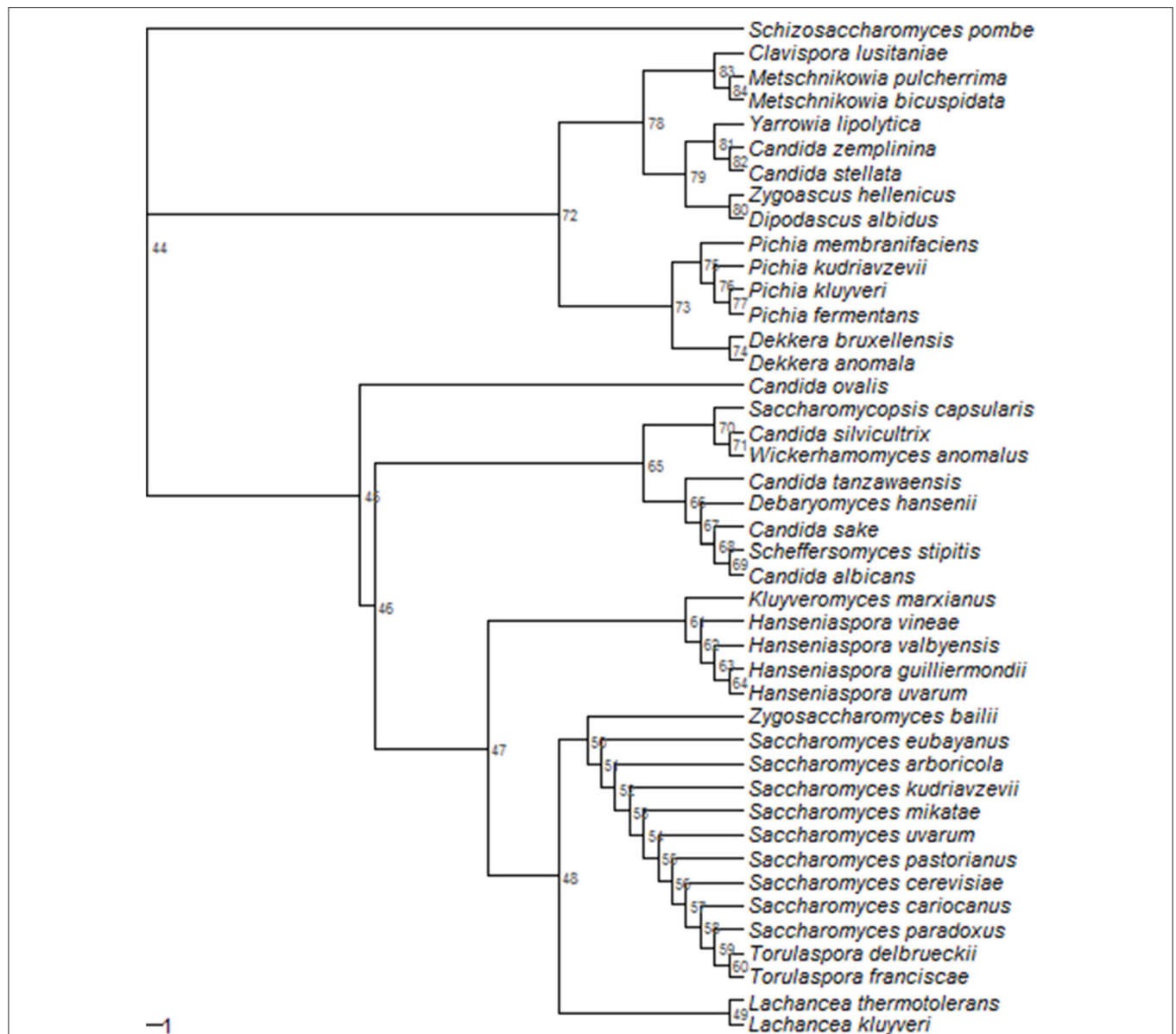


FIGURE 1 | Phylogeny of 41 species of Saccharomycetales on the basis of 18S ribosomal DNA sequence. Multiple sequence alignment (1951 bases) was performed by Clustal Omega (EMBL-EBI website). Genetic distance was computed using the K80 Kimura model (Kimura, 1980), phylogenetic tree was built using Neighbor joining clustering method and bootstrapping (1000 replicates) was used to assess the robustness of the nodes by means of R package ape (Paradis et al., 2004). *Schizosaccharomyces pombe* was used as outgroup species. The following sequences and strains (mostly type strains) were used: AB013504.1|*C. tanzawaensis* JCM 1648; AB018175.1|*C. stellata* JCM 9476; AB023473.1|*M. pulcherrima* IFO 1678; AB040997.1|*S. kudriavzevii* IFO 1802; AB040998.1|*S. mikatae* IFO 1815; AB054561.1|*C. silvicultrix* JCM 9831; AB013529.1|*C. sake* JCM 2951; AF548094.1|*S. cerevisiae* CBS 1171; AJ271813.1|*S. cariocanus* UFRJ 50816; AY046254.1|*H. valbyensis* NRRL Y-1626; AY046256.1|*H. guilliermondii* NRRL Y-1625; AY046257.1|*H. uvarum* NRRL Y-1614; AY046258.1|*H. vineae* NRRL Y-17529; S. bacillaris CBS 9494; EF550365.1|*P. membranifaciens* NRRL Y-2026; EF550372.1|*P. fermentans* Y-1619; EF550389.1|*P. kluyveri* NRRL Y-11519; EF550396.1|*D. anomala* NRRL Y-17522; EF550479.1|*Wickerhamomyces anomalus* NRRL Y-366; EU011714.1|*C. ovalis* NRRL Y-17662; EU011734.1|*D. bruxellensis* NRRL Y-12961; EU348783.1|*C. albicans* NRRL Y-12983; FJ153136.1|*L. thermotolerans* NRRL Y-8284; FJ153143.1|*T. franciscae* NRRL Y-6686; GU266277.1|*S. arboricola* AS 2.3317; GU597328.1|*Zygoascus hellenicus* CBS 5839; HQ651939.1|*Scheffersomyces stipitis* ATCC 58376; JQ698884.1|*Saccharomycopsis capsularis* NRRL Y-17639; JQ698900.1|*Clavispora lusitaniae* NRRL Y-11827; JQ698910.1|*Debaryomyces hansenii* NRRL Y-7426; JQ698926.1|*Yarrowia lipolytica* NRRL YB-423; JQ698936.1|*Schizosaccharomyces pombe* NRRL Y-12796; M55528.1|*P. kudriavzevii* MUCL 29849; S. eubayanus FM1318; S. uvarum CBS7001; X69846.1|*M. bicuspidata* MUCL 31145; X89523.1|*L. marxianus* CBS 712; X91083.1|*Zygosaccharomyces bailii* NCYC 1416; X97805.1|*S. pastorianus* NCYC 392; X97806.1|*S. paradoxus* CBS 432; X98120.1|*T. delbrueckii* CBS 1146; Z75580.1|*L. kluyveri* NCYC 543.

bioethanol, distillery, etc.) and also from wild substrates (soil, insect, plant, etc.). Isolation from clinical specimens is rarely described yet possible (yeasts being opportunistic microorganisms), and most wine yeasts are Generally Recognized As Safe (GRAS). Dissemination and transfer between the different ecological reservoirs could be performed through insects (Parle and Di Menna, 1966; Stefanini et al., 2012; Palanca et al., 2013), but also through human activities like material exchanges, etc., (Goddard et al., 2010). Indeed, although most wine yeasts are described as ubiquitous from an ecological viewpoint, some species have a restricted substrate range. This is the case of *H. guilliermondii* and *Starmerella* species for example, which are very rarely isolated from non-wine-related substrates (Masneuf-Pomarede et al., 2015). Thus, the study of most wine yeast should consider not only wine strains but also isolates from other technological processes and substrates in order to assess their biodiversity.

ADAPTATION TO WINEMAKING ENVIRONMENTS AND EVOLUTIONARY MECHANISMS

Wine environments are particularly harsh and inconstant: winemaking is a seasonal practice, so that yeasts present at the surface of grape berries at harvest suddenly have to survive in grape must containing high sugar concentrations, usually with sulfur dioxide content. Moreover, from an ecological viewpoint, the ensuing alcoholic fermentation is a rapidly fluctuating ecosystem: within a few days, grape must is depleted of nitrogen nutrients, while ethanol concentration and temperature increase steadily thanks to *Saccharomyces* spp. metabolism, thus conferring a fitness advantage for *Saccharomyces* spp. over the other wine yeasts (Goddard, 2008; Salvadó et al., 2011). In addition, the range of temperature can be quite high, with either short-term variations (daily variations) or long-term evolution (seasonal variations). As a result, within wine yeast species, some strains show specific wine-adaptation (Steensels and Verstrepen, 2014) like sulphite resistance (Divol et al., 2012), ethanol tolerance (García-Ríos et al., 2014), low pH adaptation (Pretorius, 2000), temperature adaptation (Naumov et al., 2000), etc. The underlying adaptive mechanisms vary greatly from one species to another: in *S. cerevisiae*, molecular approaches identified allelic variations as molecular causes of adaptation to the winemaking process (Aa et al., 2006; Marullo et al., 2007; Ambroset et al., 2011; Salinas et al., 2012; Jara et al., 2014). At the chromosome level, translocations were shown to be responsible for adaptation to sulfite (Zimmer et al., 2014). Polyploidy and hybridization are also major evolutionary processes that probably triggered adaptation to wine environments (Borneman et al., 2012; Erny et al., 2012) and are currently explored for biotechnological application (Timberlake et al., 2011; Plech et al., 2014; Blein-Nicolas et al., 2015; da Silva et al., 2015). Large genomic introgressions were evidenced in *S. uvarum* strains associated with human-driven fermentations, suggesting a link between introgressions and domestication (Almeida et al., 2014). Various horizontal gene transfers were

also evidenced for wine *S. cerevisiae* strains (Novo et al., 2009), and were shown to favor adaptation to the nitrogen-limited wine fermentation environment (Marsit et al., 2015). Other evolutionary mechanisms were described (Dujon et al., 2004; Barrio et al., 2006; Scannell et al., 2007), and it is highly probable that further investigations will allow the identification of additional adaptation processes in wine yeasts. In particular, it could be interesting to focus on transposon families and their possible implication in environmental adaptation (Zeyl, 2004; Liti et al., 2005; Sarilar et al., 2015), to explore the impact of mitochondrial genome variation regarding adaptation to wine environments and practices (Picazo et al., 2015; Wu et al., 2015) or to describe the landscape of gene duplication and prion involvement in fitness issues (Landry et al., 2006; Jarosz et al., 2014). However, to date, most of these data were obtained from *Saccharomyces* species and could now be obtained from non-*Saccharomyces* of interest.

POPULATION GENETICS OF YEAST SPECIES ASSOCIATED WITH WINEMAKING

Within a given species, the colonization of different ecosystems can lead to the evolutionary differentiation of the subpopulations, in relationship with their adaptation to environmental specificities. This is the case of *S. cerevisiae* species that shows genetic subgroups of wild and domestic strains associated with human activities like wine, bread, beer, sake, etc., (Fay and Benavides, 2005; Liti et al., 2009; Sicard and Legras, 2011; Almeida et al., 2015), that probably originated through multiple domestication events (Schacherer et al., 2009). In a recent study, Almeida et al. (2014) showed that *S. uvarum* was also divided in genetic subgroups, one of domestic strains used in both winemaking and cidermaking and associated with the northern hemisphere, while others subgroups were composed of wild isolates from South America and Australasia. The current hypothesis is that a Patagonian “wild” sub-population gave rise to the domestic subpopulation through a recent bottleneck (Almeida et al., 2014). Another wine species was recently described as domesticated: *T. delbrueckii* is also divided in genetic subgroups of wild and domestic strains (Albertin et al., 2014a). Moreover, the wine/grape-related group showed an increase ability to ferment sugar in oenological condition, confirming the occurrence of phenotypic domestication (Albertin et al., 2015). By contrast, no hint of domestication was recorded to date for *S. bacillaris* and *H. uvarum* whose genetic diversity is shaped by geographical localization and/or time variation (Masneuf-Pomarede et al., 2015; Albertin et al., 2016).

BIODIVERSITY IN WINEMAKING CONDITIONS

Several molecular methods were developed in order to perform intra-specific discrimination, like pulsed field electrophoresis, RAPD-PCR fingerprinting, tandem repeat-tRNA, Fourier

transform infrared spectroscopy, RFLP, etc., (Barquet et al., 2012; Tofalo et al., 2013, 2014; Pfliegler et al., 2014; Grangeteau et al., 2015). However, these approaches do not allow the establishment of the genetic relationships within a given species and subsequent population genetics studies. An alternative is the use of microsatellite genotyping. It has been successfully applied to *S. cerevisiae* (Legras et al., 2005; Richards et al., 2009), *S. uvarum* (Masneuf-Pomarede et al., 2009), *T. delbrueckii* (Albertin et al., 2014a), *S. bacillaris* (Masneuf-Pomarede et al., 2015), *H. uvarum* (Albertin et al., 2016) as well as to the spoilage wine yeast *Brettanomyces bruxellensis* (Albertin et al., 2014c), and is currently developed for additional wine species like *Meyerozyma guilliermondii* (Wrent et al., 2015). In addition to population genetic clustering, microsatellites allow measuring the genetic diversity of a given species in specific conditions. In *S. cerevisiae*, the genetic diversity varied greatly, from 0 (fully clonal populations) to 1 (fully diversified population, **Table 1**). The precise impact of *S. cerevisiae* diversity (or absence of diversity) on wine quality is still debated/studied (Egli et al., 1998; Howell et al., 2006; King et al., 2008) and the direct link between microbial diversity and wine complexity should be considered with caution. *S. uvarum* and *T. delbrueckii* showed also a large range of diversity (0.35–1 and 0–0.62). By contrast, other species show systematic high diversity (>0.9 for *H. uvarum* or *S. bacillaris*), suggesting that they are not under selective pressure in winemaking environments (Masneuf-Pomarede et al., 2015; Albertin et al., 2016).

FUTURE CHALLENGES

Definite progresses in the genetics of non-conventional yeasts were made in the last decade. However, there is still a great lack of data compared to the conventional wine yeast *S. cerevisiae*. Such knowledge is nowadays within reach thanks to the NGS revolution (Solieri et al., 2013). NGS allows the development of genome-assisted approaches like whole genome sequencing and resequencing, transcriptome profiling, ChIP-sequencing to identify DNA-structure, etc., (Solieri et al., 2013). *De novo* sequencing is greatly needed as some wine species still lack of nuclear and mitochondrial reference genomes (*S. bacillaris*, *P. fermentans*, *M. pulcherrima*, etc.). However, *de novo* assembly is sometimes difficult to conduct due to high heterozygosity level or sequence repeat, and led to draft genome with high number of contigs or scaffolds. For example, *H. uvarum* DSM 2768 genome displays 335 contigs, *P. kudriavzevii* M12 has 621 scaffolds, and *P. anomala* NRRL Y-366 shows 1932 scaffolds. Thus, the first aim of non-conventional wine yeast studies should be the completion of robust genomic sequences. Then, additional genome sequencing could be performed: genome re-sequencing using NGS captures individual genotypes and allows population genetics and ecologic studies within species. Such comparative genomics approaches were successfully applied to *S. cerevisiae* (Liti et al., 2009) and *S. uvarum* (Almeida et al., 2014), and could now address non-*Saccharomyces* yeasts of technological interest. In addition to intraspecific genomics, comparative genomics between yeast species is particularly useful to understand genome evolution (Liti and Louis, 2005). The identification

of specific metabolic pathways, gene duplications or functions between species may increase our appreciation of adaptation's mechanisms and their biotechnological interest (Blein-Nicolas et al., 2015). It has to be noted that several species genetically close to wine yeasts show no peculiar affinity with winemaking environment (**Figure 1**). This is the case of *S. paradoxus*: despite being the most closely related species to *S. cerevisiae*, *S. paradoxus* is essentially associated with wild environments and particularly trees (Sniegowski et al., 2002; Johnson et al., 2004). Comparative genomics of wine vs. non-wine yeast species could thus increase our knowledge of the common genomic requirement for grape/wine colonization, if any. Finally, NGS technologies have greatly improved genome-assisted approaches aiming at detecting genetic variants associated with phenotypes in *S. cerevisiae* (Ehrenreich et al., 2010). In particular, QTL-seq or genome-wide association studies (GWAS) could now be applied to non-conventional yeasts depending on whether classical breeding is possible (QTL-seq) or not (GWAS). These fields are blank pages waiting to be filled in the next future of oenology microbial research.

The use of mixed-cultures, combining both non-conventional yeasts and one *Saccharomyces* species able to complete AF, is increasing in winemaking. Thus, another challenge lies in understanding yeast-yeast interactions and their underlying mechanisms (Ciani et al., 2010; Ciani and Comitini, 2015). Indeed, several types of yeast-yeast interactions have been described in enological conditions: competition for nutriment, release of toxic compounds (Fleet, 2003), and even “quorum-sensing” like mechanisms (Nissen and Arneborg, 2003; Nissen et al., 2003; Renault et al., 2013). Understanding these complex interactions is of first importance as the combination of some yeast strains seems condemned to failure: for example, cell-cell contact was recently shown to be involved in the death of strains of *T. delbrueckii* and *L. thermotolerans* during mixed-culture alcoholic fermentation with *S. cerevisiae* (Renault et al., 2013; Kemsawasd et al., 2015). In some cases, yeast death was associated with the release of metabolites or killer toxin (Pérez-Nevado et al., 2006; Albergaria et al., 2010; Branco et al., 2015; Ramírez et al., 2015). The precise impact of such interactions regarding wine quality and aromas is still unclear (Ciani et al., 2006), but will have to be considered to control and optimize complex mixed oenological fermentation.

Finally, in addition to NGS-assisted approaches and interactions studies, another prospect in the field of non-conventional wine yeast lies in classical genetic approaches: indeed, one of the limits of the previously detailed approaches is their low ability in elucidating the basic life-cycle of wine yeasts, particularly regarding the occurrence and control of sexual reproduction. Still, classical breeding is one of the key issues for genetic improvement of industrial strains of *S. cerevisiae* (Pretorius, 2000; Giudici et al., 2005; Marullo et al., 2006; Steensels et al., 2014) and represents a technological barrier that must be overcome for actual improvement of non-*Saccharomyces* wine yeasts. There is an important need for traditional sporulation assays, spore microdissection attempts, subsequent segregant analyses, breeding assays, etc. In addition, genetic transformation of non-conventional wine yeasts would be a welcomed tool for subsequent functional studies (Pacheco et al.,

2009; Roberts and Oliver, 2011). These classical approaches are time-consuming and necessitate traditional yeast-manipulation know-how, sometimes viewed as old-fashioned and therefore neglected. However, these old approaches are essential for our future understanding of the genetics of non-conventional wine yeast, and are complementary to the more *en vogue* NGS-assisted approaches.

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FUNDING

This work was funded by the European Union's Seventh Framework Programme (FP7/2007–2013) under grant agreement n° 315065 WILDWINE project: “Multi-strain indigenous Yeast and Bacterial starters for “Wild-ferment” wine production.”

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Unraveling the Enzymatic Basis of Wine “Flavorome”: A Phylo-Functional Study of Wine Related Yeast Species

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OPEN ACCESS

Edited by:

Alberto Mas,
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Specialty section:

This article was submitted to
Food Microbiology,
a section of the journal
Frontiers in Microbiology

Received: 05 November 2015

Accepted: 08 January 2016

Published: 20 January 2016

Citation:

Belda I, Ruiz J, Alastruey-Izquierdo A,
Navascués E, Marquina D and
Santos A (2016) Unraveling the
Enzymatic Basis of Wine “Flavorome”:
A Phylo-Functional Study of Wine
Related Yeast Species.
Front. Microbiol. 7:12.
doi: 10.3389/fmicb.2016.00012

Non-*Saccharomyces* yeasts are a heterogeneous microbial group involved in the early stages of wine fermentation. The high enzymatic potential of these yeasts makes them a useful tool for increasing the final organoleptic characteristics of wines in spite of their low fermentative power. Their physiology and contribution to wine quality are still poorly understood, with most current knowledge being acquired empirically and in most cases based in single species and strains. This work analyzed the metabolic potential of 770 yeast isolates from different enological origins and representing 15 different species, by studying their production of enzymes of enological interest and linking phylogenetic and enzymatic data. The isolates were screened for glycosidase enzymes related to terpene aroma release, the β -lyase activity responsible for the release of volatile thiols, and sulfite reductase. Apart from these aroma-related activities, protease, polygalacturonase and cellulase activities were also studied in the entire yeast collection, being related to the improvement of different technological and sensorial features of wines. In this context, and in terms of abundance, two different groups were established, with α -L-arabinofuranosidase, polygalacturonase and cellulase being the less abundant activities. By contrast, β -glucosidase and protease activities were widespread in the yeast collection studied. A classical phylogenetic study involving the partial sequencing of 26S rDNA was conducted in conjunction with the enzymatic profiles of the 770 yeast isolates for further typing, complementing the phylogenetic relationships established by using 26S rDNA. This has rendered it possible to foresee the contribution different yeast species make to wine quality and their potential applicability as pure inocula, establishing species-specific behavior. These consistent results allowed us to design future targeted studies on the impact different non-*Saccharomyces* yeast species have on wine quality, understanding intra and interspecific enzymatic odds and, therefore, aiming to predict the most suitable application for the current non-*Saccharomyces* strains, as well as the potential future applications of new strains. This work therefore contributes to a better understanding of the concept of wine microbiome and its potential consequences for wine quality, as well as to the knowledge of non-*Saccharomyces* yeasts for their use in the wine industry.

Keywords: microbial terroir, enological enzymes, non-*Saccharomyces*, phylo-functional study, targeted yeast selection

INTRODUCTION

Microorganisms coexist and interact in many environments and processes, and this fact is of practical relevance for both the environmental and industrial fields (Ivey et al., 2013). Grape musts naturally contain a mixture of yeast species, and wine fermentation is not a "single-species" process (Fleet, 1990). Despite the dominance of *Saccharomyces cerevisiae* in fermentation, which is expected and welcomed to avoid stuck and sluggish fermentations, the indigenous non-*Saccharomyces* yeasts, already present in the musts, play a critical role during the early stages of fermentation. While these yeast species are not the ones mainly responsible for alcoholic fermentation, they can release a wide variety of hydrolytic enzymes depending on their diversity (Jolly et al., 2014). Non-*Saccharomyces* yeasts were originally held responsible for microbe-related problems in wine production due to their isolation from spoiled wines. However, in recent years both empiric and scientific knowledge has emerged concluding that, in some cases, higher microbial diversity improves wine complexity.

The concept of vineyard and wine microbiome has been addressed in recent years, obtaining extensive and meaningful results on the microbial complexity of the fermentation process (Liu et al., 2015). These population studies, carried out by both classical molecular methods and metagenomics, are currently ongoing to better understand and establish the concept of "microbial terroir" (Bokulich et al., 2013, 2014; Gilbert et al., 2014). Considering that a wide variety of yeast species have been identified in different scientific studies (Bisson and Joseph, 2009; Barata et al., 2012), the role of all these yeast species and their intraspecific variations need to be known. There is an intense debate over the pertinence of the concept of microbial terroir in vineyards and wine fermentation. Several factors have been described as determinants of microbial diversity in enological environments. Robust results reported by Bokulich et al. (2014) and Wang et al. (2015) have concluded that grape-associated microbial biogeography is non-randomly associated with regional, varietal and climatic factors across multi-scale viticultural areas. However, this concept should be studied in depth, encompassing a strain-typing level and its final influence on wine quality.

A non-*Saccharomyces* strain was first used intentionally in wine fermentation in the 1960s, when Cantarelli (1955) significantly reduced the volatile acidity of wines by using selected *Torulaspora delbrueckii* strains. Nowadays, there is a wide variety of current and expected applications of non-*Saccharomyces* yeasts whose metabolic heterogeneity not only allows overcoming certain shortcomings detected in most *S. cerevisiae*, but also enables the development of innovative fermentation processes to obtain wines with new properties in sensorial, technological and safety aspects.

Apart from reducing volatile acidity in wines (Moreno et al., 1991; Renault et al., 2009), other specific applications have been attributed to certain wine yeast species, such as alcohol reduction (Contreras et al., 2014), modulation of acidity (Gobbi et al., 2013; Benito et al., 2015), increased glycerol content (Ciani and Ferraro, 1998; Soden et al., 2000), mannoprotein

release (Belda et al., 2015), and the modulation of wine aroma profiles and other microbial products (reviewed by Jolly et al., 2014). In addition to fermentative aromas, mainly dependent on *S. cerevisiae* metabolism, non-*Saccharomyces* yeasts have long been described as a useful tool for revealing the varietal profile of certain grape varieties, whose aroma-determinant components are usually found as odorless conjugated precursors (Gunata et al., 1990; Tominaga et al., 1998). Trace amounts of terpenes and thiols could be present in grapes in a free form, although during fermentation yeasts may also release them from their corresponding odorless precursors. The cleavage of terpenic glycosides is dependent on the hydrolytic activity of glycosidases (Mateo and Di Stefano, 1997) and β -lyases for cysteine-conjugated thiols (Swiegers et al., 2009).

However, the improvement of the aromatic properties of wine is not the only aspect dependent on the enzymatic properties of yeasts, as other sensorial and technological features can be enhanced by other hydrolytic activities. Pectinolytic enzymes (mainly polygalacturonase) are widely used in enology to help degrade the plant cell wall polysaccharides of the grape skin and pulp. They can also help to improve clarification and filterability processes, releasing more color and flavor compounds entrapped in the grape skin, and facilitating the release of phenolic compounds (Lang and Dornenburg, 2000; Van Rensburg and Pretorius, 2000). Finally, the use of proteases in winemaking is not a widely extended practice at the present time, with bentonite being used more frequently to solve protein haze problems. The use of bentonite usually impairs the sensorial properties of wines, so the use of proteases for this purpose may be a potential solution (Marangon et al., 2012).

On the other hand, the presence of sulfite reductase in wine yeast strains is responsible for the production of hydrogen sulfide in wine fermentations, with the consequent appearance of the characteristic rotten egg off-flavor (Swiegers and Pretorius, 2007).

This paper explores the knowledge established between the concepts of wine microbiome and microbial terroir, linking the phylogenetic data provided with the enzymatic characteristics determined in a wide yeast collection. These results have allowed us to establish a general enzymatic phenotypical characterization of several wine-related yeast species and their intraspecific variability, predicting the impact of yeast microbiome on wine flavor. Thus, since the wine microbial terroir has been defined as the distinctive autochthonous microbiome of a wine region and it has been experimentally demonstrated as a determining feature of wine qualities (Bokulich et al., 2014), this work provides a compelling basis to understand the influence of these microbial differences on the wine flavor identity, developing the new concept of wine yeast flavorome and also providing some of its enzymatic basis.

MATERIALS AND METHODS

Grape Samples and Yeast Isolation

Grape samples were collected from three different Spanish wine appellations: Tierra de León (vineyard named in this study as G), Ribera del Duero (vineyards named as PDC and EM) and

Rueda (vineyard named as O). G is a young (20–40 years old) vineyard with vines of the Prieto Picudo variety; the PDC and EM vineyards are between 25 and 91 years old, with vines of the Tempranillo variety; and O is an ancient vineyard with pre-Phylloxera vines between 100 and 200 years old of the Verdejo variety, and also involves biodynamic agricultural practices. Representative samples were taken by analyzing a variety of different sample points depending on the particular agronomical heterogeneity of each vineyard. Three sample points were selected in vineyard G, 10 in vineyard PDC, 5 in vineyard EM and 9 in vineyard O.

Seventy-three yeasts were isolated from vineyard G during the 2012 harvest; 450 yeasts were isolated from vineyards PDC and EM during the 2013 and 2014 harvests; and finally, 247 yeasts were isolated from vineyard O during the 2013 and 2014 harvests (Table S1).

For the isolation of non-*Saccharomyces* yeasts, grape samples weighing about 0.5 kg were taken from healthy grape bunches. After pressing, to reduce the number of ubiquitous *A. pullulans* and basidiomycetous species of no interest to the enological objectives of this work, grape musts were incubated overnight at 20°C. A suitable diluted aliquot of grape must was then spread onto a lysine agar medium (Oxoid) plates at 28°C for 48 h. As stated above, 770 discrete colonies were isolated, and then restreaked on the same medium to obtain pure cultures that were cryopreserved and included in a yeast collection.

These yeast isolates were identified by partial sequencing of the 26S large subunit rRNA gene. Total genomic DNA was extracted using the isopropanol method (Querol et al., 1992), and the DNA for sequencing was amplified by using an Eppendorf Mastercycler, with forward NL-1 primer (5'-GCA TAT CAA TAA GCG GAG GAA AAG-3') and reverse NL-4 primer (5'-GGT CCG TGT TTC AAG ACG G-3') (Kurtzman and Robnett, 1997). The sequences obtained to identify yeasts were analyzed and compared by BLAST-search (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Finally, sequences were deposited in the GenBank database (<http://www.ncbi.nlm.nih.gov/genbank/>) with the accession numbers listed in Table S1.

Phylogenetic Tree Analysis

Phylogenetic analyses were conducted with InfoQuest FP Software (version 4.5 Bio-Rad Laboratories, Madrid, Spain). The clustering was performed following the Neighbor joining (NJ) method, with Kimura two-parameter correction.

Culture Media and Enzymatic Screening Procedures

Glycosidase Activities

β -Glucosidase activity was evaluated as reported by Villena et al. (2005) on a medium containing 0.5% cellobiose (4-O- β -D-glucopyranosyl-D-glucose), 0.67% yeast nitrogen base (Difco) and 2% agar. This medium was adjusted to pH 3.5 as follows. The components of the medium were sterilized separately to avoid agar hydrolysis. Agar and cellobiose were autoclaved, and the yeast nitrogen base was adjusted to pH 3.5 with HCl and

then filtered (0.22 μ m). Both fractions were subsequently mixed when the agar solution was around 60°C. A loop full of each yeast strain was spread onto the medium surface and incubated at 28°C for 3 days. Any significant growth of the colonies indicated the presence of β -glucosidase activity. A positive control (*Rhodotorula glutinis* CECT 10143) and a negative one (*Torulaspora delbrueckii* CECT 10676) were used as reference for growth determinations.

Additionally, β -D-xylosidase and α -L-arabinofuranosidase activities were evaluated using the corresponding methylumbelliferyl-conjugated substrates (methylumbelliferyl- β -D-xylopyranoside (MUX) and methylumbelliferyl- α -L-arabinofuranosidase (MUA), respectively; Sigma-Aldrich), according to the method described by Manzanares et al. (1999), with slight modifications for their development in 96-well microplates. Methylumbelliferone release was measured by detecting fluorescence using a Varioskan Flash Multimode Reader (Thermo Scientific) with an excitation wavelength at 355 nm and emission at 460 nm. Once again, *R. glutinis* CECT 10143 and *T. delbrueckii* CECT 10676 were used as positive and negative controls, respectively.

β -Lyase Activity

β -Lyase activity was evaluated on a medium containing the following: 0.1% S-methyl-L-cysteine (Sigma-Aldrich), 0.01% pyridoxal-5'-phosphate (Sigma-Aldrich), 1.2% Yeast Carbon Base (Difco, Detroit, MI, USA) and 2% agar. This medium was adjusted to pH 3.5 and sterilized as described above to avoid agar hydrolysis. The agar solution was autoclaved, and all the other components were adjusted to pH 3.5 with HCl and filtered (0.22 μ m), then both fractions were mixed when the agar solution was around 60°C. Any significant growth of the colonies after 48–72 h indicated the presence of β -lyase activity (Patent pending). *T. delbrueckii* CECT 10676 and *R. glutinis* CECT 10143 were used as positive and negative controls, respectively.

Pectinase Activities

Yeast isolates were screened for polygalacturonase activity in a polygalacturonate agar medium containing 1.25% polygalacturonic acid (Sigma), 0.67% yeast nitrogen base (YNB, Difco), 1% glucose and 2% agar, adjusted to a final pH 3.5, as previously described (Strauss et al., 2001), with slight modifications. Agar was sterilized separately by autoclaving, and all the other components were adjusted to pH 3.5 and boiled. Both solutions were mixed when agar reached a temperature of around 60°C. *Metschnikowia pulcherrima* CECT 11202 and *Lachancea thermotolerans* CECT 1951 were used as positive and negative controls, respectively.

Protease Activities

Protease activity was evaluated on YPD plates (containing 1% yeast extract, 2% peptone, 2% glucose, and 2% agar) with 2% skim milk powder (Sigma-Aldrich). The plates were incubated for 5 days at 30°C. A clear zone around the colony identified protease activity (Strauss et al., 2001). *Wickerhamomyces anomalus* PYCC 2495 and *T. delbrueckii* CECT 10676 were used as positive and negative controls, respectively.

Cellulase Activities

Cellulase production was determined on YPGE plates (containing 1% yeast extract, 2% peptone, 3% glycerol, and 2% ethanol) with 0.4% carboxymethylcellulose, as previously described (Teather and Wood, 1982). *Aureobasidium pullulans* CECT 2660 and *T. delbrueckii* CECT 10676 were used as positive and negative controls, respectively.

Sulfite Reductase Activity

Hydrogen sulfide production was evaluated by using a modification of the lead acetate method (Linderholm et al., 2008) described by Belda et al. (2015) for its use in 96-well microplates. Briefly, this method detects volatile H₂S in the headspace of a culture medium containing 1.17% yeast carbon base (Difco), 4% glucose anhydrous, and 0.5% ammonium sulfate. Yeasts were grown at 28°C for 3 days in 96-well microplates containing 200 µl of medium with orbital agitation (200 rpm). Hydrogen sulfide formation was initially detected by using paper strips (Whatman filter paper) that had been previously embedded with a 0.1 M lead acetate solution and allowed to dry at 65°C for 10 min and deposited over microplate wells. Hydrogen sulfide formation was qualitatively measured based on the degree of blackening of the lead acetate strip, and quantitatively estimated by densitometric measurement of the color intensity (Software "My Image Analysis v1.1" Thermo Scientific). *R. glutinis* CECT 10143 and *T. delbrueckii* CECT 10676 were used as positive and negative controls, respectively.

Statistical Analysis of Enzymatic Data

Enzymatic activity was coded on a scale from 1 (no activity) to 5 (highest activity) and loaded into InfoQuest FP Software (version 4.5 Bio-Rad Laboratories, Madrid, Spain) as a character type. A similarity matrix was calculated using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA). Groups were assigned according to the identification of the strains by 26S analysis. Group separation was calculated with the Jackknife method. Principal Components Analysis (PCA) was performed with InfoQuest FP Software.

The species distribution per sample site was introduced into R program (R Core Team, 2013). The function `vegdist` from the package `vegan` version 2.2-1 (Oksanen et al., 2015) was used to calculate a dissimilarity matrix between samples.

RESULTS

Description of Yeast Populations

In this work, 770 yeast isolates from grape musts of different origins were identified by partial sequencing of the 26S rRNA gene (Table S1). Fifteen different species were found among the yeast collection studied here (Figure 1), which consisted of a wide range of yeast species usually found in vineyards, and mostly having been reported to be of enological interest (Fleet, 2008; Jolly et al., 2014). *Hanseniaspora uvarum* was the most abundant species, making up more than half of the total isolates, followed by *Metschnikowia* sp. (comprising *M. pulcherrima* and *M. fructicola*) and *Lachancea thermotolerans*, with the other 12

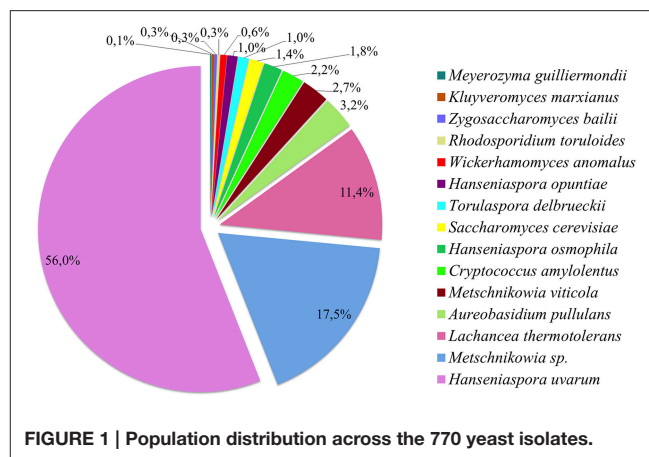


FIGURE 1 | Population distribution across the 770 yeast isolates.

yeast species only present at levels of less than 4% (Figure 1). In spite of this small diversity of species, the high sample size (770 isolates) allowed us to conduct a functional analysis of the yeast collection in question. Considering the complete yeast collection used here, a phylogenetic analysis of the 770 isolates, belonging to 15 yeast species identified on the basis of rDNA 26S sequences, was carried out in order to confirm the success of the molecular identification process (Figure S1). It should be noted that *M. fructicola* and *M. pulcherrima* could not be properly differentiated by 26S sequence analysis (Guzmán et al., 2013), and are henceforth referred to here as *Metschnikowia* sp.

Notable differences between the diversity and richness of yeast species in the different vineyards sampled were observed (Figure 2, Table S3). Furthermore, some differences could be perceived between yeast populations of different vintages from the same vineyard. Particular note should be taken of the low diversity of yeast species in the EM vineyard, which had only three yeast species, all of which were identified in both the 2013 and 2014 vintages, with *H. uvarum* accounting for more than three quarters of the total of 196 isolates, followed by *L. thermotolerans* and *Metschnikowia* sp. (Figure 2A).

In the case of the PDC vineyard (Figure 2B), a total of 254 yeast isolates, comprising eight species, were obtained. *H. uvarum*, *Metschnikowia* sp. and *L. thermotolerans* were once again the most dominant species (39, 24.8, and 19.7% of the total population, respectively). However, in this case, significant differences could be observed between vintages. There was a significant decrease in *L. thermotolerans* isolates in the 2014 vintage, and there was a higher diversity. The other species identified were *Aureobasidium pullulans*, *Cryptococcus amyloletus*, *Wickerhamomyces anomalus*, *Kluyveromyces marxianus*, and *Torulaspora delbrueckii*, jointly accounting for less than 16.6% of the PDC population and 5.4% of the total population.

Similar diversity was observed in the O vineyard, with six yeast species being identified among the 247 isolates (Figure 2C). *H. uvarum* was again the most abundant, accounting for 64.4% of the total, with the key observation being the low abundance of *L. thermotolerans* (one of 247 isolates). It should be noted that in this vineyard *M. viticola* was identified as an additional

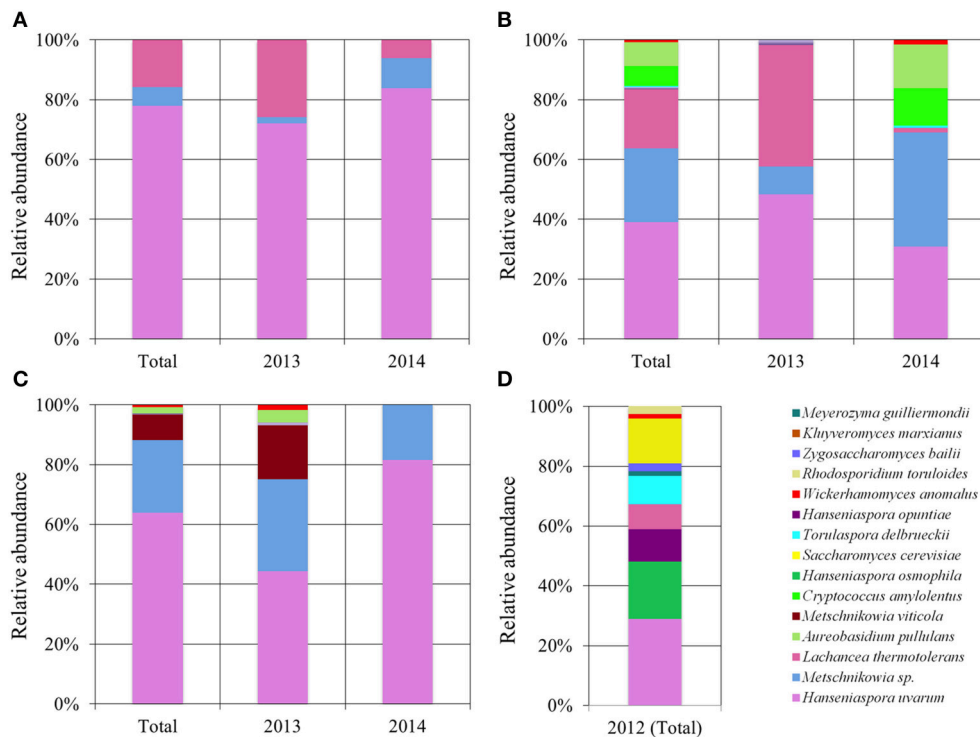


FIGURE 2 | Total and vintage-specific population distribution from the four sampled vineyards. (A) Population distribution of EM vineyard. **(B)** Population distribution of PDC vineyard. **(C)** Population distribution of O vineyard. **(D)** Population distribution of G vineyard.

Metschnikowia species. Contrary to what was observed in the PDC vineyard, a higher diversity was found in the 2013 vintage, when compared to 2014, when only *H. uvarum* and *Metschnikowia* sp. were isolated.

The G vineyard comprised 10 yeast species (nine non-*Saccharomyces* species along with some *Saccharomyces cerevisiae* isolates). *Hanseniaspora* genus was distributed among isolates of three species: *H. uvarum* (28.8%), *H. osmophila* (19.2%), and *H. opuntiae* (11%) (Figure 2D). Apart from *Hanseniaspora* species and *L. thermotolerans*, in the other vineyards the other five non-*Saccharomyces* species were either not isolated (*Meyerozyma guilliermondii*, *Zygosaccharomyces bailii*, and *Rhodospiridium toruloides*) or rarely isolated (*W. anomalus* and *T. delbrueckii*). In this case, the absence of isolates from different vintages made it impossible to establish any population trends. Finally, contrary to what was expected due to the use of a lysine medium, 11 yeast isolates were identified as *S. cerevisiae*; nevertheless, they were not removed from the collection, but instead used as a comparative control for the enzymatic study.

Phylo-Functional Study

To address a targeted use of non-*Saccharomyces* species in the wine industry, it is required a better understanding of their specific metabolic properties and their strain-dependent features. Different yeast species have been reported to modulate wine flavor and aroma, in part because of their enzymatic properties (Hernández-Orte et al., 2008; Maturano et al., 2015). The main

aim of this work was to robustly establish the wine-related enzymatic profile of a large collection of wine yeasts.

A combined analysis of phylogenetic and enzymatic data (β -glucosidase, α -L-arabinofuranosidase, β -D-xylosidase, β -lyase, protease, polygalacturonase (pectinase), cellulase, and sulfite reductase) was performed to observe whether there were any overall differences in enzyme abundances and their presence among different phylogenetic groups, inferring species-specific behaviors (Figure 3, Figure S1). In this context, two different groups of highly and less abundant enzymes could be established, highlighting α -L-arabinofuranosidase, polygalacturonase and cellulase as the least abundant activities and, on the other hand, β -glucosidase and protease as the most widespread activities throughout the yeast collection studied.

Figure 3 shows the overall abundance and activity level of the different enzymes studied in the 770 yeast isolates, and their distribution among the 15 species identified. β -Glucosidase was widespread among wine yeast species. All the strains of *Z. bailii* and *L. thermotolerans* were observed to be β -glucosidase negative, whereas most of the strains belonging to *A. pullulans*, *T. delbrueckii* and *S. cerevisiae* were also found to be β -glucosidase negative, without any species-specific behavior. On the other hand, note should be taken of the activity of *H. osmophila*, *H. opuntiae*, *M. guilliermondii*, and *R. toruloides* (Figure 3, Figure S1). Regarding the other two glycosidases, the abundance of β -D-xylosidase and α -L-arabinofuranosidase was found to be of medium and low, respectively. Special mention should be

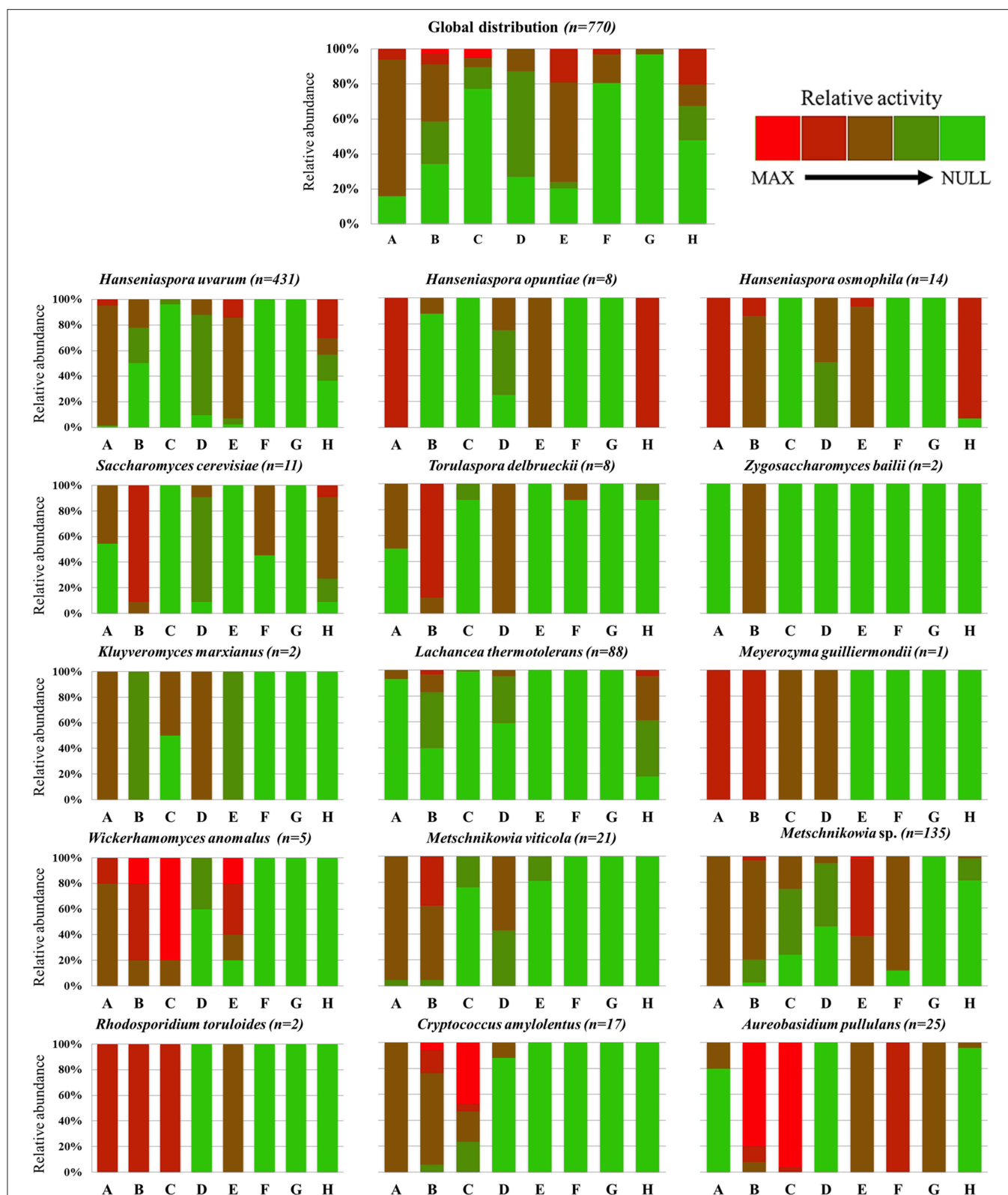


FIGURE 3 | Abundance and distribution of enzymatic activities among the total yeast collection, individually considering the 15 yeast species identified. The eight enzymatic activities evaluated were: (A) β -glucosidase; (B) β -D-xylosidase; (C) α -L-arabinofuranosidase; (D) β -lyase; (E) Protease; (F) Polygalacturonase; (G) Cellulase; (H) Hydrogen sulfide production. Enzymatic activity was determined on a scale from 1 (no activity) to 5 (highest activity) corresponding to a progressive color code from green to red.

made of the production of β -D-xylosidase in *S. cerevisiae*, *T. delbrueckii*, *M. guilliermondii*, *W. anomalus*, *R. toruloides*, and *A. pullulans*, with the production of α -L-arabinofuranosidase being only noteworthy in the three latter species, as well as in *C. amylorentus*. Overall, a glycosidase-active cluster could be observed in the basidiomycetous group (*C. amylorentus* and *R. toruloides*), together with the yeast-like fungus *A. pullulans*, all of them located at the bottom of the phylogenetic tree (Figure S1).

β -Lyase activity was widespread, albeit in most cases with moderate activity throughout the isolates. Only *T. delbrueckii*, *M. guilliermondii*, and *K. marxianus* had a wholly positive specific behavior.

Protease activity was, together with β -glucosidase, the most abundant activity in the yeast population studied. However, 40% of the yeast species (six out of 15) had no protease activity. This apparent contradiction can be explained by the small representation these species have in the total number of yeast isolates. It should be mentioned that protease activity was fully absent in the phylogenetically related species *S. cerevisiae*, *Z. bailii*, and *T. delbrueckii*, as well as in *L. thermotolerans*, *M. guilliermondii*, and *C. amylorentus* (Figure 3).

On the other hand, pectinase and cellulase activities had a restricted distribution, with pectinase having only a significant presence in *Metschnikowia* sp. and *A. pullulans*, and cellulase only in *A. pullulans*. Apart from that, almost half of *S. cerevisiae* and a few *T. delbrueckii* isolates had pectinase activity. It should be mentioned that protease and pectinase activities are the main

phenotypic differences between *M. viticola* and the other two *Metschnikowia* species isolates.

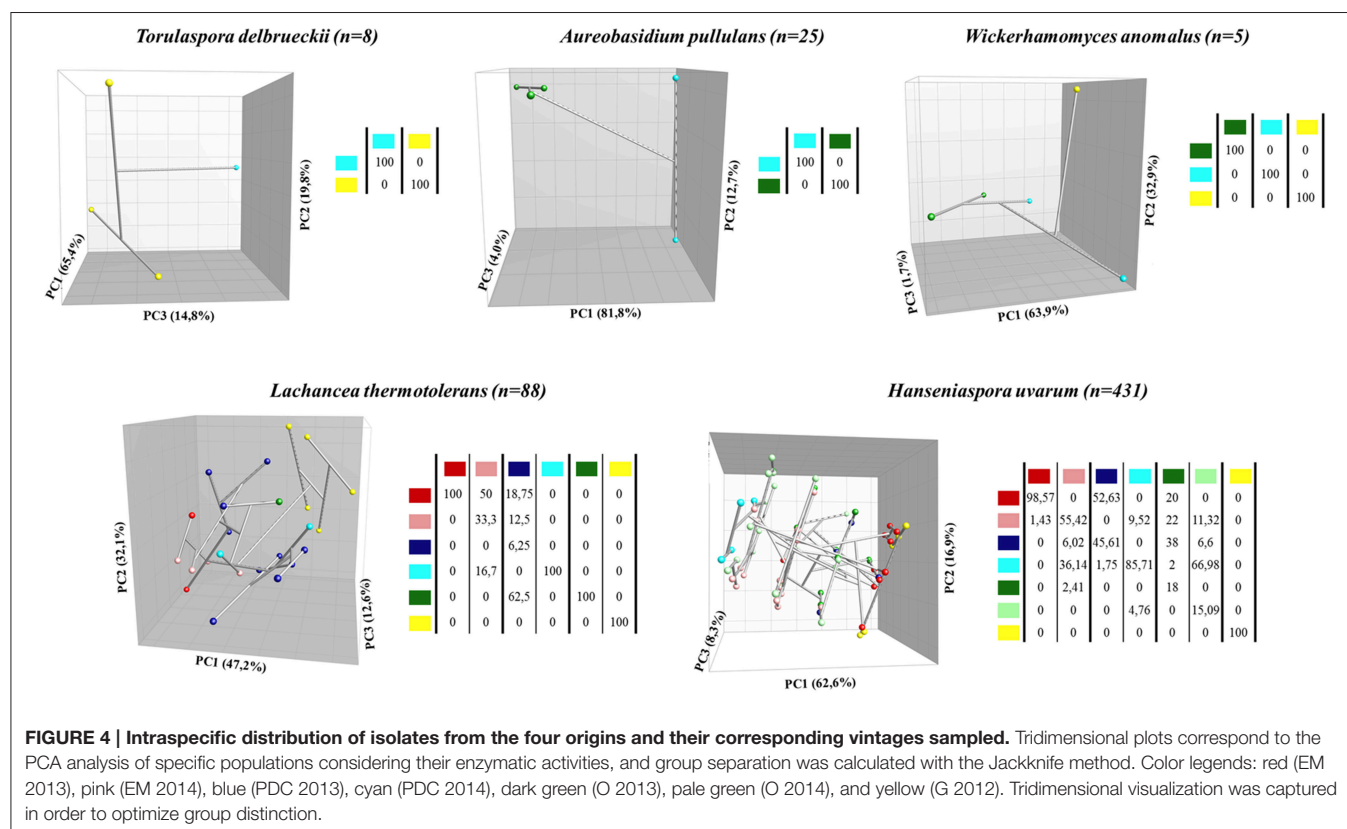
Finally, hydrogen sulfide production due to the activity of sulfite reductase was remarkably high in some *H. uvarum* and in most *H. osmophila* and *H. opuntiae* isolates, confirming a genus-related behavior. Regarding the other yeast species, only *S. cerevisiae* and *T. delbrueckii* had certain H_2S -producer strains.

Thus, Figure S1 shows an active cluster at the lower region of the phylogenetic tree composed by basidiomycetous species (*C. amylorentus* and *R. toruloides*) and by *Metschnikowia* sp. and *A. pullulans* isolates. A highly inactive cluster in enzymatic terms could also be observed in the lower-middle zone.

Origin-Dependent Intraspecific Study

In order to study the concept of microbial terroir in depth, an intraspecific analysis was conducted on the enzymatic properties associated to every strain. Figure 4 shows the intraspecific clustering of the isolates of different species (five species isolated from more than one origin) by carrying out a PCA analysis using enzymatic data.

Considering the three less abundant species analyzed (*T. delbrueckii*, *A. pullulans*, and *W. anomalus*), it was possible to clearly establish origin-dependent strain clusters composed of homogeneous populations that could be distinguished by their enzymatic profiles. *T. delbrueckii* was isolated from the G (seven isolates) and PDC (one isolate) vineyards in the 2012 and 2014 vintages, respectively. Two different groups could be



statistically identified (with two Principal Components (PCs) explaining 85.2% of the differences, and three PCs explaining 100%), showing a clear origin-dependent differentiation with β -glucosidase and pectinase mostly affecting this clustering (**Figure 4**, Table S2a). *A. pullulans* was also isolated from two vineyards: PDC (2014) and O (2013), with 20 and 5 isolates, respectively. In this case, two different groups were established depending on the isolation origin, composing 100% homogeneous population groups (**Figure 4**). The PCA analysis allowed us to statistically support this clustering, with the first two PCs explaining 94.55% of these differences, and three PCs explaining 98.51%. In this case, β -glucosidase and β -D-xylosidase were the factors mostly responsible for affecting this clustering, by greatly contributing to the first PC, which alone explains 81.84% of the established differences (Table S2b). *W. anomalus* was isolated from three different vineyards: G (2012), PDC (2014), and O (2013), with 1, 2 and 2 isolates, respectively, and these five isolates again described a phenotypic cluster according to their origin, composing three different phylo-functional groups (**Figure 4**). This clustering was again statistically significant in the PCA analysis, explaining 96.8% of the differences with the first two PCs, and 97.5% with three PCs. Protease activity was the most responsible factor, explaining the origin-dependent cluster separation, and contributing significantly to the first PC, which could explain 63.88% of the differences detected (Table S2c).

Due to their large sample size, the other two species evaluated (*L. thermotolerans* and *H. uvarum*) generate more complex clustering but, in most cases, some statistically homogeneous groups could be composed depending on the origin-dependent strain phenotype. Regarding *L. thermotolerans*, a total of 88 isolates were analyzed from G (2012), PDC (2013, 2014), EM (2013, 2014), and O (2013), with 6, 50, 31 and 1 isolates, respectively.

Clusters were established for the isolates from the four different vineyards, although a less precise separation could be established between the isolates of different years from the same vineyard. **Figure 4** shows that *L. thermotolerans* isolates from EM (2013), PDC (2014), O (2013), and G (2012) established statistically homogeneous groups, defining their own enzymatic profile. Isolates from EM (2014) did not form a homogeneous group, but 50% of these isolates could be assigned to the EM (2013) enzymatic profile. Regarding PDC (2013) isolates, it was not possible to establish a uniform profile, with most of its isolates being similar to the enzymatic profiles from other origins. Apart from that, the PCA of the enzymatic properties of the total *L. thermotolerans* population could explain 79.28% of the differences between origins, considering the first two PCs, and 91.87% considering the first three PCs. These differences could be attributed mostly to β -D-xylosidase activity, H_2S production, and β -glucosidase activity (Table S2d). Finally, regarding the largest species population in this study, the analysis of *H. uvarum* enzymatic profile generated the most complex clustering, although in some cases an origin-dependent enzymatic profile could be defined. *H. uvarum* was isolated from all the vineyards, reaching a total of 431 isolates from all sampled origins. Three origins established consistent groups: EM (2013), PDC (2014),

and G (2012). On the other hand, *H. uvarum* isolates from O (2013 and 2014) did not establish a consistent enzymatic profile of their own, with most of the isolates being statistically attributed to other origin profiles. Finally, in an intermediate situation, EM (2014) and PDC (2013) originated not-fully consistent groups, with their enzymatic profile overlapping with the profile described by other vineyards from the same appellation (EM 2014 with PDC 2014; PDC 2013 with EM 2013) (**Figure 4**), describing a wider origin-specific profile. The PCA analysis of these data gives us statistical evidence of the significance of these clustering results. Sulfite reductase and β -D-xylosidase activities contributed notably to these differences, significantly affecting PC1, which could alone explain 62.62% of the differences between groups, and also PC2, which accumulates an explanation of 79.48% of the differences (**Figure 4**, Table S2e).

DISCUSSION

Diversity and Richness of Yeast Species

The main aim of this work was to establish a large collection of non-*Saccharomyces* yeasts isolated from different Spanish wine appellations in order to perform a joint phylo-functional analysis, linking phylogenetic and phenotypic data on the enzymatic properties of the yeast species identified. Furthermore, an attempt has been made to relate certain enzymatic activities, which are usually associated with certain yeasts, to the potential role they could play in enology.

The experimental approach developed for this study was based on culture-dependent techniques in order to obtain a yeast collection of enological origin that may have a use in winemaking. From a general point of view, our population data (**Figure 1**) were in line with other studies reporting that, apart from the *Aureobasidium* and *Rhodotorula* species that were intentionally avoided in this study as described in the yeast isolation procedure, *Hanseniaspora* spp., *Metschnikowia* spp., and *L. thermotolerans* dominate yeast communities in fresh musts (Prakitchaiwattana et al., 2004; Pinto et al., 2015), with *H. uvarum* accounting for more than half of the total yeast population isolated (Beltran et al., 2002; Wang et al., 2015).

There has recently been confirmation of the major differences in population richness values between culture-dependent and independent approaches in enological environments (Wang et al., 2015). Our overall results of yeast diversity using a culture-dependent approach are wider than those obtained in other similar studies. Wang et al. (2015) have managed to identify a total of three species (*H. uvarum*, *Issatchenkia terricola*, and *Starmerella bacillaris*) from a collection of 179 yeasts isolated from nine different origins by using a lysine medium, and five species (the three previously mentioned, together with *S. cerevisiae* and *Hanseniaspora valbyensis*) in 183 isolates from the same nine samples using YPD plates. The higher diversity obtained in our work (15 vs. 5 species) could be explained by both the larger sample size (770 vs. 362 isolates) and the greater heterogeneity in sampling areas (**Figure 1**). According to data reported by Beltran et al. (2002), several differences in yeast diversity were observed between years, as shown in **Figure 2**.

Differences in the microbial composition among vintages, grape varieties, climate and location have been widely reported by Bokulich et al. (2014), and could account for the differences observed for yeast diversity found in the G vineyard compared to the diversity found in the other three vineyards studied (Figure S2, Table S3). The different microclimatic conditions, vineyard location and vine variety of this vineyard, with only the 2012 vintage sampled, could account for such a difference. The 2012 vintage in most Spanish wine appellations was characterized by low rainfall (Figure S2), which could restrict the filamentous fungi overgrowth that could displace some of the yeast species present in the grape microbial consortia (Liu et al., 2015). Additionally, as we show in this work, not only were the diversity and richness of yeast species affected by location, but also the phenotypic profile of the same yeast species differed across vineyards, and even in consecutive vintages (Figure 4).

Although most of the current population studies using culture-independent molecular methods report higher diversity values for fresh must than those reported here (Bokulich and Mills, 2013; David et al., 2014; Pinto et al., 2015), a wide variety of yeast species of enological interest (Jolly et al., 2014) were represented in the yeast collection established for their enzymatic characterization.

Enzyme Abundance and Species-Specific Distribution

Regarding enzymatic screening, eight enzymatic activities were evaluated to establish an enzymatic profile of enological interest for the 15 yeast species studied (Figure 3). A group of three glycosidases (β -glucosidase, β -D-xylosidase, and α -L-arabinofuranosidase) were determined, recording different performances in terms of activity, distribution and abundance. According to other works (Fia et al., 2005), β -glucosidase was a widespread activity among wine yeasts. Our results have highlighted the β -glucosidase production of *Hanseniaspora* species, as well as of *M. guilliermondii* and *W. anomalus*. These results are also consistent with other enzymatic screenings that additionally reported the ability of some *H. uvarum* strains to produce versatile β -glucosidase enzymes with no repression by glucose and with no significant activity decrease in a wide range of pH values (López et al., 2015). Delcroix et al. (1994) and Hernández et al. (2002) evidenced a loss of stability of β -glucosidase in *S. cerevisiae*, with a strong reduction in its enzymatic activity (about 80%) when changing from pH 5 to pH 3, while other authors have reported a notable decrease in most non-*Saccharomyces* species at pH values below 4 (Rosi et al., 1994). However, Mateo et al. (2011) have reported that *W. anomalus* reached its maximum β -glucosidase activity at pH 3.2, also recording lower rates of catabolic repression by glucose. Thus, with β -glucosidase being the final activity responsible for the release of wine terpenes from their glycosylated precursors, both *Hanseniaspora* species and *W. anomalus* seem to be a useful tool to increase wine terpenics, as suggested by Mendes-Ferreira et al. (2001) and Mateo et al. (2011), respectively.

Regarding the other two glycosidases analyzed (β -D-xylosidase and α -L-arabinofuranosidase), different abundances were observed among the yeast population studied. Contrary to

what was observed in β -glucosidase activity, *Hanseniaspora* spp. had neither β -D-xylosidase (with the exception of *H. osmophila* and a few *H. uvarum* strains) nor α -L-arabinofuranosidase activities, which was in complete agreement with previous observations reported by Manzanares et al. (1999). However, they also highlighted a remarkable β -D-xylosidase activity for some *W. anomalus* and *H. uvarum* strains at the usual enological pH values of 3–3.8, with their use also being recommended for terpene release in wine fermentation. Furthermore, lower repression levels by glucose and ethanol have been reported for *W. anomalus* glycosidase activities (Mateo et al., 2011). Regarding the other yeast isolates, a β -D-xylosidase-active cluster was observed in the phylogenetically related species *T. delbrueckii*, *Z. bailii*, and *S. cerevisiae*. However, a high glucose-dependent repression has been observed in these species (Gueguen et al., 1995; Mateo and Di Stefano, 1997; Mateo et al., 2011), restricting their use in terpene release in wine fermentation.

Finally, α -L-arabinofuranosidase, as the least distributed glycosidase, was observed in *M. guilliermondii*, *W. anomalus*, *A. pullulans*, *R. toruloides*, and *C. amylorentus*. McMahon et al. (1999) have reported the major ability *A. pullulans* glycosidases have to release wine terpene glycosides. According to Mateo et al. (2011), α -L-arabinofuranosidase, together with α -L-rhamnosidase, is the least glucose-repressed glycosidase in wine yeasts, so both are of enological interest. Regarding *Metschnikowia* spp., most of them had remarkable β -glucosidase and β -D-xylosidase activities, although a considerable number of *Metschnikowia* sp. (not considering *M. viticola* isolates) had also α -L-arabinofuranosidase activity. Along these lines, it has been reported that a commercial strain of *M. pulcherrima* could increase volatile terpenes in wine due to its α -L-arabinofuranosidase activity (Lallemand, 2013).

Overall, our results are in agreement with other works reporting that *Pichia*, *Wickerhamomyces*, and *Hanseniaspora* genera are major producers of glycosidase enzymes (Manzanares et al., 1999) and, furthermore, we report the remarkable glycosidase activity of wine-related basidiomycetes, such as *R. toruloides* and *C. amylorentus*.

β -Lyase activity, which is also directly related to varietal aroma enhancement, recorded a moderate distribution in the overall yeast collection studied. Figure 3 shows moderate β -lyase activity in the majority of yeast species, with its production being remarkable in *T. delbrueckii*, *K. marxianus*, and *M. guilliermondii*. Although this activity has been studied in depth in *S. cerevisiae* wine strains (Howell et al., 2005; Thibon et al., 2008; Roncoroni et al., 2011), actual information on the ability of non-*Saccharomyces* to release volatile thiols in wine is very scarce. Zott et al. (2011) have reported that β -lyase activity is a strain-dependent characteristic in non-*Saccharomyces* yeasts, as described in *S. cerevisiae* (Roncoroni et al., 2011; Santiago and Gardner, 2015). Accordingly, Figure 3 shows that β -lyase activity has great intraspecific variability. Zott et al. (2011) have reported that, apart from *T. delbrueckii*, some *M. pulcherrima* and *L. thermotolerans* strains have the ability to release volatile thiols in Sauvignon Blanc wines, but only a few strains of these species have recorded β -lyase activity in our *in vitro* assays. Regarding

the *Hanseniaspora* genus, and as occurred with β -D-xylosidase, *H. osmophila* recorded higher β -lyase activity compared to *H. opuntiae* and *H. uvarum* species. These phenotypical differences were consistent with the observations made in the phylogenetic tree (Figure S1), in which *H. osmophila* was distant from the *Hanseniaspora* genus cluster. Due to the high nitrogen catabolic repression affecting β -lyase activity in *S. cerevisiae*, which restricts its contribution to thiol release in wine fermentation (Thibon et al., 2008), these alternative yeasts should be studied in depth as a way to improve volatile thiol release in enological conditions.

H₂S production, as a result of sulfite reductase activity, is a rare feature among the majority of non-*Saccharomyces* species. Furthermore, as occurred with β -lyase (the other sulfur-related activity), major intraspecific variability could be observed in species such as *H. uvarum* and *L. thermotolerans*, as well as in *S. cerevisiae*, as previously reported by Linderholm et al. (2008). Given that the nitrogen composition of musts has been described to affect H₂S production by yeasts (Linderholm et al., 2008), and since non-*Saccharomyces* yeasts have high nutritional demands (Jolly et al., 2014), the lack of sulfite reductase activity in most of them is a positive characteristic for their application without the risk of wine reduction.

Protease, pectinase and cellulase have been studied for their involvement in several technological processes in winemaking. **Figure 3** shows that protease is a widespread activity when the total population of yeasts is considered, in agreement with previous works (Lagace and Bisson, 1990; Chomsri, 2008). This is caused by the protease activity of the most abundant species (*Hanseniaspora* species and *Metschnikowia* sp.), although other species of enological interest with a lower relative abundance recorded no activity (*S. cerevisiae*, *T. delbrueckii*, and *L. thermotolerans*, among others). In addition, protease and pectinase seem to be the main differential activities between *M. viticola* and the other *Metschnikowia* species isolated. The use of proteases in winemaking is not a widely extended practice at the moment, with bentonite being used more often to solve protein haze problems. The use of bentonite usually impairs the sensorial properties of wines, so the use of proteases for this purpose seems to be a potential future application (Marangon et al., 2012). Special note should be taken of the high protease activity of *W. anomalus*, especially in the NS-PDC-167 strain (**Figure 3**, Figure S1), which should be studied in depth for its application in protein haze prevention. In fact, an aspartate-protease from *M. pulcherrima* has been characterized and expressed in *S. cerevisiae* by Reid et al. (2012) for its potential wine application, but the role of proteases from yeasts in winemaking is still poorly understood.

Regarding pectinolytic activity, different studies have confirmed that most yeast species are unable to produce pectic enzymes. It should be mentioned that polygalacturonase activity has been reported in a few wine yeast isolates without establishing a species-specific behavior (Strauss et al., 2001; Merín et al., 2011). In this context, our results suggest that *M. pulcherrima*, *M. fructicola* (jointly identified here as *Metschnikowia* sp.), and *A. pullulans* are leading candidates for their use as a source of pectinase in winemaking. Following the confirmed usefulness of pectinases from *A. pullulans* in winemaking conditions (Merín and Morata de Ambrosini, 2015), the impact of *M. pulcherrima*,

improving phenolic extraction and clarification processes in some pectinase-dependent wine properties, has recently been confirmed (Belda et al., unpublished). Furthermore, in light of the behavior of *A. pullulans*, this was the only cellulase-active species in the collection studied, in contrast with data reported by Strauss et al. (2001) and Merín et al. (2015) which describe the presence of cellulase activity in some ascomycetous yeasts (*Candida stellata*, *M. pulcherrima*, and *H. uvarum*) and in the basidiomycetous yeast *Rhodotorula dairenensis*, respectively.

It has been reported that at least 75% of the *S. cerevisiae* enological strains have limited pectinolytic activity (Blanco et al., 1994). However, Merín et al. (2011) and Merín and Morata de Ambrosini (2015) have confirmed the existence of a constitutive pectinase activity not repressed by glucose in non-*Saccharomyces* species, in contrast with what occurred in *S. cerevisiae* (Radoi et al., 2005). In this context, our results confirm that the vast majority of *Metschnikowia* sp. and *A. pullulans* strains are of interest for their use as pectinase sources in enology, opening a new research line for their industrial application.

Origin-Dependent Intraspecific Phenotypic Profiles

Metagenomic approaches have allowed researchers to definitively establish the concept of microbial terroir, relating location and climatic factors to specific microbial populations in vineyards (Bokulich et al., 2014). This finding has been put forward as a determinant in the differential flavor and aroma profiles of wines from different origins (Gilbert et al., 2014). Additionally, our results confirm that significant phenotypical differences could be observed between strains of the same species from different origins, delving further into the concept of microbial terroir, for the first time at strain level.

The results shown in **Figure 4** allow us to confirm the possibility of separating single species populations based on their enzymatic properties establishing origin-dependent clusters. It has been suggested that high-throughput screening (HTS) assays are crucial for discovering interesting enzymes and new sources (Goddard and Reymond, 2004). Here, we also report the potential these techniques have to develop phylo-functional analyses of yeast communities to perform innovative ecological studies. A similar approach has recently been adopted by Zhang et al. (2015) to establish phylo-functional differences among the gut microbiota of different human populations.

The connecting lines shown in **Figure 4** have allowed us to decipher the phylogenetic relationships among groups of isolates according to their phenotypical similarities. The tridimensional plot for *T. delbrueckii*, *A. pullulans*, and *W. anomalus* shows highly defined origin-dependent clusters with significant percentages of statistical differences among groups, bearing in mind that they were scarcely isolated. The population distribution of *L. thermotolerans* and *H. uvarum* isolates shown in the tridimensional plot could be better interpreted considering numerical data for group homogeneity (**Figure 4**) because of the high number of isolates considered. The results for both species isolated from Ribera del Duero vineyards (EM and PDC) suggest that the EM population isolated in 2014 was more heterogeneous when compared with data for 2013. In contrast,

yeast populations from the PDC vineyard followed the opposite trend, with the populations being more homogeneous in 2014 for both species, as compared to 2013. These differences, together with the different behavior of EM and PDC populations shown in **Figure 2**, could be related to microclimatic determinants and to viticulture practices conditioning the health status of grapes that could determine microbial populations in them (Sipiczki, 2006; Barata et al., 2008). In the case of *H. uvarum* isolates from the O vineyard (Rueda wine appellation), the populations obtained in both the 2013 and 2014 vintages were very heterogeneous. As they were the only species analyzed for consecutive vintages in this vineyard, it is not possible to draw a wider conclusion about the intraspecific consistency in the O vineyard. It may be the case that the biodynamic practices applied in this vineyard contribute to a great microbial diversity, as suggested by Setati et al. (2012). The wide gap between the G population and the other population groups could be explained by geographic and climatic reasons, as it has been isolated in a wine appellation (Tierra de León) with several climatic and orographic differences with respect to its Ribera del Duero and Rueda counterparts, as well as in a different vintage (2012) with certain weather peculiarities (remarkably low rainfall).

In summary, the phenotypical characterization of our yeast population goes deep into the concept of microbial terroir, considering the yeast diversity at strain level as an important factor for determining the microbial influence on the flavor properties of wines. This intraspecific phenotypical clustering could not have been explored with current metagenomic approaches. However, the exponential growth of genomic data for non-*Saccharomyces* species and the versatility of high

throughput genomic techniques, together with data on the species-specific enzymatic profiles reported in this work, open new possibilities for future comparative genomic works that will allow for the targeted development of high throughput metabolic screenings.

AUTHOR CONTRIBUTIONS

AS, EN, and DM conceived the project; IB, AS, EN, and JR designed and performed the experiments; IB, AA, and AS analyzed the data, and IB and AS wrote and edited the manuscript.

ACKNOWLEDGMENTS

The funding for the research described in this paper was provided by Agrovín S.A, within the framework of the project IDI-20130192 (Centre for Industrial Technological Development-CDTI, Spain) and by Pago de Carraovejas, within the framework of the project IDI-20140448 (Centre for Industrial Technological Development-CDTI, Spain). We thank Dr. Cristina Gutiérrez for her technical support, and Rocío Ramírez for reading the paper and contributing to its final version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fmicb.2016.00012>

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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A new wine *Torulaspora delbrueckii* killer strain with broad antifungal activity and its toxin-encoding double-stranded RNA virus

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OPEN ACCESS

Edited by:

Alberto Mas,
Rovira i Virgili University, Spain

Reviewed by:

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Università Politecnica delle Marche,
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Specialty section:

This article was submitted to
Food Microbiology,
a section of the journal
Frontiers in Microbiology

Received: 31 July 2015

Accepted: 03 September 2015

Published: 15 September 2015

Citation:

Ramírez M, Velázquez R, Maqueda M,
López-Piñero A and Ribas JC (2015)
A new wine *Torulaspora delbrueckii*
killer strain with broad antifungal
activity and its toxin-encoding
double-stranded RNA virus.
Front. Microbiol. 6:983.
doi: 10.3389/fmicb.2015.00983

Wine *Torulaspora delbrueckii* strains producing a new killer toxin (Kbarr-1) were isolated and selected for wine making. They killed all the previously known *Saccharomyces cerevisiae* killer strains, in addition to other non-*Saccharomyces* yeasts. The Kbarr-1 phenotype is encoded by a medium-size 1.7 kb dsRNA, TdV-Mbarr-1, which seems to depend on a large-size 4.6 kb dsRNA virus (TdV-LAbarr) for stable maintenance and replication. The TdV-Mbarr-1 dsRNA was sequenced by new generation sequencing techniques. Its genome structure is similar to those of *S. cerevisiae* killer M dsRNAs, with a 5'-end coding region followed by an internal A-rich sequence and a 3'-end non-coding region. Mbarr-1 RNA positive strand carries *cis* acting signals at its 5' and 3' termini for transcription and replication respectively, similar to those RNAs of yeast killer viruses. The ORF at the 5' region codes for a putative preprotoxin with an N-terminal secretion signal, potential Kex2p/Kexlp processing sites, and N-glycosylation sites. No relevant sequence identity was found either between the full sequence of Mbarr-1 dsRNA and other yeast M dsRNAs, or between their respective toxin-encoded proteins. However, a relevant identity of TdV-Mbarr-1 RNA regions to the putative replication and packaging signals of most of the M-virus RNAs suggests that they are all evolutionarily related.

Keywords: wine, yeast, *Torulaspora*, killer, virus, dsRNA

Introduction

The *Saccharomyces cerevisiae* killer strains have been grouped so far into four types (K1, K2, K28, and Klus) based on their killing profiles and lack of cross-immunity. Members of each group can kill non-killer yeasts as well as killer yeasts belonging to the other killer yeast types. These killer yeasts secrete protein toxins that are also lethal to other yeast species (Rodríguez-Cousiño et al., 2011). Each killer yeast is immune to its own toxin or to toxins produced by strains of the same killer type (Schmitt and Breinig, 2006). *S. cerevisiae* killer toxins (K1, K2, K28, and Klus) are encoded by the positive strand of medium-size (1.6–2.4 kb) dsRNA of yeast viruses (M1, M2, M28, and Mlus, respectively). The RNA 5'-end region contains an ORF that codes for the toxin precursor or preprotoxin (pptox), which also provides immunity to its own killer toxin. The four toxin-coding

M dsRNAs show no sequence identity with each other (Schmitt and Tipper, 1995; Rodríguez-Cousiño et al., 2011). These M viruses depend on a second large-size (4.6 kb) dsRNA helper virus, LA, for maintenance and replication. LA provides the capsids and polymerase in which both LA and M dsRNAs are separately encapsidated and replicated, and the M dsRNAs contain some stem-loop structures that mimic those LA dsRNA signals required for genome packaging or replication [reviewed by Schmitt and Breinig (2006)]. The LA genome code for two proteins, the major coat protein Gag and a minor Gag-Pol fusion protein translated by a -1 ribosomal frameshifting mechanism and that contains all the activities required for virus propagation (Icho and Wickner, 1989; Dinman and Wickner, 1992; Fujimura et al., 1992; Park et al., 1996).

The *cis* signals required for RNA packaging and replication are located in the 3'-terminal regions of the positive strands of both LA and M RNAs (Wickner et al., 1995; Rodríguez-Cousiño et al., 2011). The signal for transcription initiation has been proposed to be present in the 5'-end first 25 nucleotides of LA RNA, probably in the very 5'-terminal sequence itself (5'-GAAAAA). This 5'-terminal recognition element is also present in the 5' end of M1, M2, M28, and Mlus RNAs (Fujimura et al., 1990; Rodríguez-Cousiño et al., 2011). The M1, M2, or M28 ORF is translated into a pptox that subsequently enters the secretory pathway for further processing and secretion as a mature toxin. The unprocessed pptox consists of an N-terminal signal sequence necessary for its import into the endoplasmic reticulum lumen, followed by the α - and β -subunits of the mature toxin separated from each other, in the case of K1 and K28, by a potentially N-glycosylated γ -sequence. The signal peptide is removed in the endoplasmic reticulum, and N-glycosylation and disulphide bond formation occurs. Then, in a late Golgi compartment, protease processing takes place involving Kex2 and Kex1 proteases. Finally, the toxin is secreted as an active α/β heterodimer, with the two subunits being covalently linked by one or more disulphide bonds (Wickner et al., 1995; Schmitt and Breinig, 2006). All this processing is also believed to occur in Klus pptox according to its predicted amino acid sequence (Rodríguez-Cousiño et al., 2011). There are some other non-*Saccharomyces* killer yeasts containing a similar set of dsRNA helper and satellite viruses responsible for their killer phenotype, such as *Hanseniaspora uvarum*, *Zygosaccharomyces bailii*, or *Ustilago maydis* (Magliani et al., 1997).

Torulaspora delbrueckii was one of the first non-*Saccharomyces* yeasts to be released onto the market, and is probably the most used for winemaking. Controlled inoculation with this yeast is broadly recommended for improving the complexity and enhancing some specific characteristics of wines (Jolly et al., 2006; Azzolini et al., 2015). However, its impact on wine quality is still far from satisfactory, mostly because of the difficulty in controlling the desired participating proportion of *T. delbrueckii* with respect to the other wine yeast species involved in the same must fermentation process, mainly *S. cerevisiae*-like yeasts. As with most non-*Saccharomyces* yeasts, *T. delbrueckii* has less fermentation vigor and slower growth rate than *S. cerevisiae* under usual wine fermentation conditions, being quickly overcome by wild or inoculated *S. cerevisiae* strains

(González-Royo et al., 2014). Additionally, cell-to-cell contact has also been suggested to explain this decline of *T. delbrueckii* when it is in the presence of *S. cerevisiae* (Nissen et al., 2003; Renault et al., 2013). Thus, knowledge of the *Saccharomyces* and *Torulaspora* wine yeast interactions during wine fermentation needs to be improved to better control each yeast's participation rate (Ciani et al., 2010). The availability of good-fermenting *T. delbrueckii* killer strains, able to kill the omnipresent wild *Saccharomyces* yeasts, would be an interesting tool to achieve the inoculated yeast's dominance of the must fermentation process, so that the result would be the desired improvement in quality of the wine. The isolation of *T. delbrueckii* killer strains has been described (Sangorrín et al., 2007a,b), but no detailed phenotype or genotype analysis of these killer strains has as yet been reported.

The objective of the present work was the phenotypic analysis and genotypic characterization of new *T. delbrueckii* killer strains (Kbarr-1) isolated from the *Barraecas* valley in Spain. We addressed the following questions: (i) antifungal spectrum of the Kbarr-1 toxin, (ii) isolation, sequencing, and characterization of Mbarr-1 dsRNA satellite viruses, and (iii) analysis of Mbarr-1 genome organization and its preprotoxin ORF as compared with the dsRNA of other M viruses. The possible evolutionary relationship between these groups of viral M dsRNAs is discussed.

Materials and Methods

Yeast Strains and Media

The new *T. delbrueckii* Kbarr wine yeasts are prototrophic strains isolated from spontaneous fermentations of grapes from vineyards of the Albarregas (*Barraecas* in Latin) river valley in Spain. The industrial use of these Kbarr yeasts is under patent application. The yeast strains used in this work are summarized in Table 1.

Standard culture media were used for yeast growth (Guthrie and Fink, 1991). YEPD contained 1% yeast extract, 2% peptone, and 2% glucose. YEPD+cyh is YEPD supplemented with cycloheximide (cyh) to a final concentration of 2 μ g/ml. Synthetic minimal medium (SD) contained 0.67% Yeast Nitrogen Base (without amino acids; with ammonium sulfate, Difco), and 2% glucose. The corresponding solid media also contained 2% agar. Standard procedures were used for sporulation of yeast cultures (Kaiser et al., 1994). Diploid cells grown on YEPD plates for 2 days at 30°C were transferred to sporulation plates (1% potassium acetate, 0.1% yeast extract, 0.05% glucose, 2% agar) and incubated for 7–30 days at 25°C until cells sporulated.

Determination of Yeast Killer Activity

Killer activity was tested on low-pH (pH 4.0 or 4.7) methylene blue plates (4 or 4.7 MB; Kaiser et al., 1994) seeded with 100 μ l of a 48-h grown culture of the sensitive strain (Ramírez et al., 2004). Depending on the experiments, the strains being tested for killer activity were either loaded as 4 μ l drops of stationary phase cultures, patched from solid cultures, or replica plated onto the seeded MB plates. Then the plates were incubated for 4–8 days at 12 or 20°C.

TABLE 1 | Yeast strains used in this study.

Strain	Genotype/relevant phenotype	Origin
Sc EX88	<i>MATa/α HO/HO cyh^S/cyh^S [K2⁺]</i>	M. Ramírez ^a (from wine)
Sc EX85	<i>MAT a/α HO/HO L-A M-2[K2⁰]</i>	M. Ramírez ^a (from wine)
Sc EX85R	<i>MAT a/α HO/HO CYH^R/cyh^S [cyh^R K2⁰]</i>	M. Ramírez ^a
Sc E7AR1	<i>MAT a/α HO/HO CYH^R/cyh^S [K2⁺]</i>	M. Ramírez ^a
Sc Rod23-1B	<i>MAT a/α HO/HO PDR5/pdr5[Rod^{PC} K2⁺]</i>	M. Ramírez ^a
Sc EX229	<i>MAT a/α HO/HO L-A M-lus [Klus⁺]</i>	M. Ramírez ^a (from wine)
Sc EX229-R1	<i>MAT a/α HO/HO CYH^R/cyh^S [cyh^R Klus⁰]</i>	M. Ramírez ^a
Sc EX33	<i>MATa/α HO/HO [L-A⁰ K1⁰ K2⁰ K28⁰ Klus⁰]</i>	M. Ramírez ^a (from wine)
Sc EX73	<i>MATa/α HO/HO L-A M2 [K2⁺]</i>	M. Ramírez ^a (from wine)
Sc F166	<i>MATα leu1 kar1 L-A-HNB M1 [K1⁺]</i>	J.C. Ribas ^b (from R. B. Wickner)
Sc F182	<i>MATα his2 ade1 leu2-2 ura3-52 ski2-2 L-A M28 [K28⁺]</i>	J. C. Ribas ^b (from M. Schmitt)
Sc EX198	<i>MATa/α HO/HO L-A Mlus [Klus⁺]</i>	M. Ramírez ^a (from wine)
Td TD291	<i>wt L-A [K2⁰]</i>	Lallemand Inc. ^c
Td EX1180	<i>wt L-A Mbarr-1 [Kbarr-1⁺]</i>	This study (from wine)
Td EX1180-11C4	<i>cyh^R L-Abarr Mbarr-1 [cyh^R Kbarr-1⁺]</i>	This study (from EX1180)
Td EX1180-2K ⁻	<i>cyh^R L-Abarr Mbarr-0 [cyh^R Kbarr⁰]</i>	This study (from EX1180)
Td EX1257	<i>wt L-Abarr Mbarr-2 [Kbarr-2⁺]</i>	This study (from wine)
Td EX1257-CYH5	<i>cyh^R L-Abarr Mbarr [cyh^R Kbarr-2⁺]</i>	This study (from EX1257)
<i>Candida albicans</i> 10231	Pathogen. 87% of membrane hydrophobicity at 37°C, and 4% at 22°C	C. López ^d
<i>C. kefir</i>	Pathogen	C. López ^d
<i>C. glabrata</i>	Pathogen	C. López ^d
<i>C. dubliniensis</i>	Pathogen	C. López ^d
<i>C. krusei</i>	Pathogen	C. López ^d
<i>C. parasitopsis</i>	Pathogen	C. López ^d
<i>C. tropicalis</i>	Pathogen	C. López ^d
<i>C. albicans</i> wt 5314C	Pathogen	J. Correa ^e
<i>C. albicans</i> CAF	(<i>wt URA3+/-</i>)/Pathogen	J. Correa ^e
<i>Yarrowia lipolytica</i> wt a		L.M. Hernández ^f
<i>Y. lipolytica mnn9 a</i>	Mutation <i>mnn9</i> truncates carbohydrate outer chain of the cell wall mannoproteins of <i>S. cerevisiae</i>	L.M. Hernández ^f
<i>Y. lipolytica</i> SA1-5 wt		A. Domínguez ^g
<i>Kluyveromyces lactis</i>	Killer phenotype encoded in a dsDNA plasmid (pGKL1)	A. Domínguez ^g
<i>Hansenula mrakii</i> wt	Killer, toxin HM1 encoded in a chromosomal gene	J.C. Ribas ^b (from T. Watanabe)
<i>Schizosaccharomyces pombe</i> wt 972h ⁻		J.C. Ribas ^b
<i>Hanseniaspora</i> sp.	Killer against <i>S. cerevisiae</i>	M. Ramírez ^a (from wine)
<i>Brettanomyces</i> sp.		M. Ramírez ^a (from wine)

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Sc, *Saccharomyces cerevisiae*; Td, *Torulaspora delbrueckii*.

Total Nucleic Acid Preparation and Nuclease Digestion

The procedure for routine dsRNA and mitochondrial DNA (mtDNA) minipreps was described previously (Maqueda et al., 2010). Basically, the cells were suspended in 10 mM Tris-HCl (pH 7.5) buffer containing 0.1 M NaCl, 10 mM EDTA and 0.2% SDS, thereafter, an equal volume of phenol (pH 8.0) was added. This mixture was incubated at room temperature

for 30 min with shaking. After centrifugation, the nucleic acids recovered in the aqueous phase were precipitated with isopropanol, washed with 70% ethanol, dried, and dissolved in TE buffer pH 8.0. Digestion of DNA was done with DNase I (RNase-free, Fermentas Life Sciences) according to the manufacturer's specifications. Digestion of RNA was performed with RNase A (Sigma-Aldrich) following the manufacturer's indications. For selective degradation of single-stranded RNA, samples were

incubated with RNase A (10 µg/ml) in the presence of 0.5 M NaCl for 30 min at 37°C. Samples were then processed through phenol/chloroform/isoamyl alcohol extraction to inactivate the enzyme before analysis through agarose gel electrophoresis (Rodríguez-Cousiño et al., 2011).

Nucleic Acid Analysis for Yeast Strain Typing and Identification of *Torulaspora* Species

The procedure for virus dsRNA analysis has been described previously (Maqueda et al., 2010). The samples (4 µl) were directly separated in 1× TAE-1% agarose gel electrophoresis for virus dsRNA analysis. Nucleic acids were visualized on a UV transilluminator after ethidium bromide staining of the gels and photographed with a Gel Doc 2000 (Bio-Rad). The data analysis was performed using Diversity Database software (Bio-Rad). The nucleic acid bands were typed by Rf, and band assignment was determined by Rf values plus or minus 2% error. The identification of *Torulaspora* species by restriction fragment length polymorphism (RFLP) analysis of the internal transcribed spacers (ITS) of ribosomal DNA was done as described previously (Esteve-Zarzoso et al., 1999).

PCR Amplification, Sequencing of 18S Ribosomal DNA (rDNA), and Yeast Identification

The PCR was performed directly from the nucleic acid minipreps with the kit pReTaq Ready-To-Go PCR Beads (Amersham Biosciences), with the 18S rDNA specific primers EukA (AACCTGGTTGATCCTGCCAGT) and EukB (TGATCCTTCTGCAGGTTACCTAC; Medlin et al., 1988; Díez et al., 2001). The thermocycler protocol was an initial denaturation step of 95°C for 2 min, followed by 35 cycles of denaturing at 95°C for 15 s, annealing at 55°C for 15 s, and extension at 72°C for 2 min; and a final extension at 72°C for 10 min. The amplification products were purified with the “Jetquick PCR purification Spin Kit” (Genomed) following the manufacturer’s recommendations. The purified rDNA PCR fragment from each isolated microorganism was sent to a sequencing service (Secugen, Madrid, Spain). The 18S rDNA gene sequences were edited with the software Chromas v. 1.45¹, and analyzed against those in GenBank using Blast (Altschul et al., 1990). Sequences of >99% similarity to previously published data available at NCBI² were binned into the same species.

Viral dsRNA Purification

Total nucleic acid preparation from *T. delbrueckii* EX1180 strain (killer Kbarr-1) was obtained by the procedures mentioned above (Maqueda et al., 2010). After 1% agarose gel electrophoresis, the slower-moving dsRNA band (4.6 kb) and the faster-moving dsRNA band (1.7 kb) were cut off from the gel and purified with RNaid Kit (Q-Biogene) following the manufacturer’s indications. This procedure was repeated until more than 20 µg of each purified dsRNA was obtained.

Preparation and Sequencing of cDNA Libraries from Purified Viral dsRNA

The purified dsRNA samples were sent to the Unidad de Genómica Cantoblanco (Fundación Parque Científico de Madrid, Spain) for cDNA library preparation and next generation sequencing (NGS). Libraries from TdV-Mbarr-1 (1.7 kb dsRNA purified band) were prepared with the “TruSeq RNA Sample Preparation kit” (Illumina) following the company’s instructions, and using 200 ng of purified dsRNA as input (quantified with Picogreen). Briefly, this protocol started at the fragmentation step, skipping the RNA purification step as the viral dsRNA had been previously purified as mentioned above. Thereafter, 15% DMSO was added to the Illumina fragment-prime solution before incubation at 94°C for 8 min to facilitate the dsRNA denaturation. The first strand of cDNA was synthesized using random primers, dTVN and dABN oligonucleotides (Isogen Life Science), and SuperScriptIII retrotranscriptase. The dTVN and dABN oligonucleotides were added to improve the retrotranscription of the expected central poly(A) region of the M virus. Thereafter, the second cDNA strand synthesis, end repair, 3'-ends adenylation, and ligation of the TruSeq adaptors were done (Illumina). These adaptor oligonucleotides included signals for further amplification and sequencing, and also included short sequences referred to as indices which allowed multiplexing in the sequencing run. An enrichment procedure based on PCR was then performed to amplify the library, ensuring that all molecules in the library included the desired adaptors at both ends. The number of PCR cycles was adjusted to 12 and the final amplified libraries were checked on a BioAnalyzer 2100 (Agilent Technology). The libraries were denatured prior to seeding on a flow cell, where clusters were formed, and sequenced using 2 × 80–2 × 150 sequencing runs on a MiSeq instrument.

dsRNA Sequence Assembling

The obtained cDNA sequences were analyzed and assembled by Biotechvana (Technological Park of Valencia, Spain). As no high-identity reference sequence was available for TdV-Mbarr-1 sequence assembly, its full-length sequence was reconstructed using the following strategy. First, SOAP deNOVO2 (Luo et al., 2012) was used to obtain a *de novo* assembly based on two Illumina libraries, trying multiple assembly attempts with scaffolding and insert size of 200 and varying the Kmer value (with 47 being the most effective Kmer in reported sequences with a minimal number of undetermined positions filled with “Ns”). This K47 assembly comprised several contigs and scaffolds, none of them showing the expected typical features of viral M RNAs such as a 5' GAAAA-like motif or a large poly(A) trait in the middle of the sequence. To filter the sample, contigs with size shorter than 300 nucleotides were removed from the contig file, while the remaining contigs were used as input to the NR database of the NCBI via the BLASTX search protocol (Altschul et al., 1997) implemented in the software GPRO 1.1 (Futami et al., 2011). The BLASTX results did not report similarity to any viral M-like RNA sequence described in the scientific literature; conversely, highly significant similarity was found between several contigs/scaffolds and some known

¹<http://www.techneysisium.com.au/chromas.html>

²<http://ncbi.nlm.nih.gov>

viral RNA sequences (LA, LBC, and others) or host transcripts. These supposed contaminating sequences were also filtered from the assembly. Assuming that our Mbarr-1 sequence may have resulted fragmented during the assembly procedure because of the low complexity in the middle of the sequence containing a putative central poly(A), a manual procedure for sequence assembly was used as follows. Bowtie2 (Langmead et al., 2009) and two *ad hoc* selected scripts were used to filter all raw reads corresponding to the contaminant sequences by mapping the two Illumina libraries against the contaminant sequences. Then we manually searched the new cleansed Illumina libraries for new raw reads containing a prominent central poly(A) trait, or its complementary poly(T), to manually construct contigs based on at least 10 reads containing the poly(A). Interestingly, only one contig passed this filter. This contig was enlarged by aligning other reads that made a contig with its 5' and 3' ends. In doing so, a manually constructed contig of 300 nucleotides with a central 78-mer poly(A), and well defined and informative 5' and 3' ends, was obtained. We then searched the K47 assembly for sequences matching these two 5' and 3' ends of the poly(A) contig with at least 100 nucleotides. Two contigs of the K47 assembly were identified under this strategy, and used to resolve a contig consisting in an almost full-length sequence of TdV-Mbarr-1 RNA containing approximately 1500 nucleotides. This sequence was also enlarged as much as possible by selecting again other raw reads (from the cleansed Illumina libraries) making a contig with both the 5' and 3' ends of the reconstructed sequence. The final sequence resulted in a 1705 nucleotide contig showing the expected 5' GAAAAA and the typical domain architecture of viral M RNAs. Finally, Bowtie2 (Langmead and Salzberg, 2012) was used to map the cleansed Illumina libraries over the reconstructed final contig in order to obtain a consensus sequence of TdV-Mbarr-1 RNA genome. Visual navigation of the resulting BAM file with the IGV browser (Thorvaldsdottir et al., 2013) showed full coverage of the whole RNA sequence thus validating the methodological approach taken in reconstructing the full-length Mbarr-1 RNA genome.

Miscellaneous

DNA manipulations (enzyme digestions, PCR, and electrophoresis) were done following standard methods according to (Sambrook et al., 1989). Most of the enzymes were from Promega or Sigma. Synthetic oligonucleotides were from Biomers.

Nucleotide Sequence Accession Number

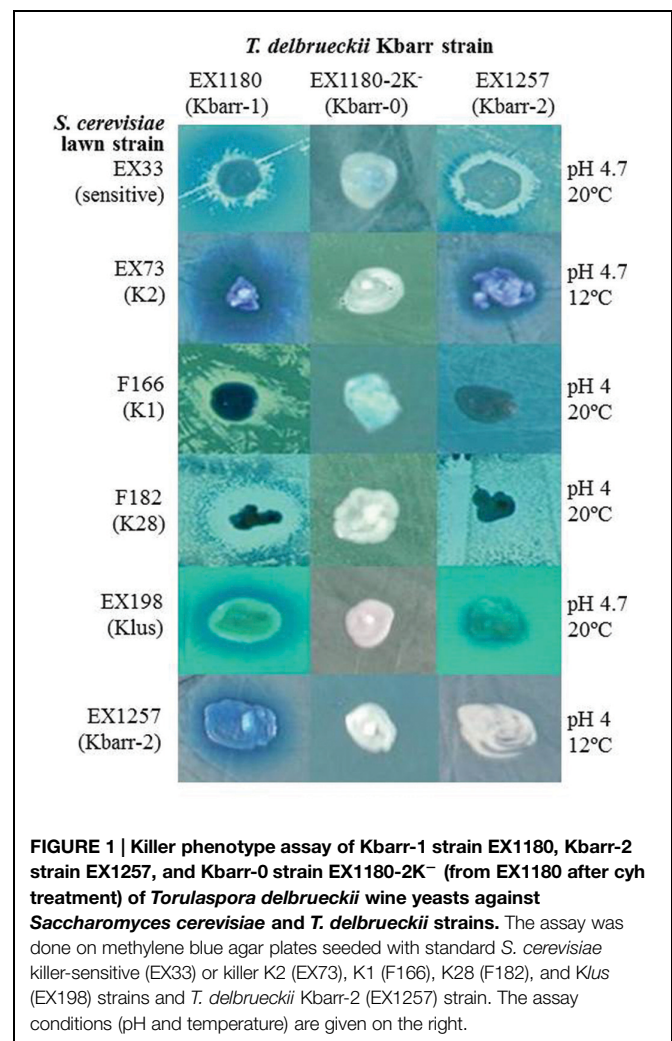
The TdV-Mbarr-1 cDNA nucleotide sequence and the encoded Mbarr-1 protein appear in NCBI/GenBank under GenBank accession number KT429819.

Results

Phenotypic Characterization of Kbarr Killer Yeasts

We analyzed the killer phenotype of 1264 *Saccharomyces*-like colonies isolated from 112 spontaneous fermentations of grapes

collected from different vineyards of the “Ribera del Guadiana” region (Spain) during several vintages. The killer strains were compared to the previously known *S. cerevisiae* K1, K2, K28 and Klus killer strains, and the EX33 non-killer strain (Table 1). New killer yeasts were found only in two fermentations of grapes from the Albarregas (*Barraecas* in Latin) river valley and named Kbarr killer strains. These Kbarr yeasts remained in more than 50% of the total viable yeast cells at the tumultuous or end fermentation stages. Two types of Kbarr phenotypes were found, Kbarr-1 and Kbarr-2. The Kbarr-1 strains killed the Kbarr-2 (Figure 1) strains, while the Kbarr-2 strains did not kill the Kbarr-1 strains (not shown). Two Kbarr-1 yeast isolates (EX1178 and EX1180) were selected for wine making according to the criteria described previously (Regodón et al., 1997), and therefore chosen for deeper killer phenotype and genotype analyses. Initially, no Kbarr-2 yeast isolate was chosen for such analysis because they showed lower killer activity than Kbarr-1 strains (Figures 1 and 2). All killer Kbarr isolates were prototrophic yeasts identified as *T. delbrueckii* according to their physiological characteristics (Kurtzman, 2011), 18S ribosomal RNA sequence (Maqueda et al., 2012), and ITS-RFLPs (Esteve-Zarzoso et al., 1999) analyses.



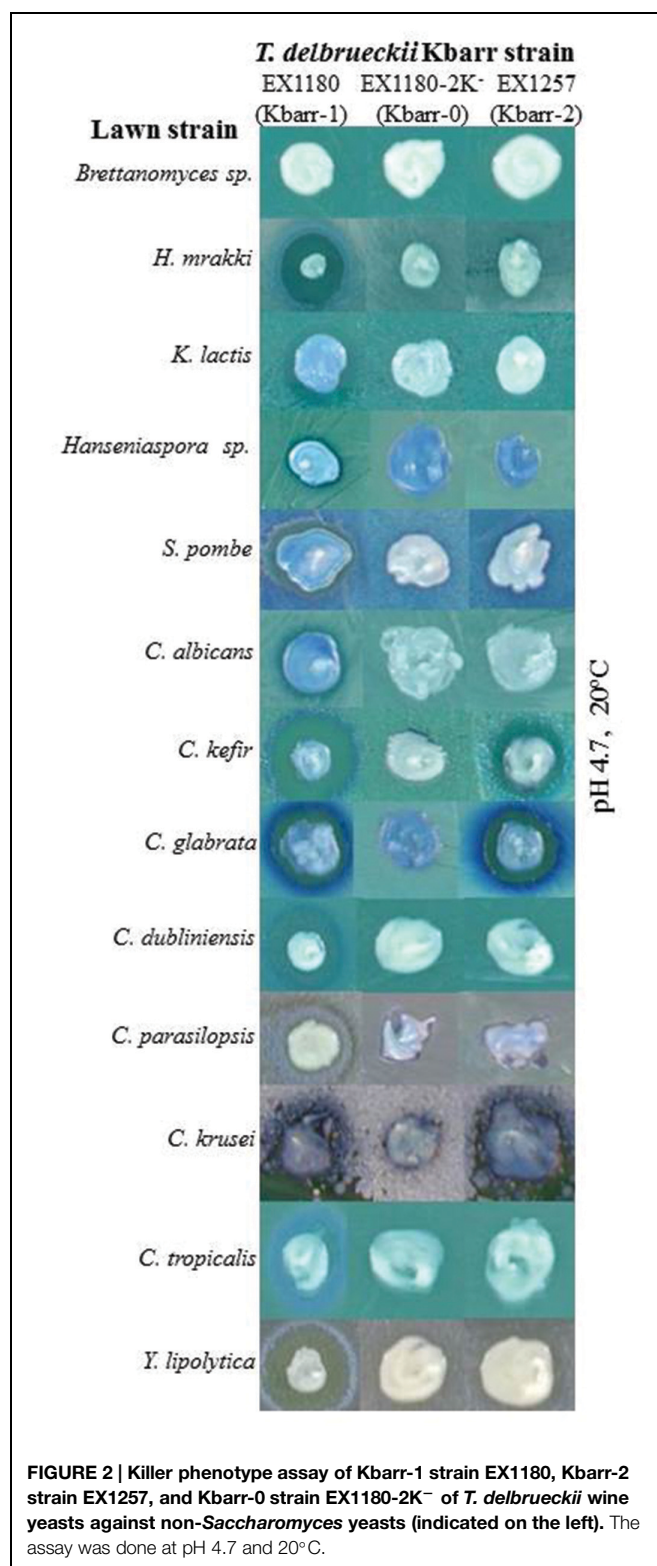
The Kbarr-1 strains killed the four known K1, K2, K28, and Klus killer strains of *S. cerevisiae* and the aforementioned *T. delbrueckii* Kbarr-2 strain, but they did not kill other Kbarr-1 yeasts (Figure 1). They were also lethal to yeast species other than *S. cerevisiae*, such as *Hanseniaspora* sp., *Kluyveromyces lactis*, *Schizosaccharomyces pombe*, *Candida albicans*, *C. tropicalis*, *C. dubliniensis*, *C. kefir*, *C. glabrata*, *C. parasilopsis*, *C. krusei*, *Yarrowia lipolytica*, and *Hansenula mrakii*, although unfortunately they did not kill the wine spoilage yeast *Brettanomyces* as it might have been desired (Figure 2). The Kbarr-1 strains were only weakly sensitive to killer toxins produced by *S. cerevisiae* Klus and *H. mrakii*, and resistant to the rest of the tested killer yeasts (not shown). The killing-activity spectrum of the Kbarr-1 yeasts was even broader than that of the recently discovered *S. cerevisiae* Klus yeasts (Rodríguez-Cousiño et al., 2011). Kbarr-1 yeasts showed stronger killer activity than K2 or Klus yeasts, and similar activity to those of *S. cerevisiae* K1, or K28 strains. The Kbarr-1's strongest activity was found at pH 4.7 and 12°C against *S. cerevisiae* K2, pH 4 and 20°C against *S. cerevisiae* K28, and pH 4.7 and 20°C against *H. mrakii*, *C. kefir*, *C. glabrata*, *C. dubliniensis*, and *Y. lipolytica* strains (Figures 1 and 2).

Genotypic Characterization of Kbarr-1 Killer Yeasts

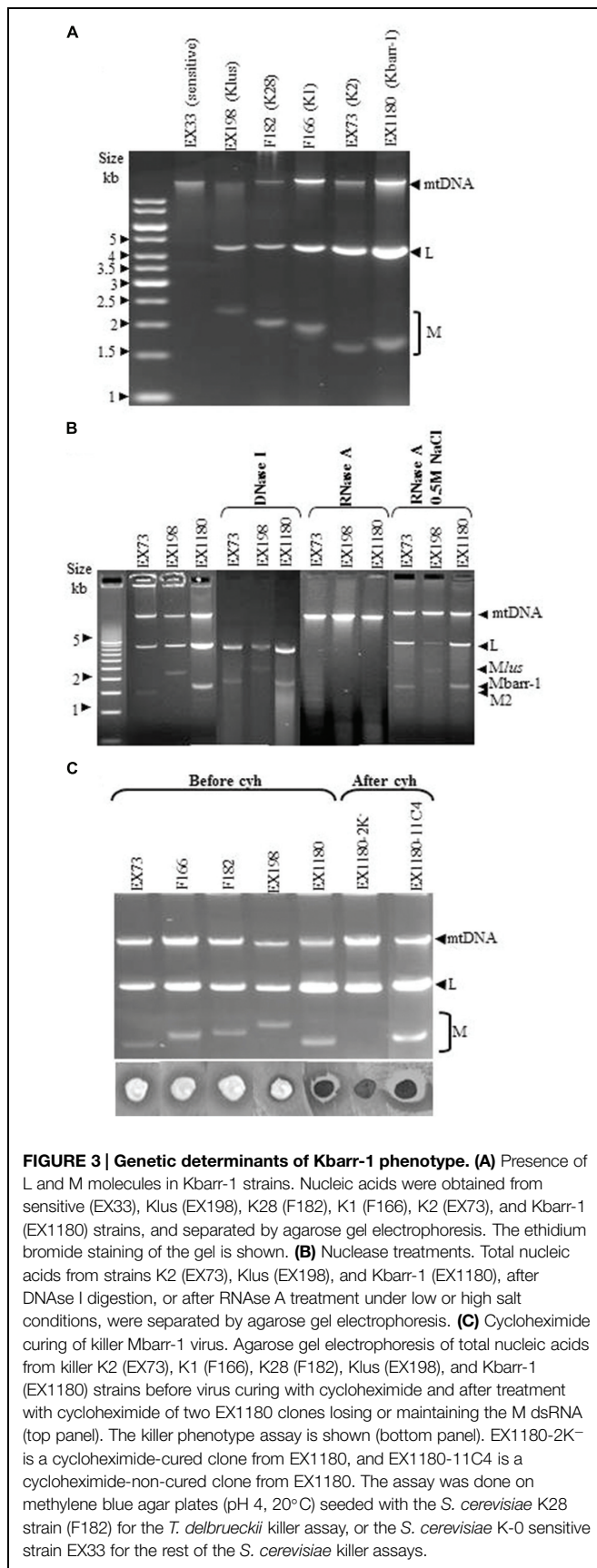
All Kbarr-1 killer strains carried two nucleic acid bands that showed an agarose-gel-electrophoresis mobility similar to those of viral dsRNAs from other killer yeasts: (1) a slower-moving band, similar in size to the dsRNA genome of ScV-LA virus (4.6 kb, now named the TdV-LAbarr band); and (2) a faster-moving band, similar to the dsRNA genome of ScV-M viruses (1.7 kb, now named the TdV-Mbarr-1 band; Figure 3A). The dsRNA nature of the two nucleic acid bands was confirmed by DNase I and RNase A treatments. The mtDNA disappeared after DNase I treatment, while LA, M2, and Mlus dsRNAs used as controls, and the bands of similar sizes present in the Kbarr-1 strains, remained unaffected (Figure 3B). Additionally, LA, M2, Mlus dsRNA bands and the Kbarr-1 strain bands disappeared after RNase A treatment, while mtDNA remained unaffected. Moreover, the RNA molecules were fairly resistant to RNase A digestion in the presence of 0.5 M NaCl, as expected for dsRNA but not for ssRNA (Berry and Bevane, 1972; Vodkin and Fink, 1973; Cansado et al., 1999; Figure 3B). Mbarr-1 dsRNA was lost during the growth of Kbarr-1 strains in the presence of cycloheximide, and a concomitant loss of its killer activity was observed (Figure 3C). This result suggests that the Kbarr-1 killer phenotype is encoded by the Mbarr-1 dsRNAs, as has been previously described for the killer toxins encoded by M1, M2, M28, and Mlus dsRNAs in *S. cerevisiae* (Berry and Bevane, 1972; Vodkin and Fink, 1973; Wickner, 1991; Rodríguez-Cousiño et al., 2011).

Analysis of TdV-Mbarr-1 dsRNA Sequence and Kbarr-1 Preprotoxin ORF

The TdV-Mbarr-1 dsRNA band present in the Kbarr-1 strain EX1180 was purified after agarose-gel electrophoresis and sequenced by NGS techniques (see Materials and Methods). The



full cDNA sequence determined was 1705 nucleotides, which is the same size as that estimated by agarose-gel electrophoresis, 1.7 kb (Figures 3 and 4; the 5'-3' orientation refers to the positive strand with coding capacity). There is a 5' GAAAAA



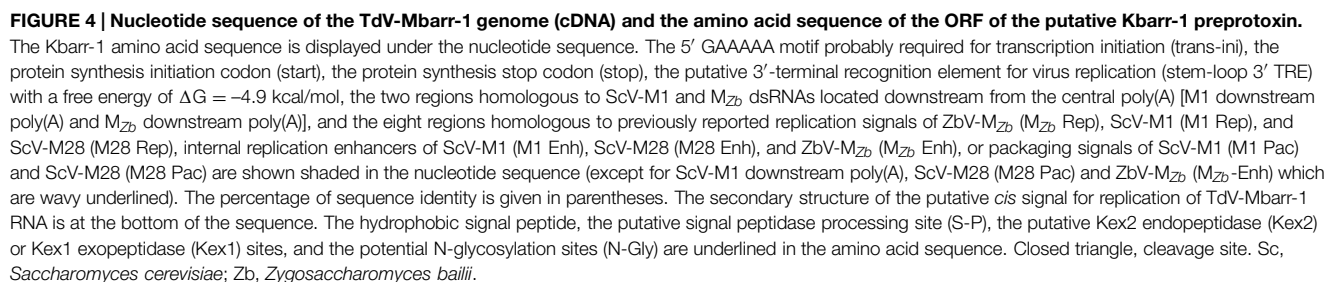
conserved motif previously found in the ScV L and M genomes and probably required for transcription initiation (Fujimura and Wickner, 1989), followed by an open reading frame for the putative preprotoxin of 271 amino acids in the 5'-most region, from nucleotides 9 to 821. The central part of the RNA molecule contains an A-rich region with 78 adenine residues. The non-coding 3'-region is presumed to provide structural *cis*-elements required for RNA replication and encapsidation. A putative 3'-terminal recognition element (3'-TRE) from nucleotides 36 to 58 (numbering from the 3' end) with a free energy of $\Delta G = -4.9$ kcal/mol was found; although it has no significant identity with previously described 3'-TRE elements required for ssRNA replication of *S. cerevisiae* viral RNAs. No putative viral binding site (VBS) with typical stem loop structure interrupted by an unpaired protruding A residue was found in this 3'-region, despite these typical VBSs having been reported in the same genome region of LA, M1, and M28 dsRNAs, but not in Mlus (Rodríguez-Cousiño et al., 2011), and being required for ssRNA binding to Gag-Pol protein and packaging (Wickner et al., 2013). However, 10 sequence stretches homologous to previously reported poly(A) downstream regions (including regions of replication, replication-enhancement, and packaging signals) of several *S. cerevisiae* and *Z. bailii* M viral genomes were found in this TdV-Mbarr1 non-coding 3'-region, but not in the rest of the molecule (Figure 4). Apart from these similarities, the TdV-Mbarr-1 genome presented no relevant sequence identity with the known M virus genomes of yeasts, which, in terms of their overall sequences, themselves share no relevant sequence identity.

The ORF located in the 5' region of Mbarr-1 contains a stretch of hydrophobic amino acids at the amino terminus according to the Kyte-Doolittle hydrophilicity plot (Kyte and Doolittle, 1982). This is a possible N-terminal secretion signal with a potential signal for peptidase cleavage. The ORF also presents six Kex2p/Kexlp processing sites and four potential sites for N-glycosylation (Figure 4). However, as it was the case for the genomes, the Kbarr-1 amino acid sequence showed no relevant sequence identity with the known yeast killer protein toxins, which, in terms of their overall sequences, themselves share no relevant amino acid sequence homology.

Discussion

Characterization of the New Kbarr-1 Killer Yeasts

The wine *T. delbrueckii* killer yeasts are less frequently isolated from spontaneous must fermentations than other wine killer yeasts such as *S. cerevisiae* K2 or Klus (Maqueda et al., 2012). This is probably because *T. delbrueckii* has slower growth rate and less fermentation vigor than *S. cerevisiae* yeasts in the grape must medium, so that *S. cerevisiae* quickly overcomes *T. delbrueckii* at the beginning of must fermentation. Also, isolation of the less frequent *T. delbrueckii* yeast may be missed because it resembles very much the colony morphology of *S. cerevisiae*, with the two being easily confused. We have succeeded probably because the intense killer phenotype of the new *T. delbrueckii* Kbarr-1 yeasts allowed them to dominate the tumultuous and end



beneficial effect during food fermentation, as it has been shown for K2 killer strains in winemaking (Pérez et al., 2001). This is especially relevant for the use of *Torulaspora* in winemaking

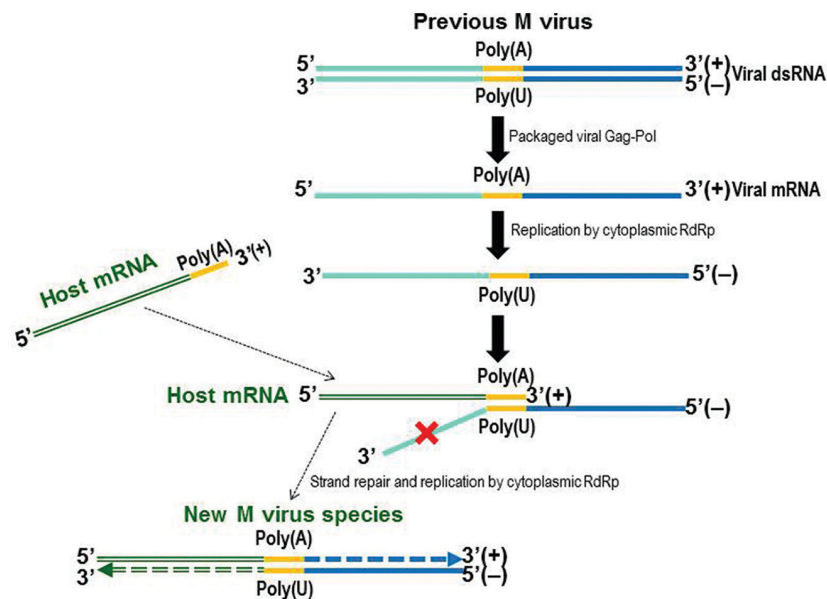


FIGURE 5 | Model of the emergence of the M virus species. Free mRNA (+) might be replicated to generate a ssRNA (–) instead of a natural dsRNA molecule, by a cytoplasmic RdRp that could be Pol of Gag-Pol of the LA virus. Complementary base pairing of the ssRNA (–) central poly(U) and the 3' poly(A) of a host mRNA (+) may generate a new incomplete hybrid dsRNA molecule that could be repaired and replicated by the RdRp to yield a new dsRNA M virus.

because this species is strongly recommended by winemaking supply companies to improve wine quality, while the non-killer strains are quickly displaced by the always present *S. cerevisiae* yeasts during must fermentation. These Kbarr-1 yeasts may also be useful to avoid undesirable potential spoilage and pathogen yeasts in food fermentation processes because they have the broadest antifungal spectrum hitherto described (Figures 1 and 2), even broader than that of the recently described Klus killer yeasts (Rodríguez-Cousiño et al., 2013), or the other *S. cerevisiae* killer yeasts (K1, K2, or K28) that mostly kill sensitive cells of the same or some congeneric species (Magliani et al., 1997). Although the intensity of the Kbarr-1 killer activity may change depending on the assay conditions (pH, temperature, and the sensitive strain tested) as it happen for other killer toxins (Pfeiffer and Radler, 1984; Young, 1987), it is active against different yeast species in the typical food fermentation environment: acidic (pH 3.5–5.5) and mild temperature (18–28°C).

Genetic Characterization of the Mbarr-1 Virus

The Kbarr-1 killer yeasts contained two dsRNA molecules corresponding to a new TdV-Mbarr-1 dsRNA and the genome of its putative helper virus TdV-LAbarr, as found in other killer yeasts (Fujimura and Esteban, 2011, 2013; Rodríguez-Cousiño et al., 2011, 2013). The putative dependency of TdV-Mbarr-1 on TdV-LAbarr (and/or TdV-LBC) is likely because we never found Mbarr-1 killer yeasts without the 4.6 kb dsRNA among the 152 isolated yeasts. We never found killer Kbarr-1 yeast free of the 1.7 kb dsRNA, suggesting that the Kbarr-1 toxin is encoded by this RNA molecule. Moreover, this was confirmed by the Kbarr-1 yeasts becoming non-killer and sensitive to Kbarr-1 killer yeasts when they lost the 1.7 kb dsRNA after cycloheximide treatment.

Mbarr-1 dsRNA Organization and the Encoded Kbarr-1 Preprotoxin

The complete cDNA sequence of Mbarr-1 dsRNA (1705 nucleotides) matches the Mbarr-1 dsRNA size estimated by agarose-gel electrophoresis (1.7 kb, Figure 4), and it includes the 5' and 3' ends, and the central A-rich region previously found in the *S. cerevisiae* M viruses. This suggests that the NGS techniques used here for dsRNA sequencing yielded more satisfactory results than those we used previously for ScV-Mlus dsRNA sequencing, where the length of the sequence obtained (2033 nucleotides) was shorter than the 2.3 kb visualized on agarose gel electrophoresis (Rodríguez-Cousiño et al., 2011). This difference was explained as due to the variable number of adenine residues in the central A-rich region, which supposedly accounts for the different-sized ScV-Mlus isotypes. This central A-rich region may facilitate sliding or jumping of either the reverse transcriptase or the Taq polymerase used in RT-PCR, yielding a slightly shorter sequence than the actual. This methodological problem is overcome by using the new NGS sequencing approach, given that it yields the same sequence length as the actual dsRNA size. TdV-Mbarr-1 contains a single continuous central A-rich region as do most of M dsRNAs (Wickner, 1996) with the exception of ScV-Mlus which contains two central A-rich regions of variable size (Rodríguez-Cousiño et al., 2011). Additionally, Mbarr-1 also resembles the ScV-M genomes in that it contains the conserved 5' GAAAAA motif probably required for transcription initiation, a putative 3'-TRE with low free energy, and 10 sequence stretches homologous to previously reported poly(A)-downstream-regions considered as putative signals for replication, replication-enhancement, and packaging, located in the non-coding 3'-region of the *S. cerevisiae*

and *Z. bailii* M viral genomes (Weiler et al., 2002; Wickner et al., 2013; **Figure 4**). Overall, the genome organization of Mbarr-1 dsRNA resembles that of other toxin-encoding satellite viruses such as ScV-M1, ScV-M2, ScV-M28, ScV-Mlus, or *Z. bailii* M_{Zb} dsRNA (Schmitt and Breinig, 2002; Weiler et al., 2002; Rodríguez-Cousiño et al., 2011). The conserved motifs located in the non-coding 3'-region suggest a close phylogenetic relationship of *T. delbrueckii*, *S. cerevisiae*, and *Z. bailii* M viruses. The coding 5'-region of the M viruses may have an independent origin, probably from host yeast genes coding for secreted proteins, given that a relevant amino acid sequence identity between the *S. cerevisiae* Klus toxin and the nuclear YFR020W ORF protein has been found (Rodríguez-Cousiño et al., 2011). Probably, the central A-rich region located between the coding 5'-region and the non-coding 3'-region of the M viruses is just a reminiscence of the mRNA 3' poly(A)-tail of a hypothetically secreted and chromosomally encoded protein, which somehow became attached to the 5' end of the viral-RNA, which in turn formed the non-coding 3'-region of the final M RNA. Thereafter, as shown in **Figure 5**, new M viruses species can be generated by annealing of the 3' poly(A)-tail of other incoming mRNA with the central poly(U) of a pre-existing viral M ssRNA(–), followed by a conservative replication of the complementary strands by a cytoplasmic RdRp to create a distinct “5' host mRNA-central poly(A)-3' viral RNA” hybrid RNA molecule. The RdRp required for this conservative replication could be provided by Pol of the Gag-Pol fusion protein of LA helper virus. Further studies will be required to confirm this hypothesis.

The AUG initiation codon of the putative Mbarr-1 preprotoxin ORF is located close to the 5' end of the RNA molecule (**Figure 4**), like in M1, M28, and M_{Zb} RNAs (Skipper et al., 1984; Meskukas, 1990; Schmitt and Tipper, 1995; Weiler et al., 2002) but different to the Mlus RNA whose AUG codon is located 112 nt away from the 5' end (Rodríguez-Cousiño et al., 2011). Also as for other M dsRNA, the Mbarr-1 ORF organization resembles that of other killer preprotoxins such as those of M1, M2, M28, or Mlus viruses. It contains a stretch of hydrophobic amino acids at the amino terminus, potential Kex2p/Kexlp processing sites, and potential sites for N-glycosylation (**Figure 4**). Proteolytic cleavage of the Kbarr-1 preprotoxin by signal peptidase and Kex2/Kex1 proteases could produce four putative peptides, the signal peptide and the α , β , and γ subunits. According to the disulphide bond prediction

(Ceroni et al., 2006), there is a potential disulphide bond between cysteine-50, located in the putative α subunit, and cysteine-189 or cysteine-238, both located in the putative β subunit of the Kbarr-1 mature toxin. This disulphide bond may be required for keeping α and β subunits together during the preprotoxin processing and secretion to yield an extracellular active α/β heterodimer.

Conclusion

The new *T. delbrueckii* Kbarr-1 wine yeasts kill all the tested *S. cerevisiae* strains as well as many other undesired yeast species in environmental conditions similar to those of the typical sugar-rich substrate fermentation, which makes them an interesting starter culture for the food fermentation industry. The killer dsRNA virus system of this wine yeast seems very similar to those previously described in *S. cerevisiae*. Although there was no relevant general sequence identity among the M genomes, the relevant identity found in 10 sequence stretches of TdV-Mbarr-1 RNA with previously reported M RNAs raises the possibility that the M viruses may have a common phylogenetic origin, at least for the non-coding 3'-region where these homologous sequences are located.

Author Contributions

MR conceived the project, MR, RV, and MM designed and performed the experiments, MR, AL-P, and JR analyzed the data, MR wrote and edited the manuscript.

Acknowledgments

This work was funded by grants GR10088 from the Extremadura Regional Government and AGL2011-25711 from the Spanish Ministry of Education and Science. RV acknowledges support by a fellowship from the Extremadura Regional Government. We thank the Unidad de Genómica Cantoblanco (Fundación Parque Científico de Madrid, Spain) for setting up the protocols for library preparation and sequencing of viral dsRNA, and the company Biotechvana S.L. (Technological Park of Valencia, Spain) for setting up the dsRNA sequence assembling strategy.

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