MICROBIOTA OF GRAPES: POSITIVE AND NEGATIVE ROLE ON WINE QUALITY

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MICROBIOTA OF GRAPES: POSITIVE AND NEGATIVE ROLE ON WINE QUALITY

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During spontaneous food/beverage fermentations, the microbiota associated with the raw material has a considerable importance: this microbial consortium evolves in reason of the nutrient content and of the physical, chemical, and biological determinants present in the food matrix, shaping fermentation dynamics with significant impacts on the 'qualities' of final productions. The selection from the indigenous micro-biodiversity of 'virtuous' ecotypes that coupled pro-technological and biotechnological aptitudes provide the basis for the formulation of 'tailored' starter cultures. In the fermenting food and beverage arena, the wine sector is generally characterized by the generation of a high added value. Together with a pronounced seasonality, this feature strongly contributes to the selection of a large group of starter cultures. In the last years, several studies contributed

to describe the complexity of grapevine-associated microbiota using both culture-dependent and culture-independent approaches. The grape-associated microbial communities continuously change during the wine-making process, with different dominances that correspond to the main biotechnological steps that take place in wine. In order to simplify, following a time trend, four major dominances can be mainly considered: non-*Saccharomyces*, *Saccharomyces*, lactic acid bacteria (LAB), and spoilage microbes. The first two dominances come in succession during the alcoholic fermentation: the impact of *Saccharomyces* (that are responsible of key enological step of ethanol production) can be complemented/integrated by the contributions of compatible non-*Saccharomyces* strains. Lactic acid bacteria constitute the malolactic consortium responsible of malolactic fermentation, a microbial bioconversion often desired in wine (especially in red wine production). Finally, the fourth dominance, the undesired microbiota, represents a panel of microorganisms that, coupling spoilage potential to the resistance to the harsh conditions typical of wine environment, can cause important economic losses. In each of these four dominances a complex microbial biodiversity has been described. The studies on the enological significance of the micro-biodiversity connected with each of the four dominances highlighted the presence of a dichotomy: in each consortia there are species/strains that, in reason of their metabolisms, are able to improve wine 'qualities' (resource of interest in starter cultures design), and species/strains that with their metabolism are responsible of depreciation of wine.

Articles describing new oenological impacts of yeasts and bacteria belonging to the four main categories above mentioned (non-*Saccharomyces*, *Saccharomycetes*, lactic acid bacteria, and spoilage microbes) are welcome. Moreover, in this Research Topic, we encourage mini-review submissions on topics of immediate interest in wine microbiology that link microbial biodiversity with positive/negative effects in wine.

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EDITORIAL
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Editorial: Microbiota of Grapes: Positive and Negative Role on Wine Quality

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Keywords: grape, wine, yeast, non-*Saccharomyces*, *Saccharomyces cerevisiae*, malolactic bacteria, safety, quality

Editorial on the Research Topic

Microbiota of Grapes: Positive and Negative Role on Wine Quality

During the vinification process, we can generally separate four main phases associated with specific microbial dominances: (i) first stages of alcoholic fermentation (AF) (non-*Saccharomyces*), (ii) most part of AF, up to the end (*Saccharomyces*), (iii) malolactic fermentation (MLF) (lactic acid bacteria or LAB), and (iv) undesired changes associated to microbial metabolism (spoilage yeasts and bacteria, microbial producers of toxic compounds). All these microorganisms can be found ecologically associated to grapevines and to the vineyard and, consequently, to the winery environment. Furthermore, it should be stressed that in some cases strains involved in the phases of pro-technological interest (AF and MLF), are even responsible of undesired production (e.g., off-flavors, compounds toxic for human health). These evidences, together with the needs for standardization, time-saving procedures and quality/safety improvements, led to the introduction of the starter cultures technologies in the wine industry. Selected strains from natural "micro-biodiversity" and/or from breeding program were selected in order to design starter cultures, in other words "a microbial preparation of large numbers of cells of at least one microorganism to be added to a raw material to produce a fermented food by accelerating and steering its fermentation process."

The research topic "Microbiota of grapes: positive and negative role on wine quality" belongs to the Food Microbiology section and covers 19 contributes: 1 review, 2 mini-reviews, and 16 original research papers. As Topic Editors, we briefly report an overview of these contributes starting with microbial consortia associated to grapes and wines. Indeed, nine of the articles focused on the description of the microbial consortia associated with specific grapes and with the corresponding (uninoculated) musts and wines. The following two studies analyzed both eukaryotic and prokaryotic microorganisms as target. Salvetti et al. described the microbial communities associated with the Italian *Vitis vinifera* L. cv. *Corvina* grape berries, used for the production of unique wines, such as Amarone, at the end of the process of "traditional withering" or "accelerated withering." Pinto et al. characterized the microbiota associated with the must from six different Portuguese wine appellations. The first phases and last stages of AF were used as target. Piao et al. investigated the bacterial community and their temporal succession during the fermentation of organically grown Riesling grapes. Moreover, six work focused only on the "eukaryotic side." Wang et al. described fungal diversity in Spanish "Carignan" and "Grenache" grape must and during wine fermentation. Sipiczki analyzed the yeast communities and their interactions in overwintering grapes (mummified on vines) in the Tokaj wine region (Hungary-Slovakia). Vigentini et al. delved into yeast biodiversity in five Georgian areas and from 22

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different native cultivars using both grapes and wines. Padilla et al. examined yeast biodiversity from uninoculated fermentations from the Priorat region, the second "Denominación de Origen Calificada" wine region in Spain, while Aponte and Blaiotta surveyed yeasts diversity and enological significance in spontaneous fermentation from Taurasi DOCG (Appellation of Controlled and Guaranteed Origin) production area. Jara et al. observed the biodiversity of non-*Saccharomyces* yeast associated with vineyards of the in Chilean valleys. All these scientific reports provide us snapshot of microbial biodiversity throughout different methodological lenses: whole metagenome sequencing (Salvetti et al.; Piao et al.; Pinto et al.; Wang et al.), quantitative PCR and DGGE (Wang et al.), cultural-dependent methods followed by molecular characterization (Sipiczki; Vigentini et al.; Padilla et al.; Aponte and Blaiotta; Jara et al.).

The grape-associated microbial communities continuously change during the winemaking process, with different dominances that correspond to the main biotechnological steps that take place in wine. With concern of this succession, the special issues reported eight studies dealing with yeast characterization/applications and one concerning simultaneous AF and MLF. Two original research papers and a review article focused on the role of *Saccharomyces* strains. Patrignani et al. proposed a non-conventional characterization including release of volatile and, particularly, of sulfur compounds, of 10 *S. cerevisiae* strains inoculated in "Trebbiano" must. Capece et al. studied the diversity of indigenous *S. cerevisiae* strains associated with geographical origin from two different Italian wine-producing regions (Tuscany and Basilicata), in order to contribute to assess the possible role of these yeasts in the regional identity of wine. Legras et al. reviewed the most recent "omics" data on the analysis of flor strains of *S. cerevisiae*, an interesting phenotype for the aging of Sherry and Sherry-like wines. On the other hand, in accordance with the recent trends regarding the use of non-*Saccharomyces* in enology, five contributes reported literature review and original data on the use of specific species/strains to improve wine quality. Ciani et al. provided a review on the explored interactions among yeast species and strains of enological interest, with a particular focus on the effect of mixed cultures on the final wine quality, which can concretely influence the stability of the final wine and its analytical and aromatic profile. Grangeteau et al. demonstrated, for the first time, the persistence of non-*Saccharomyces* yeasts (*Hanseniaspora* and *Starmerella*) from year to year in the cellar. The work by Tristezza et al. reported new insights into the oenological potential of autochthonous Apulian strains of *Hanseniaspora uvarum* and *S. cerevisiae* used in simultaneous and sequential co-fermentation for industrial wine production. Tofalo et al. tested indigenous strains of *S. cerevisiae*, *Starmerella bacillaris*, and *H. uvarum* and a co-culture of *S. cerevisiae* and *S. bacillaris* to evaluate their role in the sensory characteristic of Montepulciano d'Abruzzo wine. Canonico et al. evaluated the use of specific immobilized non-*Saccharomyces* yeasts, in sequential fermentation, in order to reduce ethanol tenor in wine. With concern of MLF, the original research paper by Bleve et al. reported the efficacy of simultaneous alcoholic and malolactic fermentations by *S. cerevisiae* and *Oenococcus oeni* cells co-immobilized in alginate beads.

Finally, Russo et al. delved into safety aspect with a review on biogenic amines and mycotoxins, among the principal toxic compounds of microbial origin in wine, offering a brief description of the main determinant involved in this phenomena, but also overviewing the prevention/correction strategies, including those biotechnological-based.

In general, several paper contribute to improve the knowledge on the shape of autochthonous microbiota and on the significance of autochthonous yeasts for different geographical enological productions, in other terms on the so-called "microbial terroir," a field that has been received considerable attention in last years.

Finally, this collection gives a flavor of the enological significance of the micro-biodiversity from grape to wine, highlighting in microbial resources the presence of a dichotomy: in each consortia there are species/strains that, in reason of their metabolisms, are able to improve wine "qualities" (resource of interest in starter cultures design), and species/strains that, with their metabolism, are responsible of depreciation of wine.

AUTHOR CONTRIBUTIONS

GS and ST drafted and revised the final version of the Editorial.

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ORIGINAL RESEARCH
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Whole-Metagenome-Sequencing-Based Community Profiles of *Vitis vinifera* L. cv. Corvina Berries Withered in Two Post-harvest Conditions

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Vitis vinifera L. cv. Corvina grape forms the basis for the production of unique wines, such as Amarone, whose distinctive sensory features are strongly linked to the post-harvest grape withering process. Indeed, this process increases sugar concentration and changes must characteristics. While microorganisms involved in must fermentation have been widely investigated, few data are available on the microbiota of withered grapes. Thus, in this paper, a whole metagenome sequencing (WMS) approach was used to analyse the microbial consortium associated with Corvina berries at the end of the withering process performed in two different conditions ("traditional withering," TW or "accelerated withering," AW), and to unveil whether changes of drying parameters could have an impact on microbial diversity. Samples of healthy undamaged berries were collected and washed, to recover microorganisms from the surface and avoid contamination with grapevine genetic material. Isolated DNA was sequenced and the data obtained were analyzed with several bioinformatics methods. The eukaryotic community was mainly composed by members of the phylum Ascomycota, including Eurotiomycetes, Sordariomycetes, and Dothideomycetes. Moreover, the distribution of the genera *Aspergillus* and *Penicillium* (class *Eurotiomycetes*) varied between the withered berry samples. Instead, *Botryotinia*, *Saccharomyces,* and other wine technologically useful microorganisms were relatively scarce in both samples. For prokaryotes, 25 phyla were identified, nine of which were common to both conditions. Environmental bacteria belonging to the class Gammaproteobacteria were dominant and, in particular, the TW sample was characterized by members of the family Pseudomonadaceae, while members of the family Enterobacteriaceae dominated the AW sample, in addition to Sphyngobacteria and Clostridia. Finally, the binning procedure discovered 15 putative genomes which dominated the microbial community of the two samples, and included representatives of genera *Erwinia, Pantoea, Pseudomonas,*

Clostridium, Paenibacillus, and of orders Lactobacillales and Actinomycetales. These results provide insights into the microbial consortium of Corvina withered berries and reveal relevant variations attributable to post-harvest withering conditions, underling how WMS could open novel perspectives in the knowledge and management of the withering process of Corvina, with an impact on the winemaking of important Italian wines.

Keywords: withered grape, post-harvest, microbiome, microbial diversity, yeasts, molds, bacteria, metagenomics

INTRODUCTION

Grape naturally hosts a reservoir of microorganisms that may be transferred into the winery and affect the vinification process, influencing wine quality and storage (Mills et al., 2007). The microbial population of sound grape berry is roughly comprised between 10^3 and 10^5 cfu/g (Barata et al., 2012), and includes filamentous fungi, yeasts, and bacteria with different physiological characteristics and different impact on the grape metabolome and final wine quality (Verginer et al., 2010; Pinto et al., 2015). Some species are found only in grapes, as saprophytic molds, like *Aspergillus* spp., *Cladosporium* spp., and *Penicillium* spp. (Martins et al., 2014), and environmental bacteria, while others are able to survive and grow in wine, constituting the wine microbial consortium (Barata et al., 2011; Liu et al., 2015), that comprises yeasts, lactic acid bacteria (LAB) and acetic acid bacteria (AAB).

Yeast species present on the berry may play important roles during the alcoholic fermentation and have significant impact on quality and aromatic properties of wine (Pretorius, 2000; Fleet et al., 2002). Species present on sound ripe berries have been reported to belong mainly to the group of oxidative basidiomycetous yeasts, such as *Cryptococcus* spp., *Rhodotorula* spp., *Sporobolomyces* spp., and *Filobasidium* spp., as well as to the dimorphic ascomycetous black yeast, *Aureobasidium pullulans* (Prakitchaiwattana et al., 2004; Magyar and Bene, 2006; Barata et al., 2008). These yeasts are ubiquitous in the vineyard environment and they are typically associated with grapes, phyllosphere, and soil (Setati et al., 2012; Gilbert et al., 2014). The oxidative ascomycetous yeasts (e.g., *Candida* spp., *Pichia* spp., and *Metschnikowia* spp.), and the fermentative ascomycetous yeasts (e.g., *Hanseniaspora*/*Kloeckera* spp.) have been found to be present at low concentrations on sound berries and appear often localized in those areas of the grape surface where some juice might escape (Nisiotou and Nychas, 2007; Čadež et al., 2010; Capozzi et al., 2015). In contrast, *Saccharomyces cerevisiae*, the most relevant fermentative wine yeast, is mostly present in low number and low frequencies, even in damaged berries (Fleet, 2003).

Grapes are considered the primary source of LAB (Nisiotou et al., 2011), which catalyse the conversion of L-malic acid to L-lactic acid with the production of CO_2 through malolactic fermentation and to impart flavor complexity (Sumby et al., 2014). Species belonging to the LAB group, such as *Lactobacillus* spp., *Leuconostoc* spp., *Pediococcus* spp., and *Oenococcus oeni*, have been frequently found in wine and winery (Pérez-Martín et al., 2014; Sumby et al., 2014), but they have been isolated from sound or damaged berries only rarely (Barata et al., 2012). Grapevine microbiota also shows a broad diversity of ubiquitous environmental bacteria belonging to the genera *Bacillus*, *Burkholderia*, *Enterobacter*, *Enterococcus*, *Pseudomonas*, *Serratia*, and *Staphylococcus* (Leveau and Tech, 2011; Martins et al., 2012; Gilbert et al., 2014), which are unable to grow in wine. In addition to LAB and environmental bacteria, the genera *Acetobacter*, *Gluconoacetobacter*, and *Gluconobacter* belonging to the AAB group have also been found on the grape surfaces (Barbe et al., 2001; Barata et al., 2012). AAB are well known for their ability to produce acetic acid from sugars and through the oxidation of the ethanol, representing a key factor in wine spoilage (Bartowsky and Henschke, 2008).

The microbiota of grapes is highly variable, mostly due to the influence of external factors, as environmental parameters, geographical location, and grape cultivars (Bokulich et al., 2013, 2014; Pinto et al., 2014). *Vitis vinifera* L. cv. Corvina is the most important red grape variety of the Verona area in north Italy, displaying good vigor, providing abundant, fairly consistent yields, and showing fair resistance to disease and hardship (Andreolli et al., 2016). The fundamental role of this grapevine variety in conferring the uniqueness wine aroma has been underlined by previous transcriptomic, proteomic, and metabolomic studies (Di Carli et al., 2011; Toffali et al., 2011; Dal Santo et al., 2013; Venturini et al., 2013). This late-ripening variety forms the basis of Verona's red wines and, despite showing a certain fragility during the drying process, it is essential in the production of Amarone wine, to which it gives structure, weight, and a surprising softness (Paronetto and Dellaglio, 2011). The distinctive features of such wine are strongly linked to the post-harvest withering process, an ancient local technique of grape semi-drying, which goes as far back as the Romans time. The grapes are partially dried in large, well-aired rooms (*fruttaio*) for 2/3 months, causing elimination of water, concentration of sugar up to about 30% (w/v) and other substances, evolution of aromatic molecules and phenolic compounds (Consonni et al., 2011; Tosi et al., 2012). Therefore, the drying process leads to a large number of changes in the grape and must characteristics, depending on environmental parameters (temperature, humidity, ventilation), time, and microbial activities. However, few studies have been carried out on the microbial communities associated with withered berries (Lorenzini et al., 2013; Rantsiou et al., 2013; Guzzon et al., 2014), and the effects of withering conditions on overall grape microbiota is still largely unknown.

The development of next-generation sequencing provides a useful tool for the description of prokaryotic and eukaryotic microbial communities associated with grape berries, leaves, must and wineries (Bokulich et al., 2013, 2014; David et al., 2014;

Pinto et al., 2014, 2015; Taylor et al., 2014; Piao et al., 2015; Wang et al., 2015). Microbial community profiling using whole metagenome sequencing (WMS) could allow an accurate and detailed investigation of the underlying microbial community, providing data also for minority species (Thomas et al., 2012). Contrary to rDNA-targeting pyrosequencing (including both 16S and 18S rRNA genes, as well as the nuclear ribosomal internal transcribed spacer regions), metagenomics offers the possibility to describe diversity at genome level, also revealing the functional gene composition of microbial communities (Sharpton, 2014). Moreover, genomic information acquired from metagenomic sampling can contribute substantially in the recognition of new taxa (Ladoukakis et al., 2014) and improve the *Candidatus* proposal, a provisional status for uncultivated novel taxa (Konstantinidis and Rosselló-Móra, 2015).

In this study, a WMS approach was carried out with the primary aim of analyzing the diversity of the microbial consortia of Corvina sound berry surfaces at the end of the withering process. Only healthy berries were considered to get information on their "natural" microbiota that might play an important role in the metabolic processes taking place inside the berries during withering. Two different post-harvest withering conditions were analyzed ("traditional," TW, and "accelerated," AW) to unveil whether changes of drying parameters could lead to relevant modifications of the microbial components in this peculiar ecological niche.

MATERIALS AND METHODS

Grape Withering and Sampling

The collection of grapevine (*V. vinifera* L. cv. Corvina) bunches was carried out at the *fruttaio* located in Gargagnago di Sant'Ambrogio di Valpolicella, Italy (45°31′20″ N, 10°50′05″ E). Grapes, harvested during the 2013 vintage, were placed on wooden racks in the *fruttaio* and subjected to two different withering conditions, i.e., "TW" and "AW." Temperature, relative humidity, and ventilation were set up to maintain conventional parameters, i.e., a gradually decreasing temperature (from 16 to 8°C) and a gradually increasing relative humidity (from 60 to 80%; http://www.appaxximento.it/eng/#fruttai) for the TW berry batch; while, a fan was placed close to another batch of grapes to promote a faster drying (AW condition). In this way, AW grapes were exposed to an average airflow of about 1 m/s that in turn contributed to remove part of the humid air stacking around the clusters, almost without affecting temperature. Temperature and relative humidity close to AW grapes were on average 0.2 ± 0.5°C and 13.5 ± 8.5% lower than TW grapes, respectively.

Grape weight loss was monitored during the withering process, and bunches were randomly sampled when grapes reached ∼30% of the weight loss (i.e., after 61 and 109 days for AW and TW conditions, respectively) and they were ready for the crushing stage. Only healthy undamaged bunches were used for the analysis. Grape bunches were placed in sterile plastic bags and transferred to the laboratory in a refrigerated container. Under aseptic conditions in the laboratory, sound berries were harvested, gentle destemming, separating stems from berries, pooled together (150 g) in order to make the sample representative, and processed as described below.

Microbial Cell Collection, Genomic DNA Extraction, and Sequencing

Berries were processed according to Renouf et al. (2005) with slight modifications. Basically, berries were placed in a sterile 500 mL flask containing a solution of Bacto Soytone (Sigma-Aldrich, St. Louis, MO, USA; 10 g/L) and Tween 80 (Sigma-Aldrich; 2 mL/L) to wash them and to release the microorganisms from the surface. This step was carried out twice at 20°C for 3 h with slow shaking. The washing solutions were then filtered through 0.45 μm Whatman nitrocellulose membrane filters (Sigma-Aldrich) and stored at 4°C until DNA extraction.

Total genomic DNA was extracted from the two filter membranes independently using the PowerWater® DNA Isolation Kit (MO BIO Laboratories Inc., Carlsbad, CA, USA), according to the manufacturer's instructions. The quantification and quality control of the DNA was determined with a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). The concentration of the DNA samples was normalized and the sequencing was carried out at the Functional Genomics Centre (University of Verona, Verona, Italy) using an Illumina HiSeq 2000 (Illumina Inc., San Diego, CA, USA) platform which generated 2×100-bp pair-end sequencing reads.

Bioinformatics Analysis

Reads Trimming and De novo Metagenome Assembly

Reads in FASTQ format were trimmed using Trimmomatic software version 0.35 (Bolger et al., 2014; considering quality encoding phred 33) with the following parameters: LEADING:3 TRAILING:3 SLIDINGWINDOW:4:15 MINLEN:30 SLIDINGWINDOW:4:15 MINLEN:30.

The presence of contaminant sequences derived from *V. vinifera* was determined aligning the filtered reads with Bowtie2 software version 2.2.6 (Langmead and Salzberg, 2012) on the reference grape genome (Jaillon et al., 2007) downloaded from NCBI database. Unaligned reads were extracted from the bam file using bedtools software package v2.17.0 (Quinlan and Hall, 2010).

Reads were assembled with MetaVelvet software version 1.2.02 (Namiki et al., 2012), using a kmer of 63 and a minimum scaffold length of 500 bp. When both paired sequences passed the trimming and quality check, they were used as paired-end (with insert size equal to 445 and SD 140), while sequences where only one pair passed the filtering step were used in the assembly as shotgun. Assembly was performed using the option "-exp_cov auto" in order to perform the final assembly step with MetaVelvet. MetaVelvet assembly was performed using the parameter "-exp_covs" and considering coverage peaks 1100, 615, 273, 110, 41, 24, and 7.5. Scaffolds were filtered and renamed using the perl script "rename_fasta_file.pl" (Campanaro et al., 2016).

Gene Finding and Annotation

Gene finding on the scaffolds obtained from the assembly process was performed running the program Prodigal in metagenomic mode (Hyatt et al., 2012). Conserved protein families and domains were identified using reverse position-specific BLAST algorithm (RPSBLAST; NCBI BLAST+) on all the proteins predicted from assembly and using COG-only (Galperin et al., 2015), Pfam (Finn et al., 2016), CDD (Marchler-Bauer et al., 2015), eggNOG (Powell et al., 2013), rpsBLAST databases. Only results with *e*-value lower than $1e^{-5}$ were considered; for COG, CDD and eggNOG only the best match was considered. KEGG annotation was performed using KEGG Automatic Annotation Server (KAAS; Moriya et al., 2007). After the binning process, scaffolds assigned to each genome bin were re-annotated using the Rapid Annotation Subsystem Technology (RAST) server (Overbeek et al., 2014). We further assigned a rough gene descriptions using BLASTp analysis performed on a database containing all the sequences of 2,765 complete bacterial genomes downloaded from NCBI database, only results with *e*-value lower than $1e^{-5}$ were considered.

Calculation of the Scaffold Coverage

Reads obtained individually for the two samples analyzed were aligned to the scaffolds larger than 500 bp with the Bowtie2 software version 2.2.6 and coverage was determined with the genomecov software of the bedtools package (Quinlan and Hall, 2010), "calculate_coverage_fromsam.pl" and "average_coverage_bedtools.pl" perl scripts (Campanaro et al., 2016). Coverage was normalized considering the number of aligned reads and using the sample with the lower number as a reference. The coverage obtained was considered for comparison between number of genes for each KEGG pathway and their average coverage.

Binning of Genomes Using Tetranucleotide Composition and Coverage

Binning process was performed using the procedure of Albertsen et al. (2013) which is based on sequence composition-independent binning and tetranucleotide binning. In the first step distinct groups of scaffolds were identified for their coverage similarity in the two samples (AW and TW). Bin selection was facilitated by coloring scaffolds according to their taxonomic affiliation. In the second step, principal component analysis (PCA) of tetranucleotide frequencies was used to separate species present in the same coverage-defined bin. Scaffolds missed in the binning process were recovered and the paired-end connections between scaffolds were checked using the script "cytoscapeviz.pl" (Albertsen et al., 2013) and "recover_interacting_scaffold.pl".

Identification of Conserved Marker Genes

A set of HMMs of essential single-copy genes (Dupont et al., 2012) were searched against the predicted open reading frames (ORFs) using HMMER3 (http://hmmer.janelia.org/) with the strategy proposed by Albertsen et al. (2013). The number of the essential genes in the genomic bins identified allowed the prediction of genome completeness and the duplication level using the script "extract_data_from_contigs_list.pl". For a proper calculation of the completeness, 105 essential genes of Firmicutes, Gammaproteobacteria, and Actinobacteria taxonomic groups were considered. The coverage of the genome bins was determined using the script "calculate_genome_coverage.pl".

Taxonomic Annotation for the Genome Bins

Taxonomic analysis of the bacterial genome bins was examined by different methods and the results were compared to obtain a consensus assignment. In this analysis, only prokaryotic species were examined because the low abundance of the eukaryotic species prevented their assembly and binning. The essential genes associated to each genome bin were checked by sequence similarity to the non-redundant (*nr*) database using BLASTn, with *e*-value threshold equal to $1e^{-5}$. Average sequence similarity of 95, 85, and 75% or better on the essential genes was used for species, genus and phylum level taxonomic annotation, respectively (Nielsen et al., 2014). The results, obtained from BLASTp performed on all the proteins predicted on the database of the complete microbial proteomes, were also checked to obtain information on the most similar species. Moreover, proteins encoded by genome bins were fed into Phylophlan version 0.99 to accurately determine their taxonomic identities (Segata et al., 2013). This software identifies hundreds of conserved proteins from a catalog of more than 3,700 finished and draft microbial genomes and uses them to build a high-resolution phylogeny. Results obtained were separated in "high," "medium," "low," and "incomplete" confidence. The high-resolution microbial tree of life with taxonomic annotations was obtained using standard parameters.

Metagenomic Analysis of the Shotgun Reads

Shotgun reads were used to profile the composition of the microbial community using MetaPhlAn version 1.7.7 (Segata et al., 2012). The software was run with standard parameters but using—sensitive-local in the Bowtie2 alignment step.

Moreover, two millions of the reads not aligned to the assembly were uploaded on the Metagenome MG Rapid Annotation using Subsystem Technology (MG-RAST) database server (http://metagenomics.anl.gov/; Meyer et al., 2008) and they were dereplicated (Gomez-Alvarez et al., 2009). The sequences which aligned to *Homo sapiens* NCBI v36 genome were removed (Langmead et al., 2009) together with the low quality sequences identified using a modified dynamic trim (Cox et al., 2010). The number of sequences obtained for each taxonomic group was determined with the MG-RAST toolkit using default parameters and selecting "RefSeq" and "GenBank" as annotation sources.

Nucleotide Sequence Accession Numbers

Shotgun reads were assigned to the study PRJNA289617 with ID number SRP063004 and were deposited to NCBI Sequence Reads Archive (SRA) with the following accession numbers: traditional withering (sample SRS1050145; experiment SRX1175002; run SRR2219805) and accelerated withering (sample SRS1050146; experiment SRX1175003; run SRR2219866). Metagenome assembly was deposited at DDBJ/ENA/GenBank under the accession LIDZ00000000. The version described in this paper

is version LIDZ01000000. The two million reads that are not represented in the metagenome assembly were deposited to MG-RAST database and are freely accessible with the following ID numbers: 4580981.3 and 4580980.3 for the TW and AW samples, respectively.

Genome bins extracted from the assembly with the binning process are available on the RAST database (http://rast.nmpdr.org/rast.cgi?page=Home).

RESULTS

Metagenomic Sequencing and Analysis of the Berry Microbiota

A total of 89,280,038 and 62,412,964 sequences were generated from the grape cv. Corvina subjected to the two different post-harvest withering conditions, AW and TW, respectively. Only 223,038 (0.25%) and 163,898 (0.26%) sequences were removed from AW and TW-derived data on the basis of quality control parameters, highlighting the success of the sequencing. Moreover, only the 0.066% (58,555 reads of AW data) and the 0.018% (11,024 reads of TW data) of the sequences showed similarity to grapevine sequences, as detected by Bowtie2-derived alignments against the reference genome of *V. vinifera* (*V. vinifera* 12X; Jaillon et al., 2007), demonstrating the very low level of plant DNA contamination due to the washing procedure used for the isolation of the berry microbiota.

Several analyses were performed to analyse the characteristics of the berry microbial communities: (i) mapping shotgun reads against MG-RAST database and a database of species-specific genomic regions; and (ii) assembling sequences into scaffolds. Since short reads could be error-prone and could contain low signal for homology search, the generation of longer sequences can simplify bioinformatics analyses. The assembly of the reads was performed with MetaVelvet including all the sequences (pair-end, and those where only forward or reverse pairs remained after filtering). A large fraction of the reads (∼96%) was assembled in scaffolds ≥500 bp (15,893 scaffolds including 86,379,522 bp; Figure S1).

A preliminary analysis of the assembly revealed that the berry microbiota was dominated by individual draft genomes belonging to prokaryotic communities. Therefore, the taxonomic diversity of the prokaryotic fraction was characterized through the analysis of the shotgun reads and the near-complete draft genomes of the species that dominated the samples obtained from the assembly. Since the scaffolds were not assigned to the eukaryotic fraction, we inferred that the fungal microbiome were not assembled due to their low abundance within the two samples, thus representing the "rare biosphere." Therefore, the taxonomic profiling of the eukaryotic population was typified examining only the reads that were not included in the assembly.

Although the primary aim of the present paper was the analysis of the microbial biodiversity, a preliminary investigation of the functional properties of the biotic consortia was carried out: genes were predicted using the program Prodigal, and ORFs were annotated through BLASTp analyses against a database composed by the protein sequences encoded by 2,765 prokaryotic genome sequences available in NCBI. In addition, the reverse position-specific BLAST algorithm (RPSBLAST) was used on all predicted proteins using COG-only and Pfam rpsBLAST databases.

The 86,425 protein encoding genes were annotated using COG, KEGG, eggNOG and Pfam; 57,333 genes (66.3%) had a match in the COG database; 23,220 (26.9%) in KEGG, and 59,401 (68.7%) had a protein domain annotated in Pfam; 63,154 had a match in the eggNOG database (73.1%), and 76,407 (88.4%) found a match in the BLASTp comparisons against the proteins encoded in the complete microbial genomes of the NCBI database (Table S1).

Analysis of the number of genes belonging to each COG and KEGG categories in the assembly was compared with the abundance (i.e., the number of gene copies) calculated considering coverage obtained for each scaffold in the two withering conditions examined. This allowed an evaluation of the relevance of the COG/KEGG classes considering the number of genes in the pathways and their abundance in the species. From these data, it is evident that some COG categories and metabolic pathways include genes with a high average coverage, which are encoded in the genomes of the most abundant species.

The coverage of the functional categories found in the two metagenomic datasets is shown in **Figure 1**. The categories had similar abundances in both samples. For all genes clustered in COG categories, the main categories were E (aminoacid metabolism and transport), G (carbohydrate metabolism and transport), K (transcription), T (signal transduction mechanisms), R (general function prediction only), and S (function unknown).

Considering all categories, those more numerically different between the AW and TW samples were represented by E (aminoacid metabolism and transport), G (carbohydrate metabolism and transport), K (transcription), U (intracellular trafficking, secretion, and vesicular transport), and V (defense mechanisms). In particular, within the E category, elements of the histidine permease ABC transporter, such as the genes *HisJ* and *HisM*, were the most abundant in both samples. Regarding the G category, the gene *araJ*, encoding for an arabinose efflux permease, was the predominant in both samples, while genes coding for the ABC-type sugar transport system, such as *UgpA*, *UgpB,* and *UgpE*, were mainly present in the TW sample respect to the AW sample. It is also interesting the presence of numerous genes involved in arginine transport and metabolism, in particular *ArtQ* (18 genes having higher coverage in TW, and 10 genes in AW), *ArtM* (17 genes in TW, and 8 genes in AW), *ArgF* (11 genes in TW, and 10 genes in AW), and *ArcC* (1 gene in TW, and 7 genes in AW).

Composition Profiling of Microbial Communities

The eukaryotic population diversity was estimated using MG-RAST software based on 2 million reads selected from each sample, which were not included into the scaffolds obtained

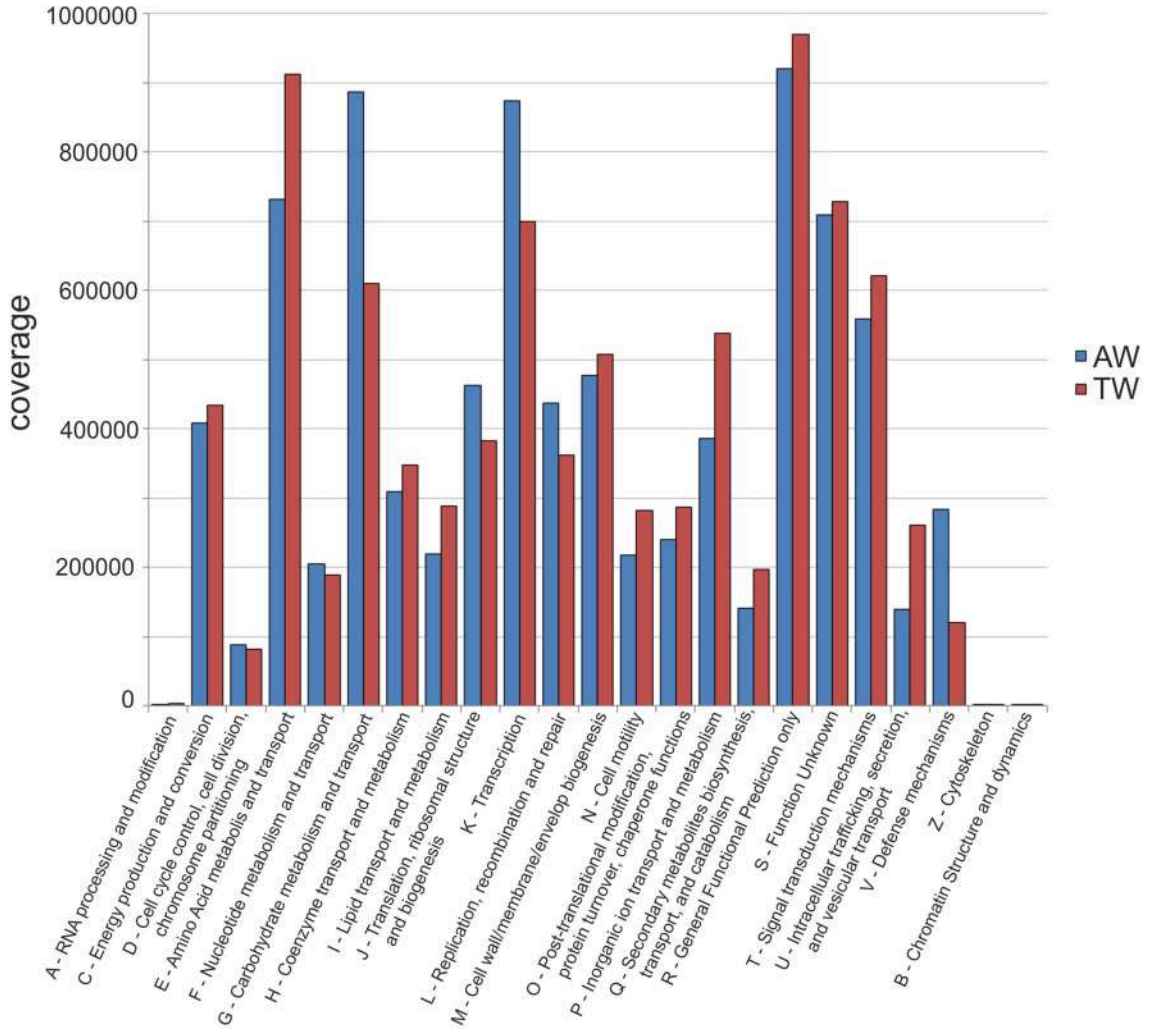

FIGURE 1 | Coverage of the functional categories found in the two metagenomic datasets. A, RNA processing and modification; B, chromatin structure and dynamics; C, energy production and conversion; D, cell cycle control, cell division, chromosome partitioning; E, amino acid transport and metabolism; F, nucleotide transport and metabolism; G, carbohydrate transport and metabolism; H, coenzyme transport and metabolism; I, lipid transport and metabolism; J, translation, ribosomal structure, and biogenesis; K, transcription; L, replication, recombination, and repair; M, cell wall/membrane/envelope biogenesis; N, cell motility; O, post-translational modification, protein turnover, and chaperones; P, inorganic ion transport and metabolism; Q, secondary metabolites biosynthesis, transport, and catabolism; R, general function prediction only; S, function unknown; T, signal transduction mechanisms; U, intracellular trafficking, secretion, and vesicular transport; V, defense mechanisms; W, extracellular structures; Y, nuclear structure; Z, cytoskeleton.

from the assembly. The unassembled reads represented the 2.9% (5,145,300 reads) for TW and 2.1% (2,598,617 reads) for AW samples. This approach revealed that the eukaryotic community was mainly composed by members of the phylum Ascomycota, 85% (33,191 reads) and 62% (3,023 reads) of reads in the TW and AW samples, respectively (**Figure 2**). This evidenced that, despite the strong sequencing effort, the absolute number of reads assigned to the fungal species remained low and this prevented their assembly and the functional analysis, which was limited to the prokaryotic fraction of the microbiome.

In detail, in both metagenomic datasets the majority of ascomycetes belong to the class Eurotiomycetes, in particular to the genera *Aspergillus* and *Penicillium*, but also to classes Sordariomycetes (principally the genera *Neurospora* and *Gibberella*), and Dothideomycetes (specifically the genera *Phaeosphaeria* and *Pyrenophora*; **Figure 3**).

The abundance of fungal population belonging to the genera *Neurospora*, *Gibberella* (whose anamorph is *Fusarium* sp.), *Phaeosphaeria,* and *Pyrenophora* was similar in the two samples. Conversely, the distribution of the genera of the class Eurotiomycetes (47% of the Ascomycota fraction for the TW berries; 15,436 reads), such as *Aspergillus* and *Penicillium*, varied between the two samples, representing, respectively, the 22% (6,428 reads) and 13% (4,212 reads) for

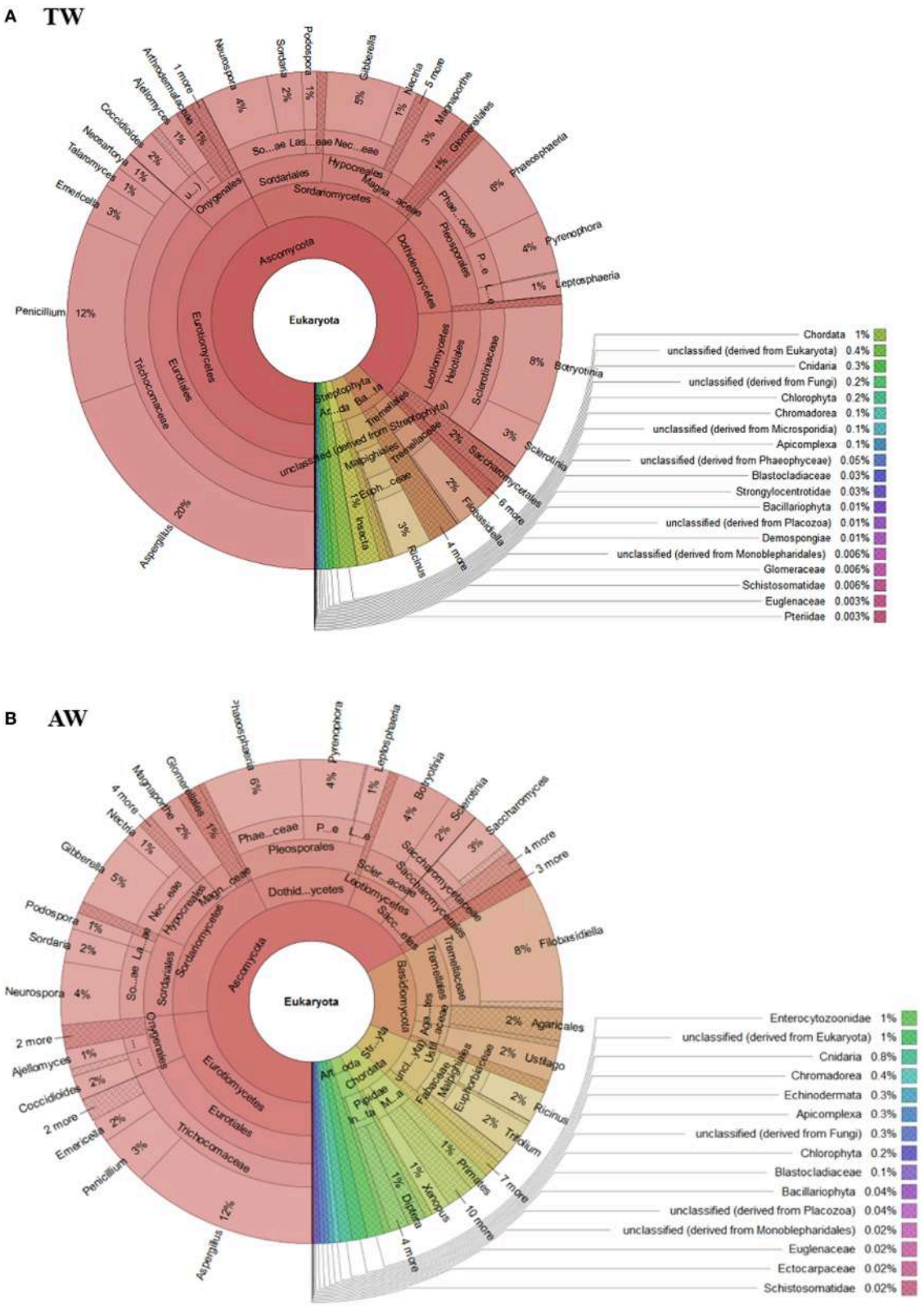

FIGURE 2 | The ecological diversity of the eukaryotic population of traditional (A-TW) and accelerated (B-AW) withered berry samples estimated using MG-RAST software based on 2 million reads selected from each samples querying the NCBI *nr* database.

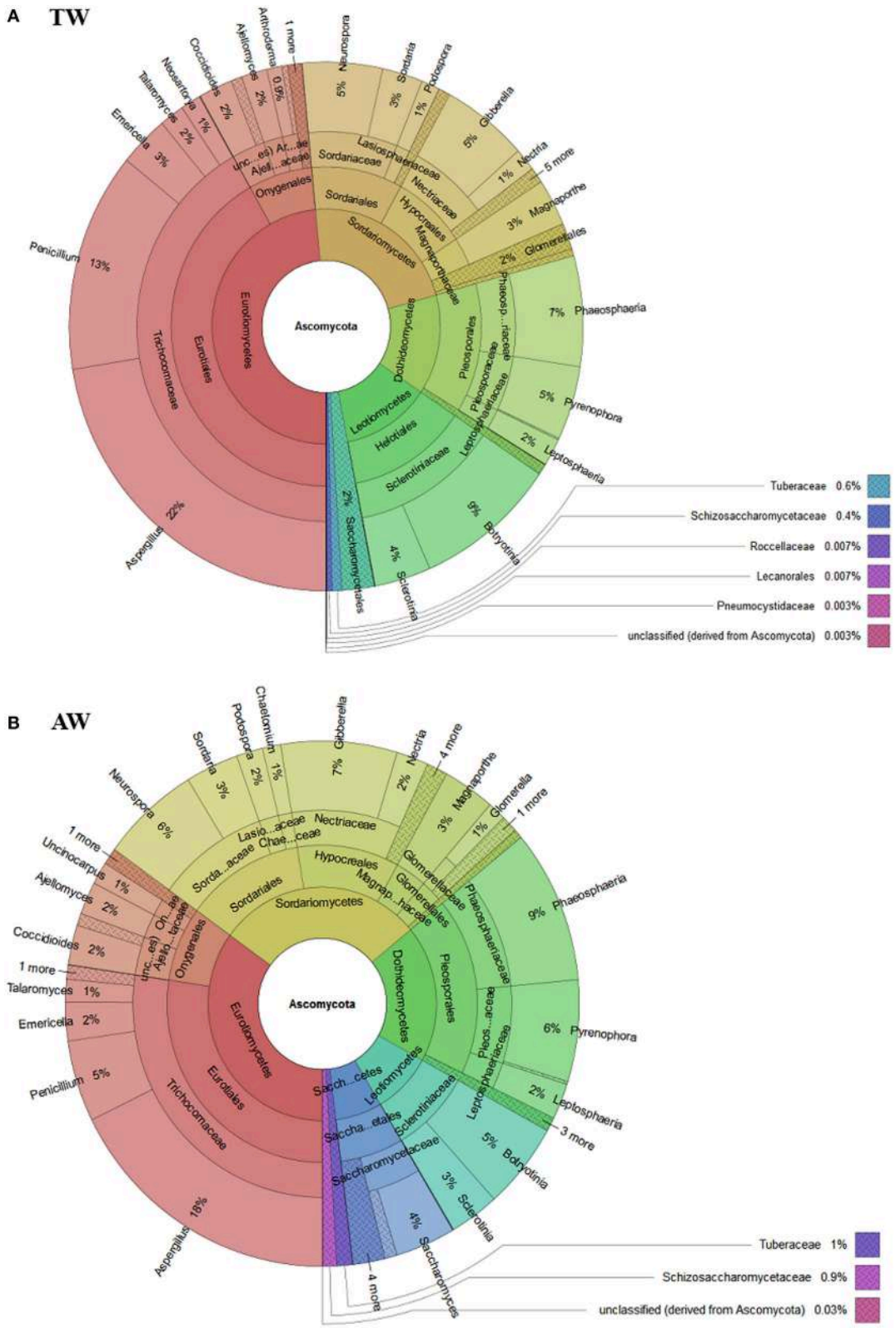

FIGURE 3 | The composition of the Ascomycota population of traditional (A-TW) and accelerated (B-AW) withered berry samples estimated using MG-RAST software based on 2 million reads selected from each samples querying the NCBI *nr* database.

the TW sample (**Figure 3A**-TW) and the 18% (463 reads) and 5% (161 reads) for the AW sample (**Figure 3B**-AW). Moreover, members belonging to the genus *Botryotinia* were detected in both samples: they constituted the 9% and 5% of the Ascomycota population of the AW and TW samples, respectively.

The bioinformatics analyses of the metadata did not reveal the presence of yeasts commonly associated with sound berries, such as *Aureobasidium*, *Cryptococcus*, *Hanseniapora, Metschnikowia*, and *Sporobolomyces*. Moreover, low amounts of wine yeast species with important role in winemaking were retrieved: members of the order Saccharomycetales represented approximately 2% (57 reads) and 1% (446 reads) of the Ascomycota fraction of the AW and TW samples, respectively. Interestingly, both samples showed the presence of members of the genus *Saccharomyces* (0.3 and 0.2% of the Ascomycota fraction of the AW and TW samples, respectively; 8 and 62 reads), which includes the most important yeasts for Amarone wine production, i.e., *S. cerevisiae* and *Saccharomyces bayanus* (Torriani et al., 1999).

The prokaryotic taxonomic diversity was characterized by aligning reads obtained from each sample against a dataset of clade-specific marker sequences, which unequivocally identified specific microbial clades at the species level or higher taxonomic ranks (Table S2; Segata et al., 2012). A total of 25 phyla were detected at the end of drying process performed in the two different conditions, of which nine phyla were found on the berry surfaces from both samples: i.e., Acidobacteria, Actinobacteria, Bacteroidetes, Chlamydiae, Chloroflexi, Cyanobacteria, Firmicutes, Proteobacteria, and Thermi (**Table 1**). Moreover, the relative abundance of each taxonomic unit was provided, revealing that Proteobacteria was the predominant phylum in both samples (97.7 and 86.1% for the TW and AW berries, respectively). Less abundant phyla were Firmicutes (7.8%), and Actinobacteria (0.7%) in the AW and TW samples, respectively; while Bacteroidetes was 4.7% in the AW and 1.2% in the TW conditions.

At the class level, the prokaryotic communities associated with berries of the TW and AW conditions were mostly characterized by Gammaproteobacteria (94.1 and 84.9%, respectively). Moreover, minor abundance of Clostridia, Sphingobacteria, and Bacilli characterized the AW sample (6.5, 4.6, 1.3%, respectively; **Table 2**).

As depicted by the nodes in the cladograms of **Figure 4**, both samples were dominated by Pseudomonadales and Enterobacteriales, belonging to the order Gammaproteobacteria, although with different relatives abundances. Indeed, members of the family Pseudomonadaceae were present in higher levels in the TW sample, and in particular the genus *Pseudomonas* accounted for 88% of the taxa, while the high incidence of Enterobacteriaceae on the AW berries was related to a relevant abundance of the genus *Pantoea* (76%) and a moderate presence of the genus *Erwinia* (3%; Table S2). Among the minority classes, Lactobacillales were detected in the AW sample, represented mainly by the genera *Enterococcus* (0.9%) and *Carnobacterium* (0.3%; Table S2).

TABLE 1 | Relative abundance of prokaryotic phyla associated with grape surfaces of the traditional and accelerated withering process obtained through the MetaPhlAn analyses.

ID	Traditional withering	Accelerated withering
k__Bacteria;p__Proteobacteria	97.6583	86.0501
k__Bacteria;p__Bacteroidetes	1.2162	4.7371
k__Bacteria;p__Actinobacteria	0.7383	0.0179
k__Bacteria;p__Chlamydiae	0.1423	0.4725
k__Bacteria;p__Firmicutes	0.0833	7.7888
k__Bacteria;p__Chloroflexi	0.0536	0.3574
k__Bacteria;p__Thermi	0.0431	0.0111
k__Bacteria;p__Cyanobacteria	0.0180	0.0009
k__Bacteria;p__Acidobacteria	0.0140	0.0128
k__Bacteria;p__Verrucomicrobia	0.0075	0.0000
k__Bacteria;p__Synergistetes	0.0061	0.0000
k__Bacteria;p__Gemmatimonadetes	0.0033	0.0000
k__Bacteria;p__Chlorobi	0.0021	0.0000
k__Bacteria;p__Planctomycetes	0.0021	0.0000
k__Bacteria;p__Lentisphaerae	0.0014	0.0000
k__Bacteria;p__Chrysiogenetes	0.0005	0.0000
k__Bacteria;p__Aquificae	0.0000	0.0042
k__Bacteria;p__Deferribacteres	0.0000	0.0028
k__Bacteria;p__Dictyoglomi	0.0000	0.0008
k__Bacteria;p__Fusobacteria	0.0000	0.2326
k__Bacteria;p__Nitrospirae	0.0000	0.0009
k__Bacteria;p__Spirochaetes	0.0000	0.0257
k__Bacteria;p__Tenericutes	0.0000	0.1460
k__Bacteria;p__Thermotogae	0.0000	0.0101
k__Bacteria;p__WWE1	0.0000	0.0001

Assembly of Individual Genomes

The assembly of reads was conducted to reconstruct the near-complete draft genomes of the bacterial species that dominate on the berries. Indeed, their abundance can be directly determined aligning the shotgun reads on the genomes and, most importantly, it is possible to infer their metabolic properties by examining their gene content (Albertsen et al., 2013).

To estimate the coverage of the scaffolds in each dataset, all the reads were aligned on the scaffolds with the Bowtie2 software: this operation revealed that the samples were effectively characterized by the presence of a relatively small amount of genomes and the populations were differentially represented in the two samples, as they had highly different coverage of scaffolds in each dataset. The tetranucleotide identity was calculated to further refine the multiple species that could be included in the same coverage-defined subset, and the conserved essential single-copy marker genes were identified (Albertsen et al., 2013).

The two coverage values were plotted against each other for all the scaffolds to achieve the binning of scaffolds into population genomes (**Figure 5**). Clusters of scaffolds showed in

TABLE 2 | Relative abundance of prokaryotic classes associated with grape surfaces of the traditional and accelerated withering process obtained through the MetaPhlAn analyses.

ID	Traditional withering	Accelerated withering
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria	94.0720	84.9150
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria	1.3884	0.1623
k__Bacteria;p__Bacteroidetes;c__Sphingobacteria	1.2101	4.6223
k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria	1.1684	0.4152
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria	1.0192	0.5203
k__Bacteria;p__Actinobacteria;c__Actinobacteria	0.7383	0.0179
k__Bacteria;p__Chlamydiae;c__Chlamydiae	0.1423	0.4725
k__Bacteria;p__Chloroflexi;c__Thermomicrobia	0.0465	0.3569
k__Bacteria;p__Firmicutes;c__Clostridia	0.0359	6.48421
k__Bacteria;p__Firmicutes;c__Bacilli	0.0349	1.29394
k__Bacteria;p__Thermi;c__Deinococci	0.0344	0.01114
k__Bacteria;p__Cyanobacteria;c__Cyanophyceae	0.0157	0.0009
k__Bacteria;p__Firmicutes;c__Negativicutes	0.0126	0.0070
k__Bacteria;p__Acidobacteria;c__Acidobacteria	0.0118	0.0128
k__Bacteria;p__Thermi;c__Thermi	0.0088	0.0000
k__Bacteria;p__Proteobacteria;c__Epsilonproteobacteria	0.0076	0.0369
k__Bacteria;p__Bacteroidetes;c__Bacteroidia	0.0061	0.0191
k__Bacteria;p__Synergistetes;c__Synergistia	0.0061	0.0000
k__Bacteria;p__Verrucomicrobia;c__Opitutae	0.0043	0.0000
k__Bacteria;p__Chloroflexi;c__Chloroflexi	0.0036	0.0000
k__Bacteria;p__Gemmatimonadetes;c__Gemmatimonadetes	0.0033	0.0000
k__Bacteria;p__Verrucomicrobia;c__Spartobacteria	0.0029	0.0000
k__Bacteria;p__Proteobacteria;c__Zetaproteobacteria	0.0028	0.0004
k__Bacteria;p__Chloroflexi;c__Anaerolineae	0.0024	0.0005
k__Bacteria;p__Cyanobacteria;c__Gloeobacteria	0.0023	0.0000
k__Bacteria;p__Acidobacteria;c__Solibacteres	0.0022	0.0000
k__Bacteria;p__Chlorobi;c__Chlorobia	0.0021	0.0000
k__Bacteria;p__Planctomycetes;c__Planctomycetacia	0.0021	0.0000
k__Bacteria;p__Lentisphaerae;c__Lentisphaerae_uncl	0.0014	0.0000
k__Bacteria;p__Chloroflexi;c__Dehalococcoidetes	0.0011	0.0000
k__Bacteria;p__Chrysiogenetes;c__Chrysiogenetes	0.0005	0.0000
k__Bacteria;p__Verrucomicrobia;c__Verrucomicrobiae	0.0003	0.0000
k__Bacteria;p__Aquificae;c__Aquificae	0.0000	0.0042
k__Bacteria;p__Bacteroidetes;c__Cytophagia	0.0000	0.0002
k__Bacteria;p__Deferribacteres;c__Deferribacteres	0.0000	0.0028
k__Bacteria;p__Dictyoglomi;c__Dictyoglomia	0.0000	0.0008
k__Bacteria;p__Firmicutes;c__Erysipelotrichi	0.0000	0.0037
k__Bacteria;p__Bacteroidetes;c__Flavobacteria	0.0000	0.0956
k__Bacteria;p__Fusobacteria;c__Fusobacteria	0.0000	0.2326
k__Bacteria;p__Tenericutes;c__Mollicutes	0.0000	0.1460
k__Bacteria;p__Nitrospirae;c__Nitrospira	0.0000	0.0009
k__Bacteria;p__Spirochaetes;c__Spirochaetes	0.0000	0.0257
k__Bacteria;p__Thermotogae;c__Thermotogae	0.0000	0.0101
k__Bacteria;p__WWE1;c__WWE1_uncl	0.0000	0.0001

Figure 5 represented putative population bins, which captured 68% of the entire assembly, 80% and 87% of all the sequenced reads in the AW and TW samples, respectively. In total, 15 population bins were identified representing three bacterial *phyla* (Proteobacteria, Firmicutes, and Actinobacteria) with extremely different abundances in the two samples: population bins 1–5 were more abundant in the sample related to the AW grape condition, while population bins 6–14 were more represented in the dataset deriving from the TW condition. Only the population bin 15 showed similar abundance in both the metagenomic datasets.

The number of conserved essential genes determined in the first stages of the binning process indicated a low level of completeness of the population bins 14 and 15 (lower than 20%). For this reason and for their small genome size they were not included in further analysis.

Genes annotated in genomes 1–13 were also used for BLASTn analyses against *nr* database reference genomes and sequence similarity values of 95, 85, and 75% were used for species, genus and phylum level taxonomic annotation, respectively (Nielsen et al., 2014). As some genomes could not be assigned to a genus or a species by DNA similarity, they were taxonomically annotated by similarity to the Uniprot database (BLASTp, best hit, $E < 0.001$). Considering these thresholds, it was possible to ascribe genomes 4, 5, and 8 at the species level, i.e., *Erwinia billingiae*, *Pantoea vagans*, and *Pseudomonas syringae*, respectively; genomes 1, 2, 6, 7, 9–11, and 13 at the genus level (1, 2, *Clostridium* spp.; 6, *Pantoea* spp.; 7, 9, 13, *Pseudomonas* spp.; 10, 11, *Paenibacillus* spp.); genomes 3 and 12 at the order level, i.e., Lactobacillales and Actinomycetales, respectively (**Table 3**).

The identified population bins represented a wide bacterial diversity, including species belonging to several families (Clostridiaceae, Enterobacteriaceae, Enterococcaceae, Microbacteriaceae, Paenibacillaceae, Pseudomonadaceae) which is also showed in the phylogenetic tree based on >400 proteins optimized from among 3,737 genomes (Segata et al., 2013; Figure S2).

Focusing on OTU classification of population bins, and their coverage in the two metagenomic datasets, the TW condition particularly promoted *Paenibacillus* spp., members of order Actinomycetales (more than 4,000 and ∼3,700 fold more abundant than the AW conditions, respectively), and *Pseudomonas* spp., while the AW condition favored Lactobacillales, *Clostridium* spp., and *Pantoea* spp. (∼4,000, ∼2,400, 740 fold more abundant than the TW condition, respectively; Table S3). The functional properties of the genome bins was investigated and the abundance of the COG categories in each genomes was reported in Table S4. In particular, the E and V categories mainly characterized *Clostridium* spp. and Lactobacillales for the AW sample and *Paenibacillus* spp. for the TW sample.

DISCUSSION

A number of studies have chiefly demonstrated that the microbial communities on grape surfaces play an important

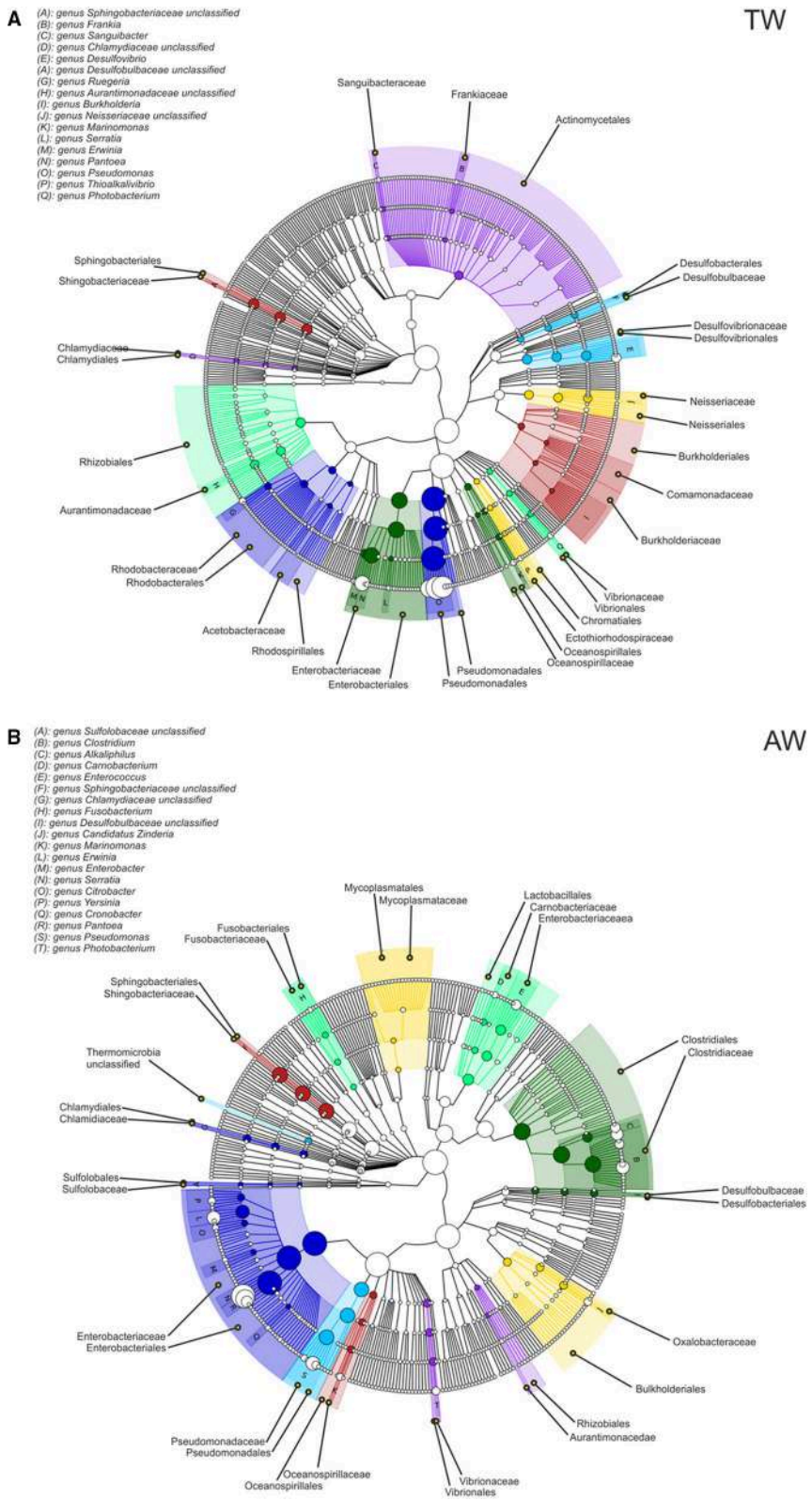

FIGURE 4 | Taxonomic cladogram reporting all clades present in the traditional (A-TW) and accelerated (B-AW) withered berry samples. Circle size is proportional to the log of the average abundance; colors represent microbial species comprised in the same taxonomic group.

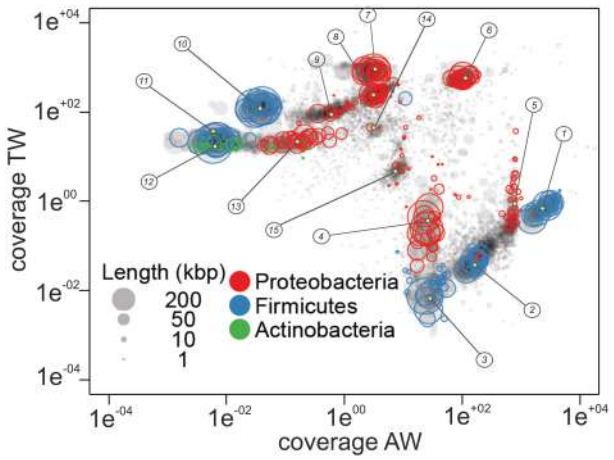

FIGURE 5 | Sequence composition-independent binning using metagenome coverage of the traditional (TW) and accelerated (AW) withered berry samples. The nodes represent scaffolds and the color of circles around nodes indicates the phylum. The circle size is proportional to the scaffold bp content. Numbered circles represent potential genome bins.

role in grape quality, yield, and in winemaking, contributing also to a regional terroir (Barata et al., 2012; Bokulich et al., 2014; Capozzi et al., 2015; Zarraonaindia et al., 2015). Indeed, the first population encountered by grape must prior to the fermentation can crucially affect the metabolic profile of wine and its quality, even when commercial starters are used (Bokulich et al., 2013). However, the composition of microbiota associated with withered berries prior to the onset of fermentation has not yet been investigated in details. WMS approach was used in this study, for the first time, to profile the microbial consortia populating the surface of cv. Corvina sound berries at the end of 2–3 months post-harvest withering process, and their diversity according to the different drying conditions of the *fruttaio*. We used only healthy undamaged berries both to avoid contamination by grapevine DNA and, especially, to have a picture of the microbial contaminants of withered berries without any "enrichment" due to the leakage of juice. It is crucial for Amarone winemakers to use healthy grape for the withering in order to avoid unwanted mold development. Indeed, the protocol applied to collect the microbiota of withered berry surface allowed to almost completely eliminate the presence of grapevine genetic material, maximizing the number of sequences useful for the analyses.

In addition, the WMS provided access to the functional gene composition of microbial communities, sequencing the majority of available genomes, and to the microbial phylogenetic profile among rare and abundant prokaryotic and eukaryotic sequence groups, avoiding the limitations associated to the PCR biases of the amplicon sequencing approach.

Considering functional analysis, only five major gene categories (E, G, K, U, and V) appeared differently abundant between the TW and AW berry communities: genes associated with defense mechanisms, and aminoacid metabolism and transport being relatively more abundant in the TW sample; transcription, carbohydrate metabolism, and transport, intracellular trafficking, secretion and vesicular transport in the AW sample. Such scarce variance in the overall composition of the COG functional classes suggests a high redundancy in the functional profiles characterizing the microbial communities in these two withering conditions, that may be more comparable than assumed from their taxonomic diversity and composition. Indeed, distinct taxa can share specific functional attributes and have similar physiologies and environmental tolerances (Fierer et al., 2012).

While limited differences in the distribution of genes and functional diversity were found in relation to the distinct conditions of withering process, greater evident differences were observed in the abundance of specific microbial groups in the two berry samples. In particular, this study revealed that the relative abundance of prokaryotic populations was considerably higher than that of eukaryotic populations in the berry microbiota.

Regarding the eukaryotic communities, the two most abundant genera in both samples were *Aspergillus* and *Penicillium*. The presence of such saprophytic filamentous fungi is likely due to their ability to rapidly colonize different grapevine tissues, including grape surfaces (Bokulich et al., 2013; Rousseaux et al., 2014). Indeed, spores of these molds are spread all over grapevine tissues and germinate when temperature and humidity are appropriate, especially when berries are injured (Barata et al., 2012). In addition, the withering in *fruttaio* easily exposes the grape to post-harvest contamination by airborne fungal spores. The substantial abundance of *Aspergillus* and *Penicillium* on withered grapes of cv. Garganega and Corvina has been previously reported using culture-dependent methods (Lorenzini et al., 2013). However, their incidence can be extremely variable, depending on seasonal conditions and withering techniques. The major presence of such fungal genera on the surfaces of berry collected from the TW sample was foreseeable, since it could be related to the longer permanence in *fruttaio* and the different storage conditions respect to the AW process.

An important genus associated with withered grape, i.e., *Botryotinia*, that includes *Botryotinia fuckeliana* (anamorph: *Botrytis cinerea*), was found at levels <10% of the Ascomycota fraction in both conditions. The great interest for this mold is due to its ambivalent nature: it is widely recognized as the causative agent of gray mold, that causes severe damage on grape, but also as "noble rot," used for processing some speciality wines (Fournier et al., 2013). Noble rot symptoms seem to depend essentially on microclimatic conditions (Blanco-Ulate et al., 2015), which has applicable consequences for the production of traditional botrytized sweet wines, like Souternes, Tokaji Aszù, and Auslese (Magyar, 2011). The effects of noble rot on the overall quality of passito red wine, like Amarone, have been less investigated. Nevertheless, it was shown that whitered Garganega and Corvina berries naturally or artificially infected with *B. cinerea* produced wines with distinctive organoleptic properties (Tosi et al., 2012; Azzolini et al., 2013; Lorenzini et al., 2013). In addition, Lorenzini et al. (2013) demonstrated that some *Penicillum* species are able to grow under withering

TABLE 3 | Assembly information of the 13 extracted genome bins.

Metagenomic dataset	Figure ID[a]	Phylogenetic affiliation	No. scaffolds	Total length (bp)	GC (%)	No. essential genes	Completeness (%)[b]	Coverage[b]	BlastP (%)[c]	BlastN (%)[c]
AW	1	*Clostridium* sp. UNIVR01 strain	112	4,456,330	30.9	105/105	100	2521.4	81.5	66.4
AW	2	*Clostridium* sp. UNIVR02 strain	103	5,268,949	29.1	105/105	100	2521.4	89.3	86.5
AW	3	Lactobacillales sp. UNIVR03 strain	270	3,864,387	42.3	72/105	69	645,474.2	83.3	62.1
AW	4	*Erwinia billingiae* UNIVR04 strain	173	4,663,534	54.7	80/105	76	137.2	99.4	98.1
AW	5	*Pantoea vagans* UNIVR05 strain	189	4,663,534	55	40/105	38	1448.1	99.2	97.2
TW	6	*Pantoea* sp. UNIVR06 strain	172	4,234,112	55.4	89/105	85	8.6	93.9	87.8
TW	7	*Pseudomonas* sp. UNIVR07 strain	233	8,444,622	60.6	66/105	63	630.3	93.1	88.1
TW	8	*Pseudomonas syringae* UNIVR08 strain	177	4,540,557	59.2	68/105	65	97	98.6	97.1
TW	9	*Pseudomonas* sp. UNIVR09 strain	224	4,145,951	59.2	14/105	13	274.4	94.1	90.05
TW	10	*Paenibacillus* sp. UNIVR10 strain	57	5,684,618	40.9	68/105	65	5042.8	81.7	86.5
TW	11	*Paenibacillus* sp. UNIVR11 strain	70	6,879,406	45.9	104/105	99	5042.8	84	63.8
TW	12	Actinomycetales sp. UNIVR12 strain	246	3,863,727	63.9	80/105	76	4390	83.2	75.7
TW	13	*Pseudomonas* sp. UNIVR13 strain	96	3,898,344	60.4	75/105	71	207.9	92.4	89.1

The number of the essential genes was estimated using 105 HMM models protein coding essential single copy genes conserved in 95% of all bacteria (Dupont et al., 2012).
*[a]Figure ID correspond to the number in **Figure 5**.*
[b]Genome bins completeness and coverage were calculated as described in the Section Materials and Methods.
[c]BlastP and BlastN similarity values based on essential genes.

conditions and have a synergic effect with *B. cinerea* on berry dehydration in simultaneous infection trials. Of particular note is also the observed antagonistic activity of *B. cinerea* vs. ocratoxin A (OTA)-producing *Aspergillus* and its capability to degrade this mycotoxin that may explain the low levels of OTA in noble wines (Valero et al., 2008). In this study, the environmental conditioning of the *fruttaio* was settled up to reproduce the traditional characteristics provided by natural drying, but assuring better control using a Natural Super Assisted Drying system, named NASA, that bring the attic to the more suitable withering conditions (Paronetto and Dellaglio, 2011). Under these conditions, the noble rot infection has been reported to occur in a limited part of the berries (Tosi et al., 2012). The detection of the genus *Botryotinia* in the two berry samples provides a steady indication of its involvement in withering process.

The other main classes characterizing the eukaryotic communities were represented by Sordariomycetes and Dothideomycetes which contain putative plant pathogens, such as *Gibberella*, *Pyrenophora*, and *Phaeosphaeria* spp. (Penton et al., 2014). However, as noted by Taylor et al. (2014), the presence of DNA from these genera does not necessarily mean that the grapes or plants had an infection, but provide an indication of potential disease load.

Microorganisms belonging to the order Saccharomycetales, that includes *Saccharomyces* and non-*Saccharomyces* yeasts of primary relevance for the wine fermentation process, represented a minority of the Ascomycota fraction of the samples. However, the identification of the genus *Saccharomyces* on the withered berry surfaces is interesting, especially because no enrichment steps were applied to collect the microbial community. Indeed, the detection of *Saccharomyces* species, especially *S. cerevisiae*, from sound berries has been reported very rarely, and only after the application of enrichment techniques (Cordero-Bueso et al., 2011; Barata et al., 2012). Although the frequency of occurrence of these yeasts increase up to 25% in heavily damaged berries, where grape juice became accessible to the yeasts through the skin lesions (Mortimer and Polsinelli, 1999), their origin are still poorly understood. In addition to grape, winery surfaces have been reported to harbor large population of *Saccharomyces*, potentially serving as vector of these yeasts in wine fermentations (Bokulich et al., 2013); however, to date no investigations have been carried out to monitor the microbial communities of the *fruttaio* environment. In addition, insects, such as bees, wasps, and *Drosophila*, as well as birds, can facilitate their dispersal on vineyard and winery environments (Francesca et al., 2012; Stefanini et al., 2012; Lam and Howell, 2015). According to Lynch and Neufeld (2015), *Saccharomyces* and other technological species, which are detected as rare viable or dormant microbial taxa in certain samples, can be defined as "conditionally rare taxa," since their abundance increases when the environmental conditions change (i.e., during fermentation).

Regarding the prokaryotic population, we found the predominance of environmental ubiquitous microorganisms, i.e., Gammaproteobacteria (Pseudomonadales and Enterobacteriales), Clostridia, Sphingobacteria, and Bacilli, rather than bacteria usually associated to wine microbial consortia. Recent ecological studies using 16S-based high-throughput sequencing techniques detected several of these taxonomic groups on fresh grape samples of cv. Grenache and Carignan (Portillo et al., 2015), in musts of Chardonnay and Cabernet Sauvignon (Bokulich et al., 2014), and also during botrytized wine fermentations (Bokulich et al., 2012). The question of whether these bacteria are truly metabolically active in wine and capable of affecting the sensory quality has been raised (Bokulich et al., 2012) but it has not been studied in depth yet. The main source of such microorganisms is likely the grapevine phyllosphere, since members belonging to the above

classes represent the usual microbiota linked to grapes, leaves, flowers, and soil of *V. vinifera* (Martins et al., 2013; Gilbert et al., 2014; Pinto et al., 2014; Rolli et al., 2015). In addition, harvest, transfer and storage of grape represent all processing stages for further contaminations (Bokulich et al., 2013), and especially the *fruttaio* habitat could be an important reservoir of environmental microbial species for withered berries.

Interestingly, the conditions of the drying process strongly influenced the relative abundance of members of the families Enterobacteriaceae and Pseudomonadaceae; indeed, the faster grape drying favored the genera *Pantoea* and *Erwinia*, while *Pseudomonas* was more abundant in the traditional drying sample. These differences revealed by shotgun reads analysis were further confirmed by the assembly and binning processes, which allowed the assignment of three genome bins to the species level: *P. vagans*, *E. billingiae,* and *P. syringae*. Species of *Pseudomonas* and *Erwinia* were recently found on grapevine leaves of the *V. vinifera* cv. Pinot gris, and were considered to represent the phyllosphere core bacterial community (Perazzolli et al., 2014). As reported in several studies, *Pseudomonas* taxa are characterized by some positive physiological features, like the capability to produce exopolysaccharides and antifungal compounds, that can contribute to the maintenance and protection of the microbial communities present on berry surfaces (Trotel-Aziz et al., 2008; Verhagen et al., 2010; Martins et al., 2012). Also the genus *Pantoea* has often been described within the grapevine microbiome and has been proposed as bacterial antagonist with biocontrol ability (Trotel-Aziz et al., 2008; Bulgari et al., 2009). In particular, the plant-associated non-pathogenic *E. billingiae* and *P. vagans* are able to compete with different plant pathogens, e.g., *Erwinia amylovora* (Kube et al., 2010; Smits et al., 2010). *P. syringae* is a common foliar bacterium that can be responsible of extensive yield losses in wine-grape production (Hall et al., 2015); however, strains of this species were also found as harmless commensals on leaf surfaces, and their capability to produce the surfactant syringomycin can improve their adaptation to phyllosphere habitat (Whipps et al., 2008). Competition for space and nutrients, production of hydrolytic enzymes, inhibition of pathogen-produced enzymes or toxins, and, in general, direct and indirect interactions between microorganisms resident on berry surface can surely affect biodiversity, favoring the survival of certain microbial species, but actually the involved factors have not yet been clearly identified.

Lactobacillales is the most important bacterial order in wine fermentation, being involved both in spoilage and malolactic activity (Bokulich et al., 2012). However, it was detected as a minor bacterial taxon (1.3%) of the AW sample and comprised only the genera *Enterococcus* and *Carnobacterium*. Enterococci are environmental ubiquitous bacteria which have been isolated, although not frequently, from the surface of grape berries at harvest (Renouf et al., 2005), and wine undergoing malolactic fermentation (Barata et al., 2012; Pérez-Martín et al., 2014). Capozzi et al. (2011) has proposed that the origin of these bacteria are the grapes, the winery equipment or practices. Conversely, the genus *Carnobacterium* was not usually associated with grape and the winemaking process, although it has been recently found at low level in Portuguese wines (Pinto et al., 2015), and strains of *Carnobacterium viridans* and *Carnobacterium inhibens* were isolated and identified from wine wooden vats (Fracchetti et al., 2015). The role of the genera *Enterococcus* and *Carnobacterium* in grape and wine is, until now, unknown perhaps as a consequence of their scarceness.

Most of the putative genomes were assigned to the genus level (*Clostridium*, *Paenibacillus*, *Pantoea*, and *Pseudomonas*), but not to the species level, likely due to the challenging in the assignment of the 16S rRNA gene to the correct genome. For this reason, the sequence of the 16S rRNA gene was not used as a phylogenetic marker, but the taxonomic assignment was entirely based on previously identified phylogenetic marker genes, either clade-specific or universal, and rarely subject to horizontal gene transfer (Dupont et al., 2012; Albertsen et al., 2013; Segata et al., 2013). Otherwise, these genome bins could represent new taxa for which the *Candidatus* provisional status may be proposed (Konstantinidis and Rosselló-Móra, 2015). A modification of the current binning strategy assisting the assignment of the 16S rRNA sequences to the genomes is probably needed. This can improve the reliability of the taxonomic assignment by taking advantage of the high number of 16S rRNA sequences present in public databases and extending the potential of the single-copy marker genes that, however, it was proven to be very good (Mende et al., 2013; Sunagawa et al., 2013). Despite this, it has to be considered that the binning approach can provide fundamental insights into physiological potential of the species identified, while the 16S rRNA analysis can only be used for the taxonomical analysis. The accessibility of these near-complete genomes could also provide valuable information about the nutritional requirements of these microorganisms in order to define a proper cultivation medium for their isolation. The availability of isolates could be useful for mainly two reasons: to evaluate whether the same strain is persistent over different vintages (contribution to the microbial component of the *terroir*) and to perform experiments of controlled inoculum on berries. This could give an important insight on the relationship between microbial component and grape metabolites produced during berry withering.

In conclusion, data presented here provide new insights into the complex microbial consortium of withered sound grape of cv. Corvina, indicating that the core microbiota associated with berry surfaces at the end of withering is mainly constituted by environmental rather than microorganisms relevant for wine production. However, "conditionally rare taxa," like *Saccharomyces*, were also detected. Interestingly, withering conditions had a strong influence on the taxonomic composition and abundance of grape microbiota, but the abundance of the functional classes did not undergo a profound modification. It could be guessed that the different abiotic factors (e.g., temperature, humidity, ventilation) applied during withering have determined more subtle damaging effects in the berries of the AW batch, leading to a release of nutrients. This in turn may impact the microbiota present on the damaged berry surface, causing a higher diversity and favoring some fermentative populations. Such microorganisms could be spread or carried by wind generated by the

fan to adjacent healthy berries, including those of the AW sample.

Further studies have to be performed to determine whether the modification of the microbial communities on grape surfaces withered under diverse conditions could lead to significant chemical variations of Corvina berry metabolites, thus influencing the final wine characteristics and sensory attributes. In this way, WMS could open novel perspectives in the knowledge and management of traditional processes, such as the withering process of Corvina grape, with an impact on the winemaking of important Italian wines.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: ES, ST, FF, GT, GF. Performed the experiments: ES, SC, AG, FF. Generated and analyzed the data: ES, SC, AG, IC, GT, GF. Wrote the paper: ES, SC, IC, ST, GF.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2016.00937

REFERENCES

Albertsen, M., Hugenholtz, P., Skarshewski, A., Nielsen, K. L., Tyson, G. W., and Nielsen, P. H. (2013). Genome sequences of rare, uncultured bacteria obtained by differential coverage binning of multiple metagenomes. *Nat. Biotechnol.* 31, 533–538. doi: 10.1038/nbt.2579

Andreolli, M., Lampis, S., Zapparoli, G., Angelini, E., and Vallini, G. (2016). Diversity of bacterial endophytes in 3 and 15 year-old grapevines of *Vitis vinifera* cv. Corvina and their potential for plant growth promotion and phytopathogen control. *Microbiol. Res.* 183, 42–52. doi: 10.1016/j.micres.2015.11.009

Azzolini, M., Tosi, E., Faccio, S., Lorenzini, M., Torriani, S., and Zapparoli, G. (2013). Selection of *Botrytis cinerea* and *Saccharomyces cerevisiae* strains for the improvement and valorization of Italian passito style wines. *FEMS Yeast Res.* 13, 540–552. doi: 10.1111/1567-1364.12054

Barata, A., González, S., Malfeito-Ferreira, M., Querol, A., and Loureiro, V. (2008). Sour rot-damaged grapes are sources of wine spoilage yeasts. *FEMS Yeast Res.* 8, 1008–1017. doi: 10.1111/j.1567-1364.2008.00399.x

Barata, A., Malfeito-Ferreira, M., and Loureiro, V. (2012). The microbial ecology of wine grape berries. *Int. J. Food Microbiol.* 153, 243–259. doi: 10.1016/j.ijfoodmicro.2011.11.025

Barata, A., Pais, A., Malfeito-Ferreira, M., and Loureiro, V. (2011). Influence of sour rotten grapes on the chemical composition and quality of grape must and wine. *Eur. Food Res. Technol.* 233, 183–184. doi: 10.1007/s00217-011-1505-x

Barbe, J. C., De Revel, G., Joyeux, A., Bertrand, A., and Lonvaud-Funel, A. (2001). Role of botrytized grape micro-organisms in SO2 binding phenomena. *J. Appl. Microbiol.* 90, 34–42. doi: 10.1046/j.1365-2672.2001.01200.x

Bartowsky, E. J., and Henschke, P. A. (2008). Acetic acid bacteria spoilage of bottled red wine - a review. *Int. J. Food Microbiol.* 125, 60–70. doi: 10.1016/j.ijfoodmicro.2007.10.016

Blanco-Ulate, B., Amrine, K. C. H., Collins, T. S., Rivero, R. M., Vicente, A. R., Morales-Cruz, A., et al. (2015). Developmental and metabolic plasticity of white-skinned grape berries in response to *Botrytis cinerea* during noble rot. *Plant Physiol.* 169, 2422–2443. doi: 10.1104/pp.15.00852

Bokulich, N. A., Joseph, C. M., Allen, G., Benson, A. K., and Mills, D. A. (2012). Next-generation sequencing reveals significant bacterial diversity of botrytized wine. *PLoS ONE* 7:e36357. doi: 10.1371/journal.pone.0036357

Bokulich, N. A., Ohta, M., Richardson, P. M., and Mills, D. A. (2013). Monitoring seasonal changes in winery-resident microbiota. *PLoS ONE* 8:e66437. doi: 10.1371/journal.pone.0066437

Bokulich, N. A., Thorngate, J. H., Richardson, P. M., and Mills, D. A. (2014). Microbial biogeography of wine grapes is conditioned by cultivar, vintage, and climate. *Proc. Natl. Acad. Sci. U.S.A.* 111, 139–148. doi: 10.1073/pnas.1317377110

Bolger, A. M., Lohse, M., and Usadel, B. (2014). Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* 30, 2114–2120. doi: 10.1093/bioinformatics/btu170

Bulgari, D., Casati, P., Brusetti, L., Quaglino, F., Brasca, M., Daffonchio, D., et al. (2009). Endophytic bacterial diversity in grapevine (*Vitis vinifera* L.) leaves described by 16S rRNA gene sequence analysis and length heterogeneity-PCR. *J. Microbiol.* 47, 393–401. doi: 10.1007/s12275-009-0082-1

Čadež, N., Zupan, J., and Raspor, P. (2010). The effect of fungicides on yeast communities associated with grape berries. *FEMS Yeast Res.* 10, 619–630. doi: 10.1111/j.1567-1364.2010.00635.x

Campanaro, S., Treu, L., Kougias, P. G., De Francisci, D., Valle, G., and Angelidaki, I. (2016). Metagenomic analysis and functional characterization of the biogas microbiome using high throughput shotgun sequencing and a novel binning strategy. *Biotechnol. Biofuels* 9, 1–17. doi: 10.1186/s13068-016-0441-1

Capozzi, V., Garofalo, C., Chiriatti, M. A., Grieco, F., and Spano, G. (2015). Microbial terroir and food innovation: the case of yeast biodiversity in wine. *Microbiol. Res.* 181, 75–83. doi: 10.1016/j.micres.2015.10.005

Capozzi, V., Ladero, V., Beneduce, L., Fernández, M., Alvarez, M. A., Benoit, B., et al. (2011). Isolation and characterization of tyramine-producing *Enterococcus faecium* strains from red wine. *Food Microbiol.* 28, 434–439. doi: 10.1016/j.fm.2010.10.005

Consonni, R., Cagliani, L. R., Guantieri, V., and Simonato, B. (2011). Identification of metabolic content of selected Amarone wine. *Food Chem.* 129, 693–699. doi: 10.1016/j.foodchem.2011.05.008

Cordero-Bueso, G., Arroyo, T., Serrano, A., Tello, J., Aporta, I., Vélez, M. D., et al. (2011). Influence of the farming system and vine variety on yeast communities associated with grape berries. *Int. J. Food Microbiol.* 145, 132–139. doi: 10.1016/j.ijfoodmicro.2010.11.040

Cox, M. P., Peterson, D. A., and Biggs, P. J. (2010). SolexaQA: At-a-glance quality assessment of Illumina second-generation sequencing data. *BMC Bioinformatics* 11:485. doi: 10.1186/1471-2105-11-485

Dal Santo, S., Tornielli, G. B., Zenoni, S., Fasoli, M., Farina, L., Anesi, A., et al. (2013). The plasticity of the grapevine berry transcriptome. *Genome Biol.* 14, 1–18. doi: 10.1186/gb-2013-14-6-r54

David, V. D., Terrat, S., Herzine, K., Claisse, O., Rousseaux, S., Tourdot-Maréchal, R., et al. (2014). High-throughput sequencing of amplicons for monitoring yeast biodiversity in must and during alcoholic fermentation. *J. Ind. Microbiol. Biotechnol.* 41, 811–821. doi: 10.1007/s10295-014-1427-2

Di Carli, M., Zamboni, A., Pè, M. E., Pezzotti, M., Lilley, K. S., Benvenuto, E., et al. (2011). Two-dimensional differential in gel electrophoresis (2D-DIGE) analysis of grape berry proteome during postharvest withering. *J. Proteome Res.* 10, 429–446. doi: 10.1021/pr1005313

Dupont, C. L., Rusch, D. B., Yooseph, S., Lombardo, M. J., Richter, R. A., Valas, R., et al. (2012). Genomic insights to SAR86, an abundant and uncultivated marine bacterial lineage. *ISME J.* 6, 1186–1199. doi: 10.1038/ismej.2011.189

Fierer, N., Leff, J. W., Adams, B. J., Nielsen, U. N., Bates, S. T., Lauber, C. L., et al. (2012). Cross-biome metagenomic analyses of soil microbial communities and

their functional attributes. *Proc. Natl. Acad. Sci. U.S.A.* 109, 21390–21395. doi: 10.1073/pnas.1215210110

Finn, R. D., Coggill, P., Eberhardt, R. Y., Eddy, S. R., Mistry, J., Mitchell, A. L., et al. (2016). The Pfam protein families database: towards a more sustainable future. *Nucleic Acids Res.* 44, D279–D285. doi: 10.1093/nar/gkv1344

Fleet, G. H. (2003). Yeast interactions and wine flavour. *Int. J. Food Microbiol.* 86, 11–22. doi: 10.1016/S0168-1605(03)00245-9

Fleet, G. H., Prakitchaiwattana, C., Heard, G., and Ciani, M. (2002). "The yeast ecology of wine grapes," in *Biodiversity and Biotechnology of Wine Yeasts*, ed M. Ciani (Trivandrum: Research Signpost), 1–17.

Fournier, E., Gladieux, P., and Giraud, T. (2013). The 'Dr Jekyll and Mr Hyde fungus': noble rot versus gray mold symptoms of *Botrytis cinerea* on grapes. *Evol. Appl.* 6, 960–969. doi: 10.1111/eva.12079

Fracchetti, F., Tebaldi, M., Felis, G. E., Del Casale, A., and Torriani, S. (2015). Ozone in the winery to control the microbial contaminants of barrels. *Industria Bevande* 260, 7–14.

Francesca, N., Canale, D. E., Settanni, L., and Moschetti, G. (2012). Dissemination of wine-related yeasts by migratory birds. *Environ. Microbiol. Rep.* 4, 105–112. doi: 10.1111/j.1758-2229.2011.00310.x

Galperin, M. Y., Makarova, K. S., Wolf, Y. I., and Koonin, E. V. (2015). Expanded microbial genome coverage and improved protein family annotation in the COG database. *Nucleic Acids Res.* 43, D261–D269. doi: 10.1093/nar/gku1223

Gilbert, J. A., van der Lelie, D., and Zarraonaindia, I. (2014). Microbial terroir for wine grapes. *Proc. Natl. Acad. Sci. U.S.A.* 111, 5–6. doi: 10.1073/pnas.1320471110

Gomez-Alvarez, V., Teal, T. K., and Schmidt, T. M. (2009). Systematic artifacts in metagenomes from complex microbial communities. *ISME J.* 3, 1314–1317. doi: 10.1038/ismej.2009.72

Guzzon, R., Franciosi, E., and Larcher, R. (2014). A new resource from traditional wines: characterisation of the microbiota of "Vino Santo" grapes as a biocontrol agent against *Botrytis cinerea*. *Eur. Food Res. Technol.* 239, 117–126. doi: 10.1007/s00217-014-2195-y

Hall, S. J., Dry, I. B., and Whitelaw-Weckert, M. A. (2015). Phylogenetic relationships of *Pseudomonas syringae* pv. syringae isolates associated with bacterial inflorescence rot in grapevine. *Plant Dis.* 100, 607–616. doi: 10.1094/PDIS-07-15-0806-RE

Hyatt, D., Locascio, P. F., Hauser, L. J., and Uberbacher, E. C. (2012). Gene and translation initiation site prediction in metagenomic sequences. *Bioinformatics* 28, 2223–2230. doi: 10.1093/bioinformatics/bts429

Jaillon, O., Aury, J. M., Noel, B., Policriti, A., Clepet, C., Casagrande, A., et al. (2007). The grapevine genome sequence suggests ancestral hexaploidization in major angiosperm phyla. *Nature* 449, 463–467. doi: 10.1038/nature06148

Konstantinidis, K. T., and Rosselló-Móra, R. (2015). Classifying the uncultivated microbial majority: a place for metagenomic data in the *Candidatus* proposal. *Syst. Appl. Microbiol.* 38, 223–230. doi: 10.1016/j.syapm.2015.01.001

Kube, M., Migdoll, A. M., Gehring, I., Heitmann, K., Mayer, Y., Kuhl, H., et al. (2010). Genome comparison of the epiphytic bacteria *Erwinia billingiae* and *E. tasmaniensis* with the pear pathogen *E. pyrifoliae*. *BMC Genomics* 11:393. doi: 10.1186/1471-2164-11-393

Ladoukakis, E., Kolisis, F. N., and Chatziioannou, A. A. (2014). Integrative workflows for metagenomic analysis. *Front. Cell Dev. Biol.* 2:70. doi: 10.3389/fcell.2014.00070

Lam, S. S., and Howell, K. S. (2015). Drosophila-associated yeast species in vineyard ecosystems. *FEMS Microbiol. Lett.* 362:fnv170. doi: 10.1093/femsle/fnv170

Langmead, B., and Salzberg, S. L. (2012). Fast gapped-read alignment with Bowtie 2. *Nat. Methods* 9, 357–359. doi: 10.1038/nmeth.1923

Langmead, B., Trapnell, C., Pop, M., and Salzberg, S. L. (2009). Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. *Genome Biol.* 10:R25. doi: 10.1186/gb-2009-10-3-r25

Leveau, J. H. J., and Tech, J. J. (2011). Grapevine microbiomics: Bacterial diversity on grape leaves and berries revealed by high-throughput sequence analysis of 16S rRNA amplicons. *Acta Hortic.* 905, 31–42. doi: 10.17660/ActaHortic.2011.905.2

Liu, Y., Rousseaux, S., Tourdot-Maréchal, R., Sadoudi, M., Gougeon, R., Schmitt-Kopplin, P., et al. (2015). Wine microbiome, a dynamic world of microbial interactions. *Crit. Rev. Food Sci. Nutr.* doi: 10.1080/10408398.2014.983591. [Epub ahead of print].

Lorenzini, M., Azzolini, M., Tosi, E., and Zapparoli, G. (2013). Postharvest grape infection of *Botrytis cinerea* and its interactions with other moulds under withering conditions to produce noble-rotten grapes. *J. Appl. Microbiol.* 114, 762–770. doi: 10.1111/jam.12075

Lynch, M. D., and Neufeld, J. D. (2015). Ecology and exploration of the rare biosphere. *Nat. Rev. Microbiol.* 13, 217–229. doi: 10.1038/nrmicro3400

Magyar, I. (2011). Botrytized wines. *Adv. Food Nutr. Res.* 63, 147–206. doi: 10.1016/B978-0-12-384927-4.00006-3

Magyar, I., and Bene, Z. (2006). Morphological and taxonomic study on mycobiota of noble rotted grapes in the Tokaj wine district. *Acta Alimentaria* 35, 237–246. doi: 10.1556/AAlim.35.2006.2.11

Marchler-Bauer, A., Derbyshire, M. K., Gonzales, N. R., Lu, S., Chitsaz, F., Geer, L. Y., et al. (2015). CDD: NCBI's conserved domain database. *Nucleic Acids Res.* 43, D222–D226. doi: 10.1093/nar/gku1221

Martins, G., Lauga, B., Miot-Sertier, C., Mercier, A., Lonvaud, A., Soulas, M. L., et al. (2013). Characterization of epiphytic bacterial communities from grapes, leaves, bark and soil of grapevine plants grown, and their relations. *PLoS ONE* 8:e73013. doi: 10.1371/journal.pone.0073013

Martins, G., Miot-Sertier, C., Lauga, B., Claisse, O., Lonvaud-Funel, A., Soulas, G., et al. (2012). Grape berry bacterial microbiota: impact of the ripening process and the farming system. *Int. J. Food Microbiol.* 158, 93–100. doi: 10.1016/j.ijfoodmicro.2012.06.013

Martins, G., Vallance, J., Mercier, A., Albertin, W., Stamatopoulos, P., Rey, P., et al. (2014). Influence of the farming system on the epiphytic yeasts and yeast-like fungi colonizing grape berries during the ripening process. *Int. J. Food Microbiol.* 77, 21–28. doi: 10.1016/j.ijfoodmicro.2014.02.002

Mende, D. R., Sunagawa, S., Zeller, G., and Bork, P. (2013). Accurate and universal delineation of prokaryotic species. *Nat Methods* 10, 881–884. doi: 10.1038/nmeth.2575

Meyer, F., Paarmann, D., D'Souza, M., Olson, R., Glass, E. M., Kubal, M., et al. (2008). The metagenomics RAST server - a public resource for the automatic phylogenetic and functional analysis of metagenomes. *BMC Bioinformatics* 9:386. doi: 10.1186/1471-2105-9-386

Mills, D. A., Phister, T., Neeley, E. T., and Johannsen, E. A. (2007). "Wine Fermentations," in *Molecular Methods and Microbial Ecology of Fermented Foods*, ed L. Cocolin and D. Ercolini (New York, NY: Elsevier), 162–192.

Moriya, Y., Itoh, M., Okuda, S., Yoshizawa, A., and Kanehisa, M. (2007). KAAS: an automatic genome annotation and pathway reconstruction server. *Nucleic Acids Res.* 35, W182–W185. doi: 10.1093/nar/gkm321

Mortimer, R., and Polsinelli, M. (1999). On the origins of wine yeast. *Res. Microbiol.* 150, 199–204. doi: 10.1016/S0923-2508(99)80036-9

Namiki, T., Hachiya, T., Tanaka, H., and Sakakibara, Y. (2012). MetaVelvet: an extension of velvet assembler to *de novo* metagenome assembly from short sequence reads. *Nucleic Acids Res.* 40, 1–12. doi: 10.1093/nar/gks678

Nielsen, H. B., Almeida, M., Juncker, A. S., Rasmussen, S., Li, J., Sunagawa, S., et al. (2014). Identification and assembly of genomes and genetic elements in complex metagenomic samples without using reference genomes. *Nat. Biotechnol.* 32, 822–828. doi: 10.1038/nbt.2939

Nisiotou, A. A., and Nychas, G. J. (2007). Yeast populations residing on healthy or *botrytis*-infected grapes from a vineyard in Attica, Greece. *Appl. Environ. Microbiol.* 73, 2765–2768. doi: 10.1128/AEM.01864-06

Nisiotou, A. A., Rantsiou, K., Iliopoulos, V., Cocolin, L., and Nychas, G. J. (2011). Bacterial species associated with sound and *Botrytis*-infected grapes from a Greek vineyard. *Int. J. Food Microbiol.* 145, 432–436. doi: 10.1016/j.ijfoodmicro.2011.01.017

Overbeek, R., Olson, R., Pusch, G. D., Olsen, G. J., Davis, J. J., Disz, T., et al. (2014). The SEED and the Rapid Annotation of microbial genomes using Subsystems Technology (RAST). *Nucleic Acids Res.* 42, D206–D214. doi: 10.1093/nar/gkt1226

Paronetto, L., and Dellaglio, F. (2011). Amarone: a modern wine coming from an ancient production technology. *Adv. Food Nutr. Res.* 63, 285–306. doi: 10.1016/B978-0-12-384927-4.00009-9

Penton, C. R., Gupta, V. V., Tiedje, J. M., Neate, S. M., Ophel-Keller, K., Gillings, M., et al. (2014). Fungal community structure in disease suppressive soils assessed by 28S LSU gene sequencing. *PLoS ONE* 9:e93893. doi: 10.1371/journal.pone.0093893

Perazzolli, M., Antonielli, L., Storari, M., Puopolo, G., Pancher, M., Giovannini, O., et al. (2014). Resilience of the natural phyllosphere microbiota of the grapevine to chemical and biological pesticides. *Appl. Environ. Microbiol.* 80, 3585–3596. doi: 10.1128/AEM.00415-14

Pérez-Martín, F., Seseña, S., Fernández-González, M., Arévalo, M., and Palop, M. L. (2014). Microbial communities in air and wine of a winery at two consecutive vintages. *Int. J. Food Microbiol.* 190, 44–53. doi: 10.1016/j.ijfoodmicro.2014.08.020

Piao, H., Hawley, E., Kopf, S., DeScenzo, R., Sealock, S., Henick-Kling, T., et al. (2015). Insights into the bacterial community and its temporal succession during the fermentation of wine grapes. *Front Microbiol.* 6:809. doi: 10.3389/fmicb.2015.00809

Pinto, C., Pinho, D., Cardoso, R., Custódio, V., Fernandes, J., Sousa, S., et al. (2015). Wine fermentation microbiome: a landscape from different Portuguese wine appellations. *Front Microbiol.* 6:905. doi: 10.3389/fmicb.2015.00905

Pinto, C., Pinho, D., Sousa, S., Pinheiro, M., Egas, C., and Gomes, A. C. (2014). Unravelling the diversity of grapevine microbiome. *PLoS ONE* 9:e85622. doi: 10.1371/journal.pone.0085622

Portillo, M. D., Franquès, J., Araque, I., Reguant, C., and Bordons, A. (2015). Bacterial diversity of Grenache and Carignan grape surface from different vineyards at Priorat wine region (Catalonia, Spain). *Int. J. Food Microbiol.* 219, 56–63. doi: 10.1016/j.ijfoodmicro.2015.12.002

Powell, S., Forslund, K., Szklarczyk, D., Trachana, K., Roth, A., Huerta-Cepas, J., et al. (2013). eggNOG v4.0: nested orthology inference across 3686 organisms. *Nucleic Acids Res.* 42, D231–D239. doi: 10.1093/nar/gkt1253

Prakitchaiwattana, C. J., Fleet, G. H., and Heard, G. M. (2004). Application and evaluation of denaturing gradient gel electrophoresis to analyse the yeast ecology of wine grapes. *FEMS Yeast Res.* 4, 865–877. doi: 10.1016/j.femsyr.2004.05.004

Pretorius, I. S. (2000). Tailoring wine yeast for the new millennium: novel approaches to the ancient art of winemaking. *Yeast* 16, 675–729. doi: 10.1002/1097-0061(20000615)16:8<675::AID-YEA585>3.0.CO;2-B

Quinlan, A. R., and Hall, I. M. (2010). BEDTools: a flexible suite of utilities for comparing genomic features. *Bioinformatics* 26, 841–842. doi: 10.1093/bioinformatics/btq033

Rantsiou, K., Campolongo, S., Alessandria, V., Rolle, R., Torchio, F., and Cocolin, L. (2013). Yeast populations associated with grapes during withering and their fate during alcoholic fermentation of high-sugar must. *Aust. J. Grape Wine Res.* 19, 40–46. doi: 10.1111/ajgw.12000

Renouf, V., Claisse, O., and Lonvaud-Funel, A. (2005). Understanding the microbial ecosystem on the grape berry surface through numeration and identification of yeast and bacteria. *Aust. J. Grape Wine Res.* 11, 316–327. doi: 10.1111/j.1755-0238.2005.tb00031.x

Rolli, E., Marasco, R., Vigani, G., Ettoumi, B., Mapelli, F., Deangelis, M. L., et al. (2015). Improved plant resistance to drought is promoted by the root-associated microbiome as a water stress-dependent trait. *Environ. Microbiol.* 17, 316–331. doi: 10.1111/1462-2920.12439

Rousseaux, S., Diguta, C. F., Radoï-Matei, F., Alexandre, H., and Guilloux-Bénatier, M. (2014). Non-*Botrytis* grape-rotting fungi responsible for earthy and moldy off-flavors and mycotoxins. *Food Microbiol.* 38, 104–121. doi: 10.1016/j.fm.2013.08.013

Segata, N., Börnigen, D., Morgan, X. C., and Huttenhower, C. (2013). PhyloPhlAn is a new method for improved phylogenetic and taxonomic placement of microbes. *Nat. Commun.* 4, 1–11. doi: 10.1038/ncomms3304

Segata, N., Waldron, L., Ballarini, A., Narasimhan, V., Jousson, O., and Huttenhower, C. (2012). Metagenomic microbial community profiling using unique clade-specific marker genes. *Nat. Methods* 9, 811–814. doi: 10.1038/nmeth.2066

Setati, M. E., Jacobson, D., Andong, U. C., and Bauer, F. F. (2012). The vineyard yeast microbiome, a mixed model microbial map. *PLoS ONE* 7:e52609. doi: 10.1371/journal.pone.0052609

Sharpton, T. J. (2014). An introduction to the analysis of shotgun metagenomic data. *Front Plant Sci.* 5:209. doi: 10.3389/fpls.2014.00209

Smits, T. H., Rezzonico, F., Kamber, T., Goesmann, A., Ishimaru, C. A., Stockwell, V. O., et al. (2010). Genome sequence of the biocontrol agent *Pantoea vagans* strain C9-1. *J. Bacteriol.* 192, 6486–6487. doi: 10.1128/JB.01122-10

Stefanini, I., Dapporto, L., Legras, J. L., Calabretta, A., Di Paola, M., De Filippo, C., et al. (2012). Role of social wasps in *Saccharomyces cerevisiae* ecology and evolution. *Proc. Natl. Acad. Sci. U.S.A.* 109, 13398–13403. doi: 10.1073/pnas.1208362109

Sumby, K. M., Grbin, P. R., and Jiranek, V. (2014). Implications of new research and technologies for malolactic fermentation in wine. *Appl. Microbiol. Biotechnol.* 98, 8111–8132. doi: 10.1007/s00253-014-5976-0

Sunagawa, S., Mende, D. R., Zeller, G., Izquierdo-Carrasco, F., Berger, S. A., Kultima, J. R., et al. (2013). Metagenomic species profiling using universal phylogenetic marker genes. *Nat Methods* 10, 1196–1199. doi: 10.1038/nmeth.2693

Taylor, M. W., Tsai, P., Anfang, N., Ross, H. A., and Goddard, M. R. (2014). Pyrosequencing reveals regional differences in fruit-associated fungal communities. *Environ. Microbiol.* 16, 2848–2858. doi: 10.1111/1462-2920.12456

Thomas, T., Gilbert, J., and Meyer, F. (2012). Metagenomics - a guide from sampling to data analysis. *Microb. Inform. Exp.* 2, 1–12. doi: 10.1186/2042-5783-2-3

Toffali, K., Zamboni, A., Anesi, A., Stocchero, M., Pezzotti, M., Levi, M., et al. (2011). Novel aspects of grape berry ripening and post-harvest withering revealed by untargeted LC-ESI-MS metabolomics analysis. *Metabolomics* 7, 424–436. doi: 10.1007/s11306-010-0259-y

Torriani, S., Zapparoli, G., and Suzzi, G. (1999). Genetic and phenotypic diversity of *Saccharomyces* sensu stricto strains isolated from Amarone wine. Diversity of Saccharomyces strains from Amarone wine. *Antonie Van Leeuwenhoek* 75, 207–215. doi: 10.1023/A:1001773916407

Tosi, E., Fedrizzi, B., Azzolini, M., Finato, F., Simonato, B., and Zapparoli, G. (2012). Effects of noble rot on must composition and aroma profile of Amarone wine produced by the traditional grape withering protocol. *Food Chem.* 130, 370–375. doi: 10.1016/j.foodchem.2011.07.053

Trotel-Aziz, P., Couderchet, M., Biagianti, S., and Aziz, A. (2008). Characterization of new bacterial biocontrol agents *Acinetobacter*, *Bacillus*, *Pantoea* and *Pseudomonas* spp. mediating grapevine resistance against Botrytis cinerea. *Environ. Exp. Bot.* 64, 21–32. doi: 10.1016/j.envexpbot.2007.12.009

Valero, A., Sanchis, V., Ramos, A. J., and Marin, S. (2008). Brief *in vitro* study on *Botrytis cinerea* and *Aspergillus carbonarius* regarding growth and ochratoxin A. *Lett. Appl. Microbiol.* 47, 327–332. doi: 10.1111/j.1472-765X.2008.02434.x

Venturini, L., Ferrarini, A., Zenoni, S., Tornielli, G. B., Fasoli, M., Dal Santo, S., et al. (2013). *De novo* transcriptome characterization of *Vitis vinifera* cv. *Corvina unveils* varietal diversity. *BMC Genomics* 14:41. doi: 10.1186/1471-2164-14-41

Verginer, M., Leitner, E., and Berg, G. (2010). Production of volatile metabolites by grape associated microorganisms. *J. Agric. Food Chem.* 58, 8344–8350. doi: 10.1021/jf100393w

Verhagen, B. W., Trotel-Aziz, P., Couderchet, M., Höfte, M., and Aziz, A. (2010). *Pseudomonas* spp.-induced systemic resistance to *Botrytis cinerea* is associated with induction and priming of defence responses in grapevine. *J. Exp. Bot.* 61, 249–260. doi: 10.1093/jxb/erp295

Wang, C., García-Fernández, D., Mas, A., and Esteve-Zarzoso, B. (2015). Fungal diversity in grape must and wine fermentation assessed by massive sequencing, quantitative PCR and DGGE. *Front Microbiol.* 6:1156. doi: 10.3389/fmicb.2015.01156

Whipps, J. M., Hand, P., Pink, D., and Bending, G. D. (2008). Phyllosphere microbiology with special reference to diversity and plant genotype. *J. Appl. Microbiol.* 105, 1744–1755. doi: 10.1111/j.1365-2672.2008.03906.x

Zarraonaindia, I., Owens, S. M., Weisenhorn, P., West, K., Hampton-Marcell, J., and Lax, S. (2015). The soil microbiome influences grapevine-associated microbiota. *mBio* 6, e02527-e02514. doi: 10.1128/mBio.02527-14

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Wine fermentation microbiome: a landscape from different Portuguese wine appellations

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Grapes and wine musts harbor a complex microbiome, which plays a crucial role in wine fermentation as it impacts on wine flavour and, consequently, on its final quality and value. Unveiling the microbiome and its dynamics, and understanding the ecological factors that explain such biodiversity, has been a challenge to oenology. In this work, we tackle this using a metagenomics approach to describe the natural microbial communities, both fungal and bacterial microorganisms, associated with spontaneous wine fermentations. For this, the wine microbiome, from six Portuguese wine appellations, was fully characterized as regards to three stages of fermentation – Initial Musts (IM), and Start and End of alcoholic fermentations (SF and EF, respectively). The wine fermentation process revealed a higher impact on fungal populations when compared with bacterial communities, and the fermentation evolution clearly caused a loss of the environmental microorganisms. Furthermore, significant differences ($p < 0.05$) were found in the fungal populations between IM, SF, and EF, and in the bacterial population between IM and SF. Fungal communities were characterized by either the presence of environmental microorganisms and phytopathogens in the IM, or yeasts associated with alcoholic fermentations in wine must samples as *Saccharomyces* and non-*Saccharomyces* yeasts (as *Lachancea, Metschnikowia, Hanseniaspora, Hyphopichia, Sporothrix, Candida,* and *Schizosaccharomyces*). Among bacterial communities, the most abundant family was Enterobacteriaceae; though families of species associated with the production of lactic acid (Lactobacillaceae, Leuconostocaceae) and acetic acid (Acetobacteriaceae) were also detected. Interestingly, a biogeographical correlation for both fungal and bacterial communities was identified between wine appellations at IM suggesting that each wine region contains specific and embedded microbial communities which may contribute to the uniqueness of regional wines.

Keywords: grape microbiology, wine spontaneous fermentation microbiome, industrial metagenomics

Introduction

The knowledge and the understanding of the microbial *terroir* – how the microbiome contributes to the natural environment of grapes and to the identity of wine, is a process that starts at the vineyards, at the harvest of grapes, and then evolves along the different stages of fermentation (Van Leeuwen and Seguin, 2006; Bokulich et al., 2013). Indeed, it is known that grapes harbor

a complex microbiome, including a high range of filamentous fungi, yeasts and bacteria with different physiological and metabolic characteristics (Pretorius, 2000; Fleet, 2003; Barata et al., 2012). The microflora of the grapes is highly variable, mostly due to the influence of external factors as environmental parameters, geographical location, grape cultivars and application of phytochemicals on the vineyards (Pretorius, 2000; Cadez et al., 2010; Pinto et al., 2014). These microbial communities play an important role during the winemaking process, as they metabolize the sugars from the grapes and produce a whole set of secondary metabolites that influence the wine aromatic quality (Fleet, 2003). In fact, the natural diversity of those metabolic pathways, and the contribution of the different microorganisms involved on the fermentation process, is well documented (Setati et al., 2012). Therefore, unveiling the microbial biodiversity of grapes and during their fermentation will expand our understanding on fermentation dynamics, on its control (Bisson, 1999; Bisson and Butzke, 2000) and may also contribute to the identification of novel starter cultures (Fleet, 2008; Ciani et al., 2010).

The spontaneous wine fermentation is carried out by indigenous microbiota (Heard, 1999; Pretorius, 2000; Ciani et al., 2006; Renouf et al., 2007). Species of *Metschnikowia, Candida, Hanseniaspora*, *Pichia, Lachancea* (*Kluyveromyces*), and *Saccharomyces* are often present at the initial stages of wine fermentations and form the dominant consortium (Cocolin et al., 2000; Mills et al., 2002; Fleet, 2008). However, during the wine fermentation, the ethanol content increases and *Saccharomyces cerevisiae* strains dominate the alcoholic fermentation (AF; Fleet, 2008). Additionally, a deacidification may occur, by conversion of malic acid into lactic acid. This process is known as malolactic fermentation (MLF) and is due to the activity of lactic acid bacteria (LAB; Lonvaud-Funel, 1999; Lerm et al., 2011). The LAB species associated with MLF generally belong to the *Oenococcus, Pediococcus, Lactobacillus,* and *Leuconostoc* genera (Lonvaud-Funel, 1999). Indeed, MLF mainly influences the organoleptic characteristics and the aging of wines (Lonvaud-Funel, 1999). On the other hand, acetic acid bacteria (AAB) may cause a negative impact on the winemaking process, due to the production of undesirable metabolites, as acetic acid, thus affect negatively the quality of wine and so are considered spoilage microorganisms (Zoecklein et al., 2000).

The majority of the wine microbiology studies focus on the characterization of *S. cerevisiae* strains (Pretorius, 2000; Fleet, 2008; Nisiotou et al., 2011). Nevertheless, recent studies based on culture-independent methods, started to explore the microbial communities associated with wine grapes (Bokulich et al., 2013; Taylor et al., 2014). It is widely accepted that unveiling the indigenous microbial community associated with particular grape varieties, from specific locations, could represent an important source of distinctive metabolites and introduce an authenticity *terroir* to the region (Heard, 1999; Jolly et al., 2006; Fleet, 2008). The biogeographical distribution of the wine associated microorganisms has been recently investigated in vineyards from different regions of California (Bokulich et al., 2013), New Zealand (Taylor et al., 2014), and in conventional, biodynamic, and integrated vineyards of South Africa (Setati et al., 2012). These studies allowed for a better spatial and temporal characterization of the wine grapes microbiome and brought new insights of its dynamics and biodiversity. Also, other biogeography wine studies have been previously published focusing on *S. cerevisiae* (Schuller et al., 2012). Nevertheless, there is still a lack of knowledge on the diversity and the dynamics of microbial communities as a whole– from the wine grapes until the wine fermentation, which can now be obtained using high-throughput sequencing technologies and metagenomics approaches that allow for the identification of both non-cultivable microorganisms, and of less represented species.

In this work, a total of six different Portuguese wine appellations were considered to analysis and high-throughput sequencing was used to unveil the wine microbiome present at initial musts (IM), and start and end of alcoholic fermentations (SF and EF, respectively). This work aims to understand the dynamics of microbial communities across spontaneous wine fermentations and also to reveal the biogeographic distribution of grape and wine microbiomes of Portuguese wine appellations.

Materials and Methods

Grape Sampling, Laboratory-Scale Fermentation, and DNA Extraction

The grape samples were collected during the 2010 vintage, from six different Portuguese appellations, namely, Minho (Mi), Douro (Dr), Dão (D), Bairrada (B), Estremadura (E), and Alentejo (Al). For each appellation, the three most representative grape varieties were considered for sampling, with exception of Minho where only two grape varieties were considered (**Supplementary Figure S1**). For all regions, the sampling was carried out 1 day prior the harvest. The sampling was authorized by private wine producers, who are fully acknowledged in this paper, and no specific permissions were required for this activity. Also, the field study did not involve any endangered or protected species.

For each appellation, one vineyard (farm) with different grape varieties was selected, and for each grape variety, 2 kg of healthy and undamaged grapes were collected. Grapes were collected from multiple bunches of different grapevines, randomly distributed across the vineyard in order to assure the representativeness of the sampling. These samples were collected into sterile plastic bags and transported to the laboratory chilled on ice. In total, 17 grape samples were collected, crushed and allowed for laboratory-scale fermentation (spontaneous AF) under aseptic conditions and acclimatised at 21°C, at the Genomics Unit from Biocant. For each sample, the microbial diversity was analyzed at three stages: IM, corresponding to the juice of crushed grapes; start of alcoholic fermentation (SF) and end of alcoholic fermentation (EF), which corresponded to the weight loss of 5 and 70 g/L of sugar, respectively. The SF and EF where daily monitored through weighting. At each stage, 50 mL of wine must were collected and centrifuged at 4000 rpm for 10 min. The respective microbial pellets were collected, washed twice with 0.9% NaCl and re-suspended with glycerol. A total

of 51 samples ($n = 17 \times 3$ fermentation stages) were stored at −80°C for DNA extraction. The DNA from each individual sample was extracted using the DNeasy Plant mini kit (QIAGEN, USA), according to the manufacturer's instructions, with a prior cell rupture using glass beads in Tissue Lyser (Qiagen, USA), to assure full disruption of microbial cells.

rDNA Library Construction and Pyrosequencing

A PCR amplicon library was built for each individual sample. For a better discrimination of the entire microbial community present during the fermentation process, rDNA sequences from both prokaryotic and eukaryotic microorganisms were amplified, using PCR primers that were designed to target three distinct regions. The V6 hypervariable region of the 16S rRNA was used for the identification of prokaryotic microorganisms (Sogin et al., 2006) and the D2, from the 26S rRNA, and ITS2 regions (White et al., 1990) for eukaryotic identification. The sequence-specific portions of the used primers were: V6_F 5′-ATGCAACGCGAAGAACCT-3′ and V6_R 5′-TAGCGATTCCGACTTCA-3′ of V6 region; D2_F 5′-AAGMACTTTGRAAAGAGAG-3′ and D2_R 5′-GGTCCGTGT TTCAAGACG-3′ of D2 region; and ITS2_F 5′-GCATCGATG AAGAACGC-3′ and ITS2_R 5′-CCTCCGCTTATTGATAT GC-3′ of ITS2 region. Additionally, the fusion primers also contained a specific Roche 454 adaptor sequence and a multiplex identifier sequence with eight nucleotides, which allows the pooling of amplicons.

All PCR reactions were carried out in 30 μL reaction mix containing 2 μL of DNA template, 1.5 units of FastStart High Fidelity Taq DNA polymerase (Roche, USA), 1x reaction buffer with $MgCl_2$ (1.8 mM) incorporate (Roche, USA), 0.2 mM dNTPs (Bioron, Germany) and 0.8 μM of the forward and reverse primers for V6 region or 0.4 μM of forward and reverse primers for D2 and ITS2 regions. For prokaryotes amplification, cycling conditions consisted in a first denaturation step at 94°C for 5 min followed by 20 cycles with a denaturation step at 94°C for 35 s, annealing at 50°C for 35 s and an extension at 72°C for 40 s. A final extension cycle at 72°C for 5 min was applied. The cycling conditions applied for eukaryotic microorganisms were the same, but the PCR consisted in 25 cycles. The amplification success was assessed by electrophoresis using the HT DNA 5K/RNA LabChip for the LabChip 90 (Caliper Life Sciences, USA). The PCR reaction products were then purified with the High Pure 96 UF Cleanup Plates (Roche, USA) and quantified using the PicoGreen® dsDNA quantitation kit (Invitrogen, USA). Samples were pooled together according to the number of DNA molecules, in equimolar concentrations and submitted for pyrosequencing using the GS FLX Titanium platform (454 Life Sciences, Roche) at Biocant, Portugal. The raw data obtained was deposited in NCBI platform with the accession number SRA097159.

Bioinformatic Data Analysis

Raw sequence reads were processed with MetaBiodiverse, an automatic annotation pipeline fully implemented at Genoinseq of Biocant (Vaz-Moreira et al., 2011; Egas et al., 2012; Pinto et al., 2014). Briefly, the raw data obtained was split through the identification of barcode sequences and quality filters were applied to remove low quality reads. Thus, (*i*) short sequences (<120 bp), (*ii*) sequences containing more than two undetermined nucleotides (N), (*iii*) masked sequences with more 50% of low complexity areas (Sogin et al., 2006) and (*iv*) chimera sequences, detected using UChime were removed (Edgar et al., 2011). All sequences with a distance value below 0.03, which corresponds to the species-level threshold (Sharpton et al., 2011), were grouped in operational taxonomic units (OTUs) through USearch, version 6.0.307 (Edgar, 2010). The Mothur package (Schloss et al., 2009) was used to generate rarefaction curves (richness of population analysis) and to calculate the population diversity analysis estimator Chao1 (α diversity). For the taxonomic annotation, each generated consensus sequences were queried by BLAST on curated databases. The Ribosomal Database Project II (RDP; Cole et al., 2009) was used for prokaryotic microorganisms assignment and the nt@ncbi/SILVA database for eukaryotic classification. After BLAST, the best hits were selected and subjected to another quality control. All sequences with an alignment of less than 40% or with an *E*-value greater than $1e^{-50}$ were rejected. Sequences that passed the quality check were subjected to a bootstrap test with 100 replicates, using the seqBoot application from the Phylip package (Felsenstein, 1989). The OTU identification process implemented provided a high level of confidence in taxon assignment of each sequence. The process assessed the correct *E*-values scores, went through the taxonomy path and identified the lowest common taxonomy level in the bootstrap process. Only those sequences with an identity greater than 70% were reported, while all the others went up the taxonomy levels until reached 70%.

Statistical Analyses

To determine the minimum significant difference ($p < 0.05$) in the biodiversity (Chao1) of IM, SF and EF samples, one-way analysis of variance (ANOVA) was performed using SPSS 20.0 (IBM, US). Shapiro-Wilk normality tests were carried out for each eukaryotic and prokaryotic phylogenetic group. As most groups did not follow the normal distribution, Friedman and Sign tests (pairwise comparisons) were used. The microbial communities were compared at family level for prokaryotic population and at genus level for eukaryotic population through the sequence reads analysis. Thus, microbial population comparisons were carried out using these taxa.

Sequence reads data matrixes of the 97% similarity grouped bacterial and fungal OTUs, produced by Metabiodiverse, were normalized by the total reads obtained for each analyzed sample, and then log(X+1) transformed and used to calculate a Bray–Curtis resemblance matrixes. The data obtained for the three fermentation stages were (i) explored by principal coordinate analysis (PCO), (ii) tested by Analysis of Similarities (ANOSIM) for significant differences and (iii) analyzed by SIMPER to identify the taxa responsible for similarity between samples within each group and dissimilarities between groups, using Primer E software version 6 (Clarke and Gorley, 2006). The same analyses were performed to explore and test the influence of wine appellations on microbiome although, for each fermentation

stage, individual matrixes were created in order to remove the "fermentation stage" variable.

Results

Diversity and Richness of Microbial Communities

In this study, we assessed and compared the microbial community of IM, and the Start and End of wine alcoholic fermentations (SF and EF, respectively), from six Portuguese appellations by DNA massive parallel sequencing of 16S rDNA for bacteria, and both, ITS2 and D2 for fungal analysis. Two target regions were used for the fungal population identification as previous experiments demonstrated that these combination would allow for the highest coverage of eukaryotic organisms (Pinto et al., 2014).

The deep sequencing of microbial communities generated a total of 1,180,106 sequences of ITS2, D2, and V6 regions from IM, SF, and EF (**Table 1** and **Supplementary Table S1**). A total of 1,160,482 sequences passed the quality control parameters, representing an acceptance of 98.3% of high quality sequences (723,474 eukaryotic sequences: 313,919 reads for ITS2 region and 409,555 for D2 region; and 437,008 prokaryotic sequences). The clustering of the sequences at a phylogenetic distance of 3% generated a total of 1,034 OTUs for ITS2, 1,099 for D2, and 1,461 for V6. The number of OTUs from both eukaryotic and prokaryotic communities decreased along the fermentation.

The diversity of microbial community was compared by rarefaction curve analysis (**Supplementary Figure S2**) and the ratio between the number of the obtained and the expected OTUs (predicted by Chao1) was used to determine the coverage for the microbial communities: it was of 73.7 ± 2.0% for ITS2 region, 71.7 ± 1.9% for D2 region and 65.1 ± 1.9% for V6 region (**Supplementary Table S1**).

In order to assess the variations of microbial biodiversity, the Chao1 richness estimator was used to compare the three fermentation stages at both domain and phylum levels. In general, and as expected, a decrease of richness was observed over the spontaneous wine fermentation for both fungi and bacteria, at the analyzed taxonomical levels (domain and phylum; **Figure 1**). Considering the domain (**Figure 1A**), no significant differences were found for the three rDNA regions. At the phylum level, significant differences ($p < 0.05$) in the Basidiomycota between all stages of fermentation were observed (both for ITS2 and D2 regions), and in the Ascomycota population differences were between SF and EF, but not between IM and SF (**Figure 1B**). For the bacterial population, a decrease in biodiversity was observed but no significant differences were detected (V6 rDNA region). A clear relationship was observed between the microbial community biodiversity and the stage of fermentation. Interestingly, the variations of biodiversity, which were observed along the fermentation stages, revealed a higher impact on the structure of the eukaryotic population, when compared with the prokaryotic communities. Moreover, regarding the microbial biodiversity, the prokaryotic population was richer than the eukaryotic population.

General Characterization of Microbial Communities

The dominant phylum across the entire eukaryotic population was Ascomycota (42.4%), though it also contained Basidiomycota (17.7%), and other fungi, as Chytridiomycota phylum (0.2%) and *basal fungal lineages* (5.6%). Also, a considerable number of unidentified microorganisms (34.1%) were mostly present at IM (**Figure 2A**).

TABLE 1 | Total sequences obtained for eukaryotic (ITS2 and D2) and prokaryotic (V6) microbial community for IM, SF, and EF samples.

		No. Reads		0.03 distance		
Sampling point	**Target region**	**Total**	**High quality**	**OTU obtained (mean ± SEM)**	**Estimated species (mean ± SEM)**	**Coverage (mean ± SEM)**
IM	ITS2	119876	116064	68 ± 6	100 ± 9	68.83 ± 2.26%
	D2	131837	129652	71 ± 6	110 ± 10	66.54 ± 2.52%
	V6	145796	145051	78 ± 12	134 ± 21	60.30 ± 3.19%
SF	ITS2	114993	111075	33 ± 3	47 ± 5	74.44 ± 3.62%
	D2	145559	143100	36 ± 3	56 ± 7	68.63 ± 3.29%
	V6	159940	159054	56 ± 9	83 ± 13	66.92 ± 3.28%
EF	ITS2	90207	86780	20 ± 1	29 ± 4	77.74 ± 4.10%
	D2	138156	136803	19 ± 2	25 ± 2	79.82 ± 3.23%
	V6	133742	132903	54 ± 9	81 ± 12	68.15 ± 3.48%
	Eukaryotic	740628	723474			
	Prokaryotic	439478	437008			
	Total	**1180106**	**1160482**			

Operational taxonomic units (OTUs) and estimated species (chao1) were determined at a genetic distance of 3% using Mothur. The coverage obtained was also determined as being the ratio between the observed OTUs and estimated Chao1 (OTUs/Chao1). A detailed table with indication of the samples origin is provided as ***Supplementary Table S1****.*

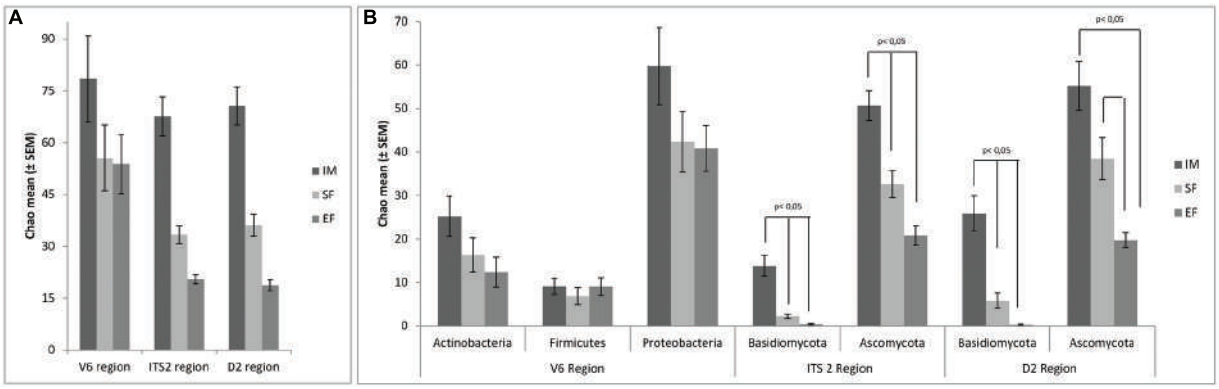

FIGURE 1 | Biodiversity dynamics associated with V6, ITS2, and D2 region, at domain (A) and phylum level (B). The mean of Chao1 index ± SEM are represented in the graph. Significance was assessed with Friedman test and signal test. $p < 0.05$ was set as statistic significant level.

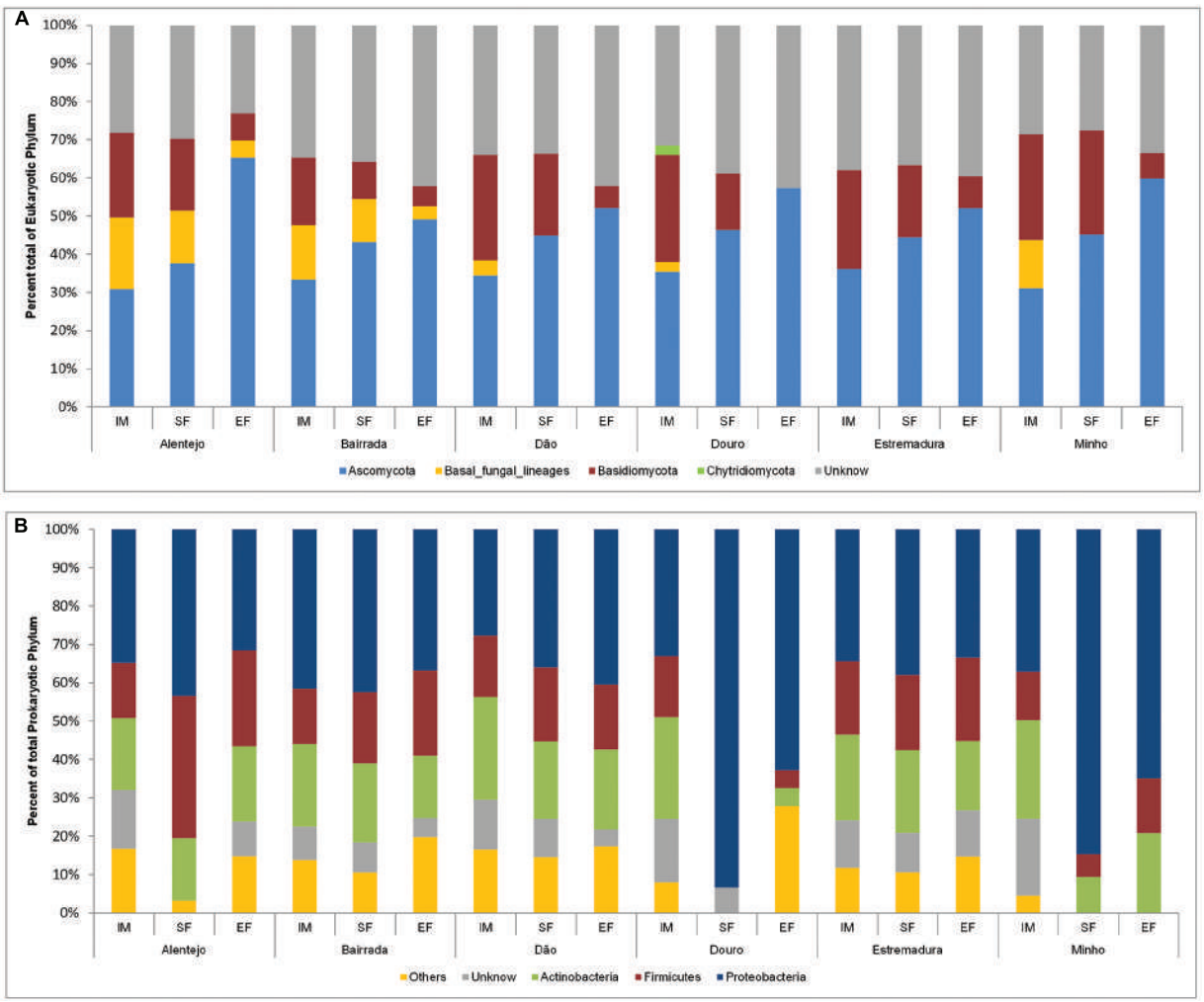

FIGURE 2 | Eukaryotic (A) and prokaryotic (B) community distribution over IM, SF, and EF from Portuguese appellations at the phylum level. Relative abundance of the eukaryotic **(A)** and prokaryotic **(B)** community through phylum analysis. For the whole figure, "Unknown" represents unclassified sequences. The prokaryotic members of rare population phyla were placed in an artificial group designed as "Others" and included Acidobacteria, Bacteroidetes, Chloroflexi, Cyanobacteria, Deinococcus-Thermus, Gemmatimonadetes, Nitrospirae, Planctomycetes, Tenericutes, and Verrumicrobia.

In all samples, the dynamics of microbial populations at phylum level were very similar. Nevertheless, the relative abundances varied along the fermentation and across Portuguese appellations (**Figure 2A**). Microorganisms belonging to Basidiomycota phylum decreased during the fermentation process. To better understand such population dynamics, the relative abundance at class level was analyzed. The entire microbial community was mostly characterized by Saccharomycetes (22.9%), Dothideomycetes (16.2%), Leotiomycetes (12.9%), Microbotryomycetes (9.6%), and Schizosaccharomycetes (7.7%; **Figure 3A**).

Concerning the prokaryotic communities, the dominant phyla were Proteobacteria (41.6%), Actinobacteria (19.2%), and Firmicutes (17.9%; **Figure 2B**). The members of under-represented phyla were grouped together in the artificial group "Other" (12.4%) and included Acidobacteria, Bacteroidetes, Chloroflexi, Cyanobacteria, Deinococcus-Thermus, Gemmatimonadetes, Nitrospirae, Planctomycetes, Tenericutes, and Verrumicrobia. As a reflection of the microbial community dynamics, and as seen in eukaryotic microorganisms, the relative abundances of all prokaryotic communities varied in both time and space. Along the spontaneous wine fermentations, it was possible to observe an increase of microorganisms belonging to the Proteobacteria phylum (**Figure 2B**), thus indicating that samples are losing their environmental characteristics. Regarding the prokaryotic classes, microorganisms from Gammaproteobacteria (27.9%), Betaproteobacteria (15.9%), Alphaproteobacteria (14.8%), Actinobacteria (13.2%), and Bacilli (11.5%) were identified (**Figure 3B**).

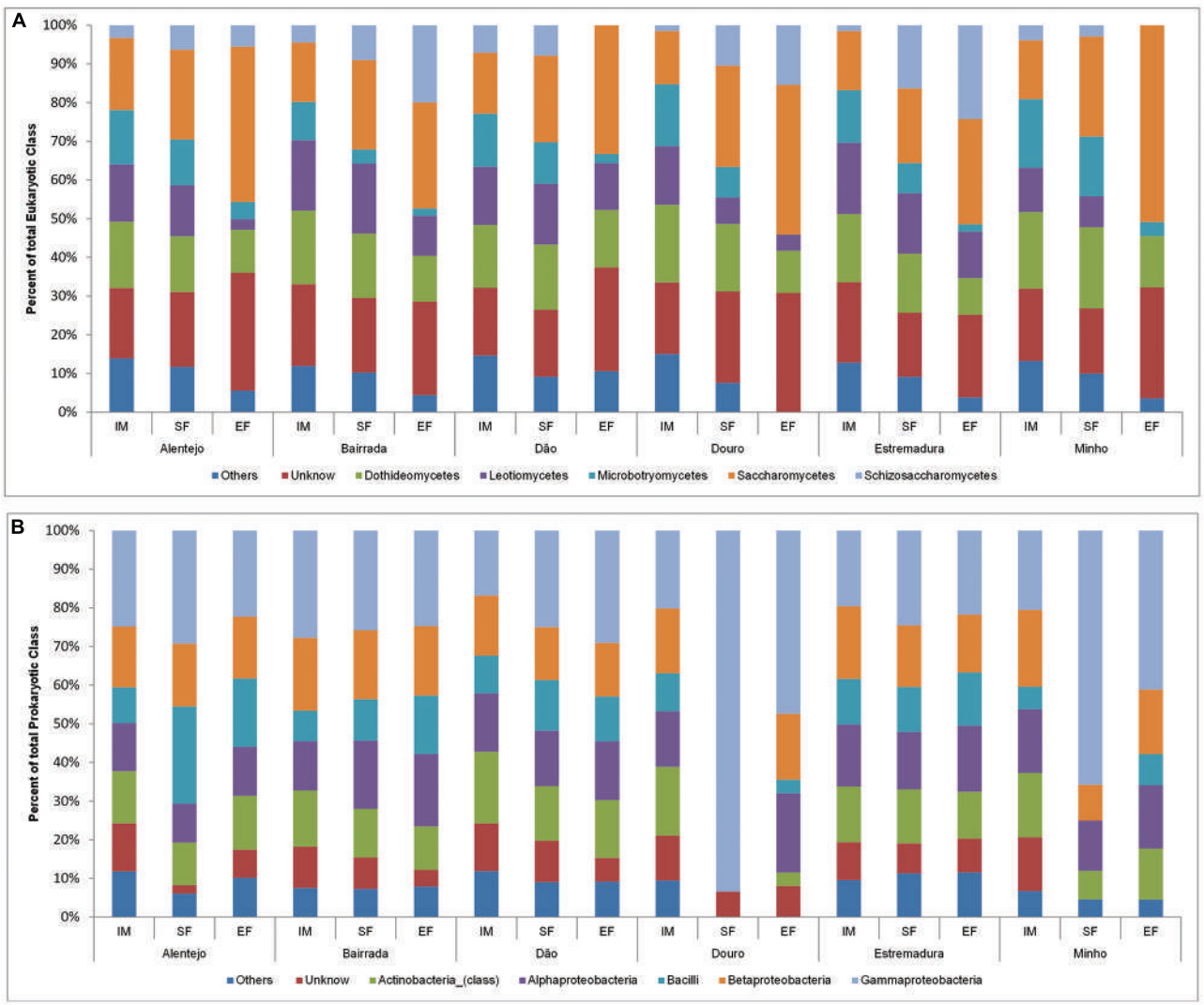

FIGURE 3 | Eukaryotic (A) and prokaryotic (B) community distribution over IM, SF, and EF from Portuguese appellations at the class level. Relative abundance of the eukaryotic **(A)** and prokaryotic **(B)** community through the class analysis. The members of rare population phyla were placed in an artificial group designed as "Others."

The Landscape of Microbial Communities Throughout Wine Fermentation

The dynamics of microbial communities present at IM, SF, and EF of samples from different Portuguese wine appellations were explored by principal coordinates analysis (PCO; **Figure 4**). For both fungal (**Figure 4A**) and bacterial communities (**Figure 4B**), samples were grouped according to their fermentative stage, where the first axis explains 48 and 52.3% of the total variation, respectively. Interestingly, SF samples were mixed with both IM and EF and, indeed this stage is a transition between IM and EF. As expected, the distribution of the microbial community composition is affected by fermentation. Significant differences (Fungi: $R_{ANOSIM} = 0.512$, $p = 0.001$; Bacteria: $R_{ANOSIM} = 0.170$, $p = 0.002$) between IM, SF, and EF samples were observed for a global test. Conversely, no significant differences were observed between SF and EF samples of the bacterial communities ($R_{ANOSIM} = 0.155$, $p = 0.954$) when analyzed by pairwise tests.

The fungal and bacterial microorganisms responsible for the similarities within each group, and the dissimilarity between the different stages of fermentation, were analyzed using SIMPER analysis (**Supplementary Table S2**). The average of similarity within each group increased over the fermentation process for both fungal (IM: 39.84%; SF: 42.27%; EF: 64.19%) and bacterial community (IM: 42.64%; SF: 48.36%; EF: 46.96%). Further, the fungal communities of IM samples were mainly characterized by the environmental yeasts *Aureobasidium* and *Rhodotorula*, which contributed with 64.55% for the group similarity. Other microorganisms, such as *Hanseniaspora*,

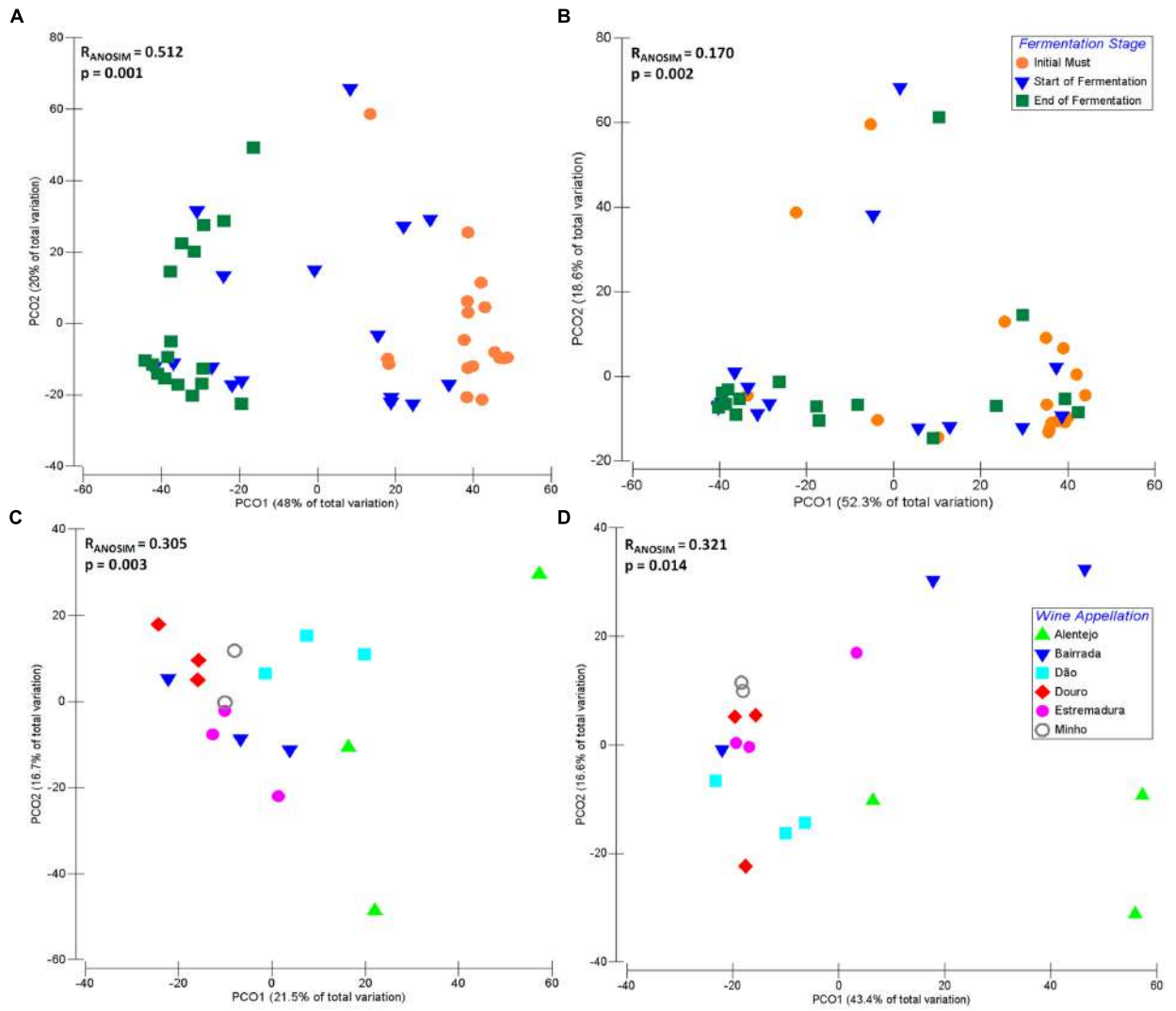

FIGURE 4 | Principal coordinate analysis (PCO) biplot diagram of microbial community during fermentation process, based on sequence abundance of eukaryotic genus and bacterial family. Principal coordinates analysis (showing the first and second components) of fungal **(A)** and bacterial **(B)** communities across the fermentation stage namely, initial musts (IM), start of fermentation (SF) and end of fermentation (EF) for Portuguese appellations. Biogeographical distribution of fungal **(C)** and bacterial **(D)** microorganisms at IM across the six Portuguese wine appellations namely, Alentejo, Bairrada, Dão, Douro, Estremadura, and Minho.

Saccharomyces, *Lachancea*, *Botryotinia*, *Alternaria*, *Aspergillus*, *Metschnikowia*, *Filobasidiella,* and *Candida* contributed with 25.80% for the group similarity. Regarding the bacterial community at IM, Enterobacteriaceae, Pseudomonadaceae, Microbacteriaceae, Comamonadaceae families contributed with 52.68% for group similarity, followed by Oxalobacteraceae, Sphingomonadaceae, Xanthomonadaceae, Nocardioidaceae, Methylobacteriaceae, Halomonadaceae, Propionibacteriaceae, Rhodobacteraceae, Micrococcaceae, Acetobacteraceae, which all together contributed with 38.25%.

The analysis of similarity of the fungal community at SF and EF revealed that fewer microorganisms contributed to the similarity of groups when compared with IM, which is explained by the evolution of the fermentative process. In fact, the microbial community tended to be more similar and less diverse at EF. At SF, the microorganisms *Saccharomyces*, *Hanseniaspora*, *Aureobasidium,* and *Lachancea* contibuted with 91.91% for group similarity, and at EF the *Saccharomyces* and *Hanseniaspora* microorganisms contributed with 91.19%. The same behavior was observed for bacterial communities where Enterobacteriaceae, Halomonadaceae, Comamonadaceae, Pseudomonadaceae, and Xanthomonadaceae families contributed with 91.44% of similarity for SF group, whereas Enterobacteriaceae, Comamonadaceae, Acetobacteraceae, Xanthomonadaceae, Pseudomonadaceae, and Oxalobacteraceae families contributed with 91.44% for EF group similarity.

Regarding the comparison between IM, SF, and EF groups of fungal communities, a higher dissimilarity value was obtained for IM vs. EF (86.53%) followed by IM vs. SF (73.84%) and SF vs. EF (53.44%), where microorganisms belonging to the *Lachancea, Saccharomyces, Hanseniaspora, Aureobasidium, Schizosaccharomyces, Candida, Metschnikowia, Torulaspora, Rhodotorula,* and *Alternaria* genera contributed for the dissimilarity of the groups. Furthermore, the diferences of the dissimilary were less pronounced for the bacterial community when compared with fungal population: IM vs EF (66.09%), IM vs SF (66.05%), and SF vs EF (50.51%). Micoorganisms belonging to the Halomonadaceae, Enterobacteriaceae, Pseudomonadaceae, Comamonadaceae, Oxalobacteraceae, Microbacteriaceae, Sphingomonadaceae, Acetobacteraceae, and Xanthomonadaceae familes were those that mostly contributed for the dissimilarity of groups (**Supplementary Table S2**).

Microbiome of Wine Appellations

In order to understand the biogeographical distribution of microbial populations, the microbiome associated with the six Portuguese appellations was individually compared for IM, SF, and EF, for both bacterial and fungal communities (**Figures 4C,D**). Significant differences were observed across wine appellations for IM samples (Fungi: $R_{ANOSIM} = 0.305$, $p = 0.003$; Bacteria: $R_{ANOSIM} = 0.321$, $p = 0.014$). For both fungal (**Figure 4C**) and bacterial communities (**Figure 4D**), samples were grouped according to their similarity, where the first axis explain 21.5 and 43.4% of the total variation, respectively. The SIMPER analysis (**Supplementary Table S2**) revealed that the average of similarity within each wine appellation was higher at Minho for both bacterial (76.20%) and fungal (63.21%) communities, followed by Estremadura (50.49 and 51.99% for bacterial and fungal populations, respectively), Bairrada (40.81 and 51.77%), Douro (49.68 and 50.68%), Dão (59.74 and 45.29%), and Alentejo (51.54 and 23.98%). The SF samples (fungi: $R_{ANOSIM} = 0.060$, $p = 0.320$; bacteria: $R_{ANOSIM} = 0.073$, $p = 0.271$) and EF samples (fungi: $R_{ANOSIM} = -0.039$, $p = 0.596$; bacteria: $R_{ANOSIM} = 0.093$, $p = 0.199$) did not show any significant differences.

Regarding the fungal microorganisms that contributed for each wine appellation, the genus *Aureobasidium* dominated and contributed for an average of 44.39% appellations similarity (**Supplementary Table S2**). Interestingly, it was observed a regional effect on the contribution of other microorganisms: at Alentejo appellation *Lachancea* prevailed, contributing for 21.44% of region's similarity; in the Estremadura appellation *Rhodotorula* and *Botryotinia* contributed for 37.96% of the similarity; the Bairrada appellation was characterized by the presence of *Hanseniaspora* and *Ramularia,* who contributed for 18.86% of the regional similarity; the Dão appellation was characterized by the presence of microorganisms from the *Lachancea* and *Rhodotorula* genera (29.07% of similarity); within Douro appellation, *Rhodotorula* and *Erysiphe* contributed with 21.29% for the similarity; and finally, the Minho appellation was characterized by *Rhodotorula* and *Alternaria* (40% of similarity; **Supplementary Table S2**). In general, the fungal populations of IM were characterized by ubiquitous genera as *Aureobasidium, Rhodotorula, Hanseniaspora, Alternaria, Metschnikowia, Saccharomyces, Candida, Ramularia, Penicillium, Lewia, Filobasidiella, Leptosphaerulina,* and *Schizosaccharomyces*, forming the principal structure of the microbial populations (**Figure 5A**).

In SF samples, an increase of *Saccharomyces* population was observed in all regions. Nevertheless, Alentejo had the highest abundance of *Lachancea* and Minho was characterized by having the richest biodiversity, which included *Hanseniaspora, Lachancea, Metschnikowia,* and *Aureobasidium.* Expectedly at EF the dominant genus was *Saccharomyces*, but still some regional differences were observed: samples from Alentejo, Douro, and Minho presented a similar composition (*Saccharomyces* and *Lachancea*), while Bairrada and Dão were mostly composed by *Saccharomyces*. Samples from Estremadura region contained high amounts of both *Saccharomyces* and *Schizosaccharomyces*.

Regarding the bacterial community, the families of Halomonadaceae and Enterobacteriaceae contributed with 91.93% for the Alentejo appellation similarity whereas at Bairrada region, Enterobacteriaceae and Pseudomonadaceae contributed with 75.78%. At Dão appellation, Microbacteriaceae, Oxalobacteraceae, and Enterobacteriaceae contributed with 36.83% and Comamonadaceae, Enterobacteriaceae, Oxalobacteraceae, and Microbacteriaceae families with 52.35% for Douro region similarity. Finally, at Estremadura, Enterobacteriaceae, contributed with 22.47% and at Minho appellation, Oxalobacteraceae, Pseudomonadaceae, and or Enterobacteriaceae with 45.39% for the similarity. It is interesting to notice that the bacterial families responsible for the regional similarities were mostly environmental, and are not related with the oenological process.

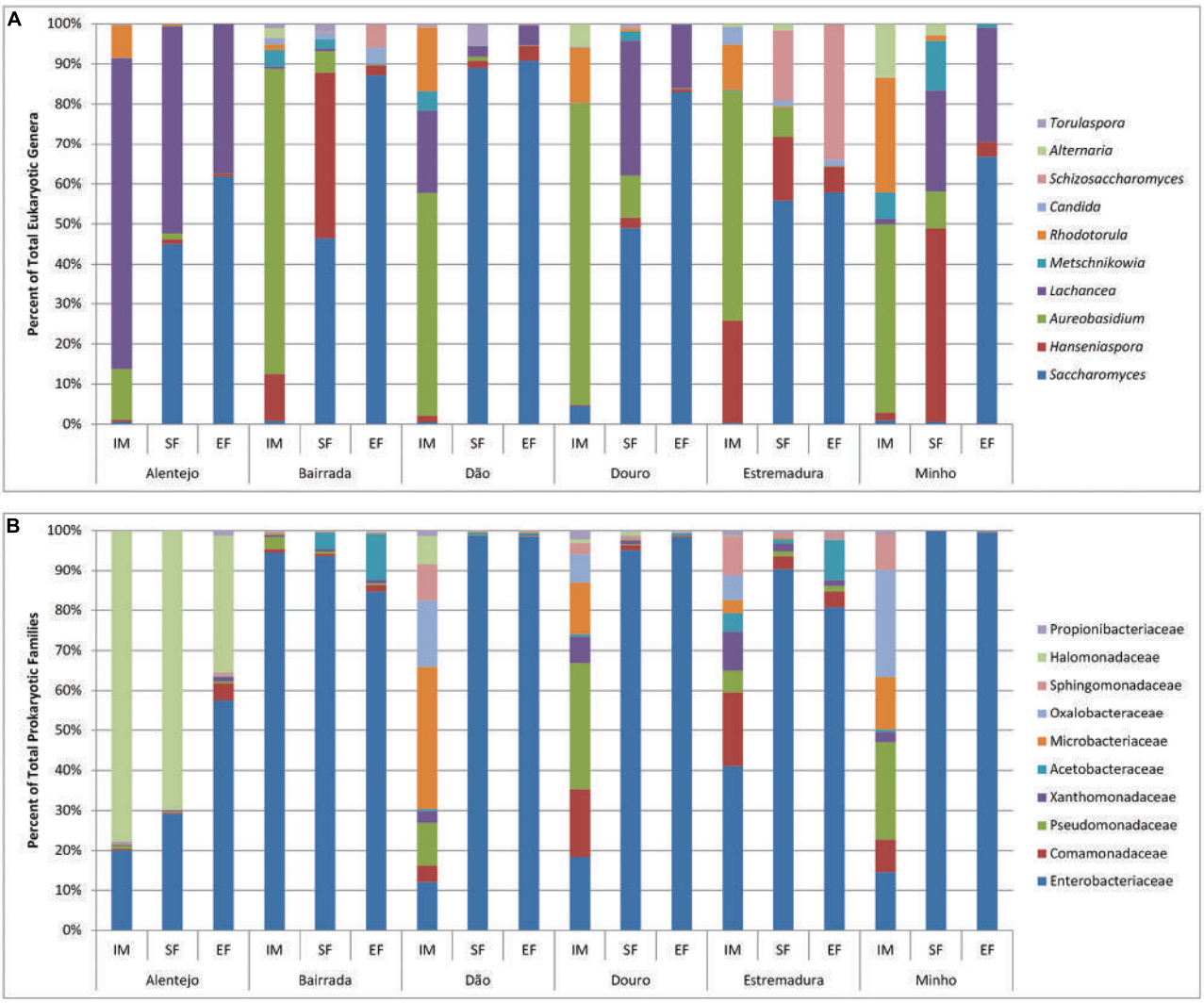

FIGURE 5 | Eukaryotic (A) and prokaryotic (B) microbial community distribution over IM, SF and EF of the Portuguese appellations. Relative abundance of the 10 most abundant eukaryotic **(A)** and prokaryotic **(B)** microorganisms through the genus and family analysis, respectively.

In general, the bacterial community was observed to differ across the appellations at IM samples. Additionally, grapes from Alentejo and Bairrada appellations presented the most distinct bacterial profiles (**Figure 5B**). Regarding SF and EF samples, Enterobacteriaceae was ubiquitous to all appellations. Bairrada and Estremadura were also characterized by high amounts of Acetobacteriaceae, while samples from Alentejo presented a unique microbiome characterized by the Halomonadaceae family (**Figure 5B**).

Regarding the most abundant bacterial family, Enterobacteriaceae, microorganisms from the genus *Pantoea* were found in all samples, whereas *Klebsiella* was only detected at IM and SF, and *Tatumella* was only identified at SF and EF samples. Also, bacteria belonging to the Microbacteriaceae family as *Curtobacterium* and *Frigobacterium* were detected in all samples and *Leifsonia* only at IM samples. Concerning all samples, the bacterial genera *Gluconobacter* (Acetobacteraceae) and *Leuconostoc* (Leuconostocaceae) were also abundant, which was expected as they have been long related with wine fermentations. *Variovorax* (Comamonadaceae); *Carnimonas*, *Halotalea,* and *Zymobacter* (Halomonadaceae); *Massilia* (Oxalobacteraceae); *Pseudomonas* (Pseudomonadaceae); and *Sphingomonas* (Sphingomonadaceae) were also extensively detected in all samples.

Discussion

The aims of this work were to characterize and to compare the diversity of the microbial communities during spontaneous wine fermentations and across different wine Portuguese appellations. To achieve this, high-throughput sequencing was used to fully characterize both eukaryotic and prokaryotic communities from samples collected from six Portuguese wine regions.

Wine fermentations are known to harbor a heterogeneous population of microorganisms. In this work, a diverse set of microbial communities was identified, where the most abundant phyla were Proteobacteria and Ascomycota from prokaryotic and eukaryotic populations, respectively. As expected, a clear relationship was observed between the microbial community and fermentation stage. The biodiversity across the fermentation process decreased for both prokaryotic and eukaryotic communities as a result of the selective environment created over the spontaneous wine fermentation. Interestingly, the variations of biodiversity along this process revealed a higher impact on the fungal community structure, when compared with the bacterial populations. Furthermore, the prokaryotic populations were more diverse than the eukaryotic populations.

In this study, the most abundant eukaryotic microorganisms at IMs were *Aureobasidium* (*A. pullulans*), *Rhodothorula* (*R. nothofagi*), *Hanseniaspora* (*H.uvarum*), and *Lachancea* (*L. thermotolerans*). A diverse set of bacterial population was also uncovered, where Enterobacteriaceae (namely, *Pantoea,* and *Klebsiella*) and Pseudomonadaceae (namely, *Cellvibrio,* and *Pseudomonas*) were the most abundant families. This is in line with the previous reported by Bokulich et al. (2013), where microorganisms as *Cladosporium* spp., *A. pullulans, H. uvarum* were detected as the major eukaryotic population in the IMs, and as regards to prokaryotic population, Lactobacillales, Pseudomonadales, or Enterobacteriales were also identified.

The high microbial biodiversity within IM samples was mostly due to environmental microorganisms derived from vineyard. Indeed, several detected microorganisms, namely, *Botryotinia, Phomopsis, Aspergillus, Penicillium, Aureobasidium, Rhodotorula,* Enterobacteriaceae, or *Sphingomonas,* were previously described on grapevine leafs and grape surfaces and some of them are even refereed as inhabitant of grapes (Mills et al., 2008; Martins, 2012; Bokulich et al., 2013; Pinto et al., 2014). Also, *Saccharomyces* was detected at IMs, which suggests that this community comes from grapes, reinforcing findings from Bokulich et al. (2013), Pinto et al. (2014), and Taylor et al., 2014.

Regarding the origin of spoilage microorganisms, there has been a vivid discussion on whether or not these are present at the vineyards, where grapes are the principal source for wine contamination and deterioration (Renouf et al., 2005), or otherwise, winemaking equipment is the source of spoilage microorganisms (Couto et al., 2005). For instance, it is considered that *Dekkera*/*Brettanomyces*, the lactic and AAB are the most important wine spoilage microorganisms (Bartowsky et al., 2003; Beneduce et al., 2004; Cocolin et al., 2004). In this study, *Dekkera*/*Brettanomyces bruxellensis* was not detected, which is in line with the study of Suárez et al. (2007), who reported that this spoilage yeast is mainly present in winemaking equipment with deficient cleaning; and is opposed to the findings reported by Renouf and Lonvaud-Funel (2007). Still, these results *per se* do not yet allow for a clear conclusion on their origin. In the other hand, LAB and AAB were detected at low abundances, but *Oenococcus oeni*, a LAB extensively used to carry out the MLF, was not detected. Additionally, filamentous fungi (molds) were identified on IMs: *Alternaria, Aspergillus, Botrytis, Cladosporium, Penicillium,* or *Rhizopus*, which are undesirable for wine quality (Toit and Pretorius, 2000). *Aspergillus* (*A. niger*) and *Penicillium* (*P. glabrum* and *P. brevicompactum*) were found in all the appellations considered in this work. However, and along fermentations, these molds disappeared, which supports the observations that they are sensitive to the wine fermentation conditions (Blesa et al., 2006).

From the IM to the wine, sequential stages of microbial development were observed, as result of fermentation activities (Fleet et al., 1984; Jolly et al., 2003). An initial growth of non-*Saccharomyces,* such as *Hanseniaspora, Torulaspora*, *Metschnikowia,* and *Pichia* at SF was followed by a decrease or even a disappearance of these yeasts at the EF and, conversely, the increase of *S. cerevisiae* was evidenced. A similar kinetic pattern was also observed on prokaryotic community, where in transition from IM to SF, Enterobacteriaceae family increased, and then decreased from SF to EF, specifically in Bairrada, Dão, and Estremadura appellations.

In spontaneous wine fermentations, *S. cerevisiae* was dominant despite the high abundance of *Hanseniaspora* and *Lachancea*. Yeasts associated with wine fermentation such as *Metschnikowia* (*M. pulcherrima and M. viticola*), *Torulaspora* (*T. delbrueckii*), *Schizosaccharomyces* (*S. japonicus*), *Candida* (*C. zemplinina*), *Issatchenkia* (*I. terricola*), and, less frequently, *Pichia* (*P. kluyveri* and *P. kudriavzevii*) were also detected. However, their relative abundances varied according to their appellation of origin. Indeed, each appellation presented characteristic microbial communities, with different abundances of non-*Saccharomyces* and specific patterns of microbial communities. Interestingly, *Schizosaccharomyces* (*S. japonicus*) was also detected, even at later stages, and was present at higher abundances in the Estremadura region. This yeast is characterized by having a high fermentative capacity at high temperatures (optimal growth around 30°C), and by being resistant to SO_2 and to the stringent conditions of fermentation (Torija et al., 2001). Regarding *Torulaspora delbrueckii,* it was found until EF, and it has been previously reported to survive until later stages of fermentation and to produce lower levels of acetic acid (Ciani et al., 2006). Interestingly, samples which presented higher abundance of this microorganism also generally had higher abundance of AAB namely, *Gluconobacter* (*G. oxydans*).

Among bacterial communities, during the fermentation, Enterobacteriaceae was the most abundant family (namely, *Tatumella* sp.). Nisiotou et al. (2011) also showed that Enterobacteriaceae persists in fermentation, and Ruiz et al. (2010) also confirmed its prevalence at beginning, mid and final stages of MLFs in different Spanish wineries. This raises the question if these bacteria interact with fermenting yeasts and, if so, in what degree can this microbial population influence (negatively or positively) the organoleptic proprieties of wine. The bacterial populations were found to be less dynamic than the eukaryotic populations in the later stages of fermentation process, and their geographic profiles were more similar: it was observed a clear dominance of Enterobactereaceae family at all appellations but Alentejo, where microorganisms from Halomonadaceae family were also presented with high abundance. The Bairrada and Estremadura appellations were also characterized by

the presence of microorganisms from the Acetobacteraceae family. Among the LAB, high amounts of *Lactobacillus* (Lactobacillaceae), *Leuconostoc* (Leuconostocaceae), *Lactococcus,* and *Streptococcus* (Streptococcaceae) were detected. Additionally, *Facklamia* (Aerococcaceae), *Carnobacterium, Dolosigranulum, Granulicatella,* and *Trichococcus* from Carnobacteriaceae family, *Enterococcus* (Enterococcaceae) and *Weisella* as *W. cibaria* (Leuconostocaceae) were also detected, but at lower abundances. Interestingly, and with exception of *Weisella*, those specific microorganisms had not been previously isolated from musts and wines (König and Fröhlich, 2009).

To investigate whether or not there is a geographic imprint on the wine fermentation microbiome, a PCO was performed for each fermentation stage in order to evaluate differences according to wine appellation. Interestingly, significant differences ($p < 0.05$) were observed for both fungal and bacterial microbial communities at IM between wine appellations. These results are consistent with those reported by Bokulich et al. (2013), who observed differences in the microbial community structure across wine appellations from California. Over the fermentation process, the initial microbiome associated with each wine appellation disappears and, as a consequence, the biogeographic profile was lost (no significant differences were observed for SF and EF). As observed, this microbiome is characterized by the presence of environmental microorganisms, which constituted a signature of each Portuguese wine regions. Moreover, these results also suggested that the initial microbial community could strongly contribute to the uniqueness of the wines derived from each specific wine appellation. Furthermore, each wine appellation presented its own pattern of biodiversity that varied in terms of the microbial abundance. This finding is of special interest when considering the non-saccharomyces population at the SF, whom have been acknowledged for their metabolic contribution to the final wine sensorial properties (Romano et al., 2003; Jolly et al., 2014), which reinforces their role on the regional attributes of wines. These findings open new horizons to dissect how microbiomes affect wine properties and support the need to unveil the endogenous microflora of such regions and explore its natural microbial populations in order to produce valuable wines styles.

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Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.00905

Figure S1 | Portugal map with the appellations and grape varieties chosen for study. Sample collection was done in 6 appellations – Minho (M), Douro (Dr), Dão (D), Bairrada (B), Estremadura (E), and Alentejo (Al). The grave varieties collected were Al, Alvarinho; Ax, Alfrocheiro; B, Baga; J, Jean; L, Loureiro; P, Piriquita; T, Trincadeira; TF, Touriga Franca; TN, Touriga Nacional; and TR, Tinta Roriz (also known Aragonez).

Figure S2 | Rarefaction curves at a genetic distance of 3% for each sample (IM, SF, and EF). D2 **(A)** and ITS2 **(B)** sequences both from the analysis of 26S rRNA and ITS regions of eukaryotic population present in the sample and V6 sequences **(C)** from the analysis of 16S rRNA of prokaryotic diversity. The IM, start fermentation (SF) and end of fermentation (EF) are represented by the blue, yellow and green color, respectively.

Table S1 | Total sequences obtained for eukaryotic (ITS2 and D2) and prokaryotic (V6) microbial communities for IM, SF, and EF from different wine appellations. Operational taxonomic units (OTUs) and estimated species (Chao1) were determined at a genetic distance of 3% using Mothur. The coverage obtained was also determined as being the ratio between the observed OTUs and the estimated Chao1 (OTUs/Chao1).

Table S2 | Analysis of the similarity and dissimilarity across wine fermentation stages and wine appellations. The similarity and dissimilarity across wine fermentation stages namely, initial musts (IM), start of fermentation (SF), and end of fermentation (EF) and wine appellations were calculated through the SIMPER analysis.

References

Barata, A., Malfeito-Ferreira, M., and Loureiro, V. (2012). The microbial ecology of wine grape berries. *Int. J. Food Microbiol.* 153, 243–259. doi: 10.1016/j.ijfoodmicro.2011.11.025

Bartowsky, E., Xia, D., Gibson, R., Fleet, G., and Henschke, P. (2003). Spoilage of bottled red wine by acetic acid bacteria. *Lett. Appl. Microbiol.* 36, 307–314. doi: 10.1046/j.1472-765X.2003.01314.x

Beneduce, L., Spano, G., Vernile, A., Tarantino, D., and Massa, S. (2004). Molecular characterization of lactic acid populations associated with wine spoilage. *J. Basic Microbiol.* 44, 10–16. doi: 10.1002/jobm.200310281

Bisson, L. (1999). Stuck and sluggish fermentations. *Am. J. Enol. Viticulture* 50, 107–119.

Bisson, L., and Butzke, C. (2000). Diagnosis and rectification of stuck and sluggish fermentations. *Am. J. Enol. Viticulture* 51, 168–177.

Blesa, J., Soriano, J., Moltó, J., and Mañes, J. (2006). Factors affecting the presence of ochratoxin a in wines. *Crit. Rev. Food Sci. Nutr.* 46, 473–478. doi: 10.1080/10408390500215803

Bokulich, N. A., Thorngate, J. H., Richardson, P. M., and Mills, D. A. (2013). Microbial biogeography of wine grapes is conditioned by cultivar, vintage, and climate. *Proc. Natl. Acad. Sci. U.S.A.* 11, E139–E148. doi: 10.1073/pnas.1317377110

Cadez, N., Zupan, J., and Raspor, P. (2010). The effect of fungicides on yeast communities associated with grape berries. *FEMS Yeast Res.* 10, 619–630. doi: 10.1111/j.1567-1364.2010.00635.x

Ciani, M., Beco, L., and Comitini, F. (2006). Fermentation behaviour and metabolic interactions of multistarter wine yeast fermentation. *Int. J. Food Microbiol.* 108, 239–245. doi: 10.1016/j.ijfoodmicro.2005.11.012

Ciani, M., Comitini, F., Mannazzu, I., and Domizio, P. (2010). Controlled mixed culture fermentation: a new perspective on the use of non-*Saccharomyces* yeasts in winemaking. *FEMS Yeast Res.* 10, 123–133. doi: 10.1111/j.1567-1364.2009.00579.x

Clarke, K. R., and Gorley, R. N. (2006). *PRIMER v6: User Manual/Tutorial*. Plymouth: PRIMER-E, 192.

Cocolin, L., Bisson, L., and Mills, D. (2000). Direct profiling of the yeast dynamics in wine fermentations. *FEMS Microbiol. Lett.* 189, 81–87. doi: 10.1016/S0378-1097(00)00257-3

Cocolin, L., Rantsiou, K., Iacumin, L., Zironi, R., and Comi, G. (2004). Molecular detection and identification of *Brettanomyces/Dekkera bruxellensis* and *Brettanomyces/Dekkera anomalus* in spoiled wines. *Appl. Environ. Microbiol.* 70, 1347–1355. doi: 10.1128/AEM.70.3.1347-1355.2004

Cole, J., Wang, Q., Cardenas, E., Fish, J., Chai, B., Farris, R. J., et al. (2009). The ribosomal database project: improved alignments and new tools for rrna analysis. *Nucleic Acids Res.* 37, D141–D145. doi: 10.1093/nar/gkn879

Couto, J., Neves, F., Campos, F., and Hogg, T. (2005). Thermal inactivation of the wine spoilage yeasts *Dekkera/Brettanomyces*. *Int. J. Food Microbiol.* 104, 337–344. doi: 10.1016/j.ijfoodmicro.2005.03.014

Edgar, R. (2010). Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* 26, 2460–2461. doi: 10.1093/bioinformatics/btq461

Edgar, R., Haas, B., Clemente, J., Quince, C., and Knight, R. (2011). UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics* 27, 2194–2200. doi: 10.1093/bioinformatics/btr381

Egas, C., Pinheiro, M., Gomes, P., Barroso, C., and Bettencourt, R. (2012). The transcriptome of bathymodiolus azoricus gill reveals expression of genes from endosymbionts and free-living deep-sea bacteria. *Mar. Drugs* 10, 1765–1783. doi: 10.3390/md10081765

Felsenstein, J. (1989). PHYLIP-Phylogeny inference package (version 3.2). *Cladistics* 5, 164–166.

Fleet, G. (2003). Yeast interactions and wine flavour. *Int. J. Food Microbiol.* 86, 11–22. doi: 10.1016/S0168-1605(03)00245-9

Fleet, G. (2008). Wine yeasts for the future. *FEMS Yeast Res.* 8, 979–995. doi: 10.1111/j.1567-1364.2008.00427.x

Fleet, G., Lafon-Lafourcade, S., and Ribéreau-Gayon, P. (1984). Evolution of yeasts and lactic acid bacteria during fermentation and storage of *Bordeaux* wines. *Appl. Environ. Microbiol.* 48, 1034–1038.

Heard, G. (1999). Novel yeasts in winemaking - looking to the future. *Food Aust.* 51, 347–352.

Jolly, N., Augustyns, O., and Pretorius, I. (2003). The effect of non-Saccharomyces on fermentation and wine quality. *S. Afr. J. Enol. Vitic.* 24, 55–62.

Jolly, N., Augustyn, O., and Pretorius, I. (2006). The role and use of non-*Saccharomyces* yeasts in wine production. *S. Afr. J. Enol. Vitic.* 27, 15–39.

Jolly, N. P., Varela, C., and Pretorius, I. S. (2014). Not your ordinary yeast: non-*Saccharomyces* yeasts in wine production uncovered. *FEMS Yeast Res.* 14, 215–237. doi: 10.1111/1567-1364.12111

König, H., and Fröhlich, J. (2009). "Lactic acid bacteria," in *Biology of Microorganisms on Grapes, in Must and in Wine*, eds H. König, G. Unden, and J. Fröhlich (Verlag Berlin Heidelberg: Springer), 3–30.

Lerm, E., Engelbrecht, L., and Du Toit, M. (2011). Selection and characterisation of *Oenococcus oeni* and *Lactobacillus plantarum* south african wine isolates for use as malolactic fermentation starter cultures. *S. Afr. J. Enol. Viticulture* 32, 280–295.

Lonvaud-Funel, A. (1999). Lactic acid bacteria in the quality improvement and depreciation of wine. *Antonie Van Leeuwenhoek* 76, 317–331. doi: 10.1023/A:1002088931106

Martins, G. (2012). *Communautés Microbiennes de la Baie de Raisin: Incidence des Facteurs Biotiques et Abiotiques*. Ph.D. theis, Université de Bourdeaux, Bourdeaux.

Mills, D., Johannsen, E., and Cocolin, L. (2002). Yeast diversity and persistence in botrytis-affected wine fermentations. *Appl. Environ. Microbiol.* 68, 4884–4893. doi: 10.1128/AEM.68.10.4884-4893.2002

Mills, D., Phister, T., Neeley, E., and Johannsen, E. (2008). "Wine fermentation," in *Molecular Techniques in the Microbial Ecology of Fermented Foods*, eds L. Cocolin and D. Ercolini (New York, NY: Springer), 162–192. doi: 10.1007/978-0-387-74520-6_6

Nisiotou, A., Rantsiou, K., Iliopoulos, V., Cocolin, L., and Nychas, G.-J. (2011). Bacterial species associated with sound and *Botrytis*-infected grapes from a Greek vineyard. *Int. J. Food Microbiol.* 145, 432–436. doi: 10.1016/j.ijfoodmicro.2011.01.017

Pinto, C., Pinho, D., Sousa, S., Pinheiro, M., Egas, C., and Gomes, A. (2014). Unravelling the diversity of grapevine microbiome. *PLoS ONE* 9:e85622. doi: 10.1371/journal.pone.0085622

Pretorius, I. (2000). Tailoring wine yeast for the new millennium: novel approaches to the ancient art of winemaking. *Yeast* 16, 675–729. doi: 10.1002/1097-0061(20000615)16:8<675::AID-YEA585>3.0.CO;2-B

Renouf, V., Claisse, O., and Lonvaud-Funel, A. (2005). Understanding the microbial ecosystem on the grape berry surface through numeration and identification of yeast and bacteria. *Aust. J. Grape Wine Res.* 11, 316–327. doi: 10.1111/j.1755-0238.2005.tb00031.x

Renouf, V., Claisse, O., and Lonvaud-Funel, A. (2007). Inventory and monitoring of wine microbial consortia. *Appl. Microbiol. Cell Physiol.* 75, 149–164. doi: 10.1007/s00253-006-0798-3

Renouf, V., and Lonvaud-Funel, A. (2007). Development of an enrichment medium to detect *Dekkera/Brettanomyces bruxellensis*, a spoilage wine yeast, on the surface of grape berries. *Microbiol. Res.* 162, 154–167. doi: 10.1016/j.micres.2006.02.006

Romano, P., Fiore, C., Paraggio, M., Caruso, M., and Capece, A. (2003). Function of yeast species and strains in wine flavour. *Int. J. Food Microbiol.* 86, 169–180. doi: 10.1016/S0168-1605(03)00290-3

Ruiz, P., Seseña, S., Izquierdo, P. M., and Palop, M. L. (2010). Bacterial biodiversity and dynamics during malolactic fermentation of Tempranillo wines as determined by a culture-independent method (PCR-DGGE). *Appl. Microbiol. Biotechnol.* 86, 1555–1562. doi: 10.1007/s00253-010-2492-8

Schloss, P., Westcott, S., Ryabin, T., Hall, J. R., Hartmann, M., Hollister, E. B., et al. (2009). Introducing mothur: open-Source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl. Environ. Microbiol.* 75, 7537–7541. doi: 10.1128/AEM.01541-09

Schuller, D., Cardoso, F., Sousa, S., Gomes, P., Gomes, A. C., Santos, M. A. S., et al. (2012). Genetic diversity and population structure of *Saccharomyces cerevisiae* strains isolated from different grape varieties and winemaking regions. *PLoS ONE* 7:e32507. doi: 10.1371/journal.pone.0032507

Setati, M., Jacobson, D., Andong, U., and Bauer, F. (2012). The vineyard yeast microbiome, a mixed model microbial map. *PLoS ONE* 7:e52609. doi: 10.1371/journal.pone.0052609

Sharpton, T., Riesenfeld, S., Kembel, S., Ladau, J., O'Dwyer, J. P., Green, J. L., et al. (2011). PhylOTU: a high-throughput procedure quantifies microbial community diversity and resolves novel taxa from metagenomic data. *PLoS Comput. Biol.* 7:e1001061. doi: 10.1371/journal.pcbi.1001061

Sogin, M., Morrison, H., Huber, J., Mark Welch, D., Huse, S. M., Neal, P. R., et al. (2006). Microbial diversity in the deep sea and the underexplored "rare biosphere." *Proc. Natl. Acad. Sci. U.S.A.* 103, 12115–12120. doi: 10.1073/pnas.0605127103

Suárez, R., Suárez-Lepe, J. A., Morata, A., and Calderón, F. (2007). The production of ethylphenols in wine by yeasts of the genera *Brettanomyces and Dekkera*: a review. *Food Chemis.* 102, 10–21. doi:10.1016/j.foodchem.2006.03.030

Taylor, M. W., Tsai, P., Anfang, N., Ross, H. A., and Goddard, M. R. (2014). Pyrosequencing reveals regional differences in fruit-associated fungal communities. *Environ. Microbiol.* 16, 2848–2858. doi: 10.1111/1462-2920.12456.

Toit, M., and Pretorius, I. (2000). Microbial spoilage and preservation of wine: using weapons from nature's own arsenal - a review. *S. Afr. J. Enol. Viticulture* 21, 74–96.

Torija, M., Rozès, N., Poblet, M., Guillamón, J., and Mas, A. (2001). Yeast population dynamics in spontaneous fermentations: comparison between two different wine-producing areas over a period of three years. *Antonie Van Leeuwenhoek* 79, 345–352. doi: 10.1023/A:1012027718701

Van Leeuwen, C., and Seguin, G. (2006). The concept of terroir in viticulture. *J. Wine Res.* 17, 1–10. doi: 10.1080/09571260600630631

Vaz-Moreira, I., Egas, C., Nunes, O., and Manaia, C. (2011). Culture-dependent and culture-independent diversity surveys target different bacteria: a case study in a freshwater sample. *Antonie Van Leeuwenhoek* 100, 245–257. doi: 10.1007/s10482-011-9583-0

White, T., Bruns, T., Lee, S., and Taylor, J. (1990). "Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics," in *Pcr Protocols: A Guide to Methods and Applications*, eds M. Innis, D. Gelfand, J. Sninsky, and T. White (New York, NY: Academic Press), 315–322.

Zoecklein, B., Williams, J., and Duncan, S. (2000). Effect of sour rot on the composition of white riesling (*Vitis vinifera* l.) grapes. *Small Fruits Rev*. 1, 63–77. doi: 10.1300/J301v01n01_08

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Insights into the bacterial community and its temporal succession during the fermentation of wine grapes

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Grapes harbor complex microbial communities. It is well known that yeasts, typically *Saccharomyces cerevisiae*, and bacteria, commonly the lactic acid fermenting *Oenococcus oeni*, work sequentially during primary and secondary wine fermentation. In addition to these main players, several microbes, often with undesirable effects on wine quality, have been found in grapes and during wine fermentation. However, still little is known about the dynamics of the microbial community during the fermentation process. In previous studies culture dependent methods were applied to detect and identify microbial organisms associated with grapes and grape products, which resulted in a picture that neglected the non-culturable fraction of the microbes. To obtain a more complete picture of how microbial communities change during grape fermentation and how different fermentation techniques might affect the microbial community composition, we employed next-generation sequencing (NGS)—a culture-independent method. A better understanding of the microbial dynamics and their effect on the final product is of great importance to help winemakers produce wine styles of consistent and high quality. In this study, we focused on the bacterial community dynamics during wine vinification by amplifying and sequencing the hypervariable V1–V3 region of the 16S rRNA gene—a phylogenetic marker gene that is ubiquitous within prokaryotes. Bacterial communities and their temporal succession was observed for communities associated with organically and conventionally produced wines. In addition, we analyzed the chemical characteristics of the grape musts during the organic and conventional fermentation process. These analyses revealed distinct bacterial population with specific temporal changes as well as different chemical profiles for the organically and conventionally produced wines. In summary these results suggest a possible correlation between the temporal succession of the bacterial population and the chemical wine profiles.

Keywords: wine bacteria, wine fermentation, temporal succession, organic grape products, 16S rRNA gene profile, next-generation sequencing

Introduction

Wine is an alcoholic beverage that is produced by fermenting grapes and represents a heterogeneous mixture of complex compounds. Many of the wines' compounds contribute to their characteristic color, aroma, and flavor (Styger et al., 2011; González-Barreiro et al., 2015), and are released during the fermentation process. The metabolic conversion of grape juice into wine is a complex process of alcoholic fermentation and malolactic fermentation (MLF) and involves a mixture of different microorganisms (Fugelsang and Edwards, 2007). Yeasts play important roles during the alcoholic fermentation step and have significant impact on wine quality. Although bacteria are not the main driving force behind wine characteristics and quality, they do have a significant effect on the final product. For example, lactic acid bacteria are known to convert L-malic acid to lactic acid through MLF and to impart flavor complexity, while acetic acid bacteria (AAB) produce acetic acid, which is a key factor in wine spoilage. MLF is important in winemaking by regulating deacidification and microbial stability. MLF usually occurs after the alcoholic fermentation but it may occur during the alcoholic fermentation process. It is possible that monitoring bacterial community profiles during alcoholic fermentation might allow predicting and controlling wine quality more efficiently. Microorganisms that are present during the various stages of vinification have significant impact on the wine quality both positively and negatively (Fleet, 1993; Fugelsang and Edwards, 2007). To ensure consistent high quality wines and allow reliable risk management, it is essential to monitor the microbial populations throughout the vinification process. NGS represents a fast and precise approach to obtain high-resolution insights into the population dynamics.

In past years, several microorganisms have been found in association with wine grapes and wine musts using culture-dependent techniques (Cappello et al., 2004). These conventional microbiology methods facilitated the isolation of a number of yeasts (e.g., *Brettanomyces/Dekkera, Issatchenkia, Zygoascus*, and *Zygosaccharomyces*) (Curtin et al., 2007; Barata et al., 2012; Di Toro et al., 2015), AAB (e.g., *Acetobacter and Gluconacetobacter*) (Barata et al., 2012), and lactic acid bacteria (e.g., *Enterococcus, Lactobacillus, Lactococcus, Oenococcus,* and *Pediococcus*) (Beneduce et al., 2004; Bae et al., 2006; Capozzi et al., 2010; Garofalo et al., 2015). Due to the viable but non-culturable nature of many wine microorganisms or the dominance of a few organisms that grow very well under laboratory conditions, these conventional microbiology approaches resulted in a rather incomplete and biased picture of the microbial community that is involved in the fermentation process (Millet and Lonvaud-Funel, 2000; Oliver, 2005; Cocolin et al., 2013). In more recent years, a culture-independent method called PCR-DGGE, which combines polymerase chain reaction (PCR) with denaturing gradient gel electrophoresis (DGGE), has been frequently used for detecting specific microorganisms during different stages of the wine fermentation process (Renouf et al., 2007; Spano et al., 2007; Andorrá et al., 2008; Laforgue et al., 2009; Pérez-Martín et al., 2014). Although PCR-DGGE remains a useful tool to detect and discriminate microbial organisms potentially present in wine grapes and musts without cultivation, it has its limitation due to the challenge of distinguishing co-migrating bands from multiplexed PCR products and requirement of intensive bands (Laforgue et al., 2009; Cocolin et al., 2013). With next-generation sequencing (NGS) technologies being a commodity now, powerful tools for high-throughput analysis of complex microbial communities via amplification and subsequent sequencing of the 16S ribosomal RNA (rRNA) hypervariable regions are now available (Sinclair et al., 2015). NGS have been applied widely and resulted in new insights into microbial community dynamics from diverse environmental samples (Piao et al., 2014; Trexler et al., 2014; Nguyen and Landfald, 2015; Pessoa-Filho et al., 2015) including grape and botrytized wine (Bokulich et al., 2012, 2014), but it is still not well known how the microbial communities associated with different grapes change over time and how these changes affect the final quality of the fermentation products.

There has been a fast growing demand for organic foods and beverages and the market for organically produced wines has experienced a significant boost. To obtain an enhanced understanding of how the different winemaking techniques affect bacterial community dynamics and further find out the bacterial community dynamics affect wine fermentation, we analyzed the temporal succession of the bacterial community and its effects on the changes of chemical characteristics during organic and conventional wine fermentation using 16S rRNA amplicon sequencing. The obtained results revealed a broad bacterial diversity in wine including known wine bacteria. Many of the identified organisms have to our knowledge not been reported to date. By analyzing the dynamics of the bacterial population during the fermentation process, it was possible to detect bacteria that were previously not associated with wine fermentation. The chemical characteristics of the wines, combined with the results of bacterial community profiles, indicated that there might be a possible link between specific bacteria, their succession and some wine characteristics.

Materials and Methods

Sample Collection

Both organic and conventional pied-de-cuve (PDC) were obtained by stomping and fermenting hand-harvested organically grown Riesling grapes in a 200 gallon tote. No sulfur dioxide (SO_2) was added to the organic PDC fermentation, whereas SO_2 (55.8 mg/L) was added during the conventional PDC fermentation process. For organic and conventional bulk fermentation, the organically grown Riesling grapes were machine pressed and transferred to a 15,000 gallon fermentation tank. Juice was allowed to settle for 36 h before heavy solids were removed. When sugar content of the organic or conventional PDC reached approximately 10 Brix, the PDCs were transferred to bulk fermentation tanks. Fermentation temperature was maintained between 10 and 13°C. Neither SO_2 nor fining agents were added to the organic musts during primary fermentation, while SO_2 (38.5 mg/L) and bentonite were added to the conventional musts. Yeast assimilable nitrogen was added in the

form of autolyzed yeast product and diammonium phosphate (DAP) to the organic and conventional wine respectively. Brix and ethanol measurements were taken to monitor fermentation progress and fermentation was terminated when a Brix of 2.5 and 6.9 was reached for organic and conventional wine, respectively.

DNA Extraction and 16S rRNA Gene Amplification

Total microbial DNA was extracted from 500 mg of the organic and conventional wine samples using a FastDNA SPIN Kit for Soil (MP Biomedical, Solon, OH) according to the manufacturer's instructions. Extracted DNA was quantified with a spectrophotometer (Nanodrop ND1000; Thermo Scientific, USA). The hypervariable V1–V3 region of the 16S rRNA gene was amplified from the environmental DNA using the primer set 28F/519R (28F: 5′-ccatctcatccctgcgtgtctccgactcagxxxxxxxxGAG TTTGATCNTGGCTCAG-3′ and 519R: 5′-cctatcccctgtgtgccttg gcagtctcagGTNTTACNGCGGCKGCTG-3′). Primer sequences were modified by the addition of 454 A or B adapter sequences (lower case) and ended with the sequencing key "TCAG" (underlined). In addition, the forward primer included a 8 bp barcode, indicated by xxxxxxxx in the forward primer sequence above, for multiplexing of samples during sequencing. The barcode sequence for each sample is listed in Table S1.

The V1–V3 region of the 16S rRNA genes was amplified with primer pair 28F/519R by emulsion PCR. Subsequent PCR reactions were performed using the Roche Live amplification mix (according to the Roche protocol) with the following PCR conditions: initial denaturation for 1 min at 94°C, followed by 50 amplification cycles of (30 s at 94°C, 4.5 min at 58°C, and 30 s at 68°C), and hold at 10°C. Emulsion PCR and sequencing of the PCR amplicons were performed following the Roche 454 GS FLX Titanium technology instructions provided by the manufacturer.

Data Analysis

Raw pyrosequencing data were demultiplexed and processed using QIIME version 1.7.0 (Caporaso et al., 2010b). Sequencing primers and barcodes were removed from the raw sequence reads by allowing 1.5 mismatches to the barcode and 2 mismatches to the primer sequence. Sequences were removed if they had homopolymeric regions of more than 6 nt, were smaller than 200 nt, had quality scores lower than 25, or if they were identified as being chimeric. This resulted in a total of 16,142 and 28,490 high quality 16S rRNA gene sequences from organic and conventional wine samples, respectively.

Quality filtered sequences were clustered into operational taxonomic units (OTUs) at a 97% sequence identity cut-off using UCLUST (Edgar, 2010). The most abundant sequence of each OTU was picked as representative sequence. Singleton and doubleton abundance, Shannon, Simpson, and Chao1 estimators were calculated using the QIIME software. Representative sequences were aligned using the PyNAST algorithm (Caporaso et al., 2010a) and the alignment was filtered to remove common gaps. Following the quality filtering and grouping steps, 1340 unique sequences (representing 44,632 total sequences) were aligned and taxonomically classified using the RDP classifier program (Wang et al., 2007) with 80% confidence rating against the Greengenes database (McDonald et al., 2012).

Chemical Analysis

Chemical analyses of the wine samples were performed at ETS Laboratories (Saint Helena, CA) using an Agilent 7700 inductively coupled plasma-mass spectrometer according to manufacturer's instructions and as described by Hopfer et al. (2013).

Results

Bacterial Community Profile of Organically and Conventionally Produced Wine

To determine bacterial community dynamics and their effects on wine components, we compared the profiles of the bacterial community in wines that were produced using organic and conventional fermentation protocols. Grape juice was inoculated with indigenous yeasts from the grape skins by adding PDC. This traditional wine making technique reduces the needs for commercial yeast and usually increases wine complexity. Samples for bacterial community profiling were collected from the PDC (0 day) and must at different fermentation stages after PDC was added to the grape juice. Environmental DNA was extracted from PDC and must followed by pyrosequencing of the hypervariable V1–V3 region of the 16S rRNA genes. The quality-filtered pyrotag reads were clustered into OTUs at a 97% of sequence identity level, which resulted in 529 and 1099 distinct OTUs, representing 16,142 and 28,490 sequences from organic and conventional wine, respectively (**Table 1**). Analysis of OTUs profiles suggests that community richness within organic wine was stable at early stage of fermentation (0, 2, and 3 days; **Table 1**; Table S2). Continuing the fermentation process, increased community richness at 10 days was measured, whereas decreased community richness was observed afterwards (**Table 1**; Table S2). Compared to organically producing wine, bacterial community richness increased significantly at 6 days of fermentation (**Table 1**; Table S2) then decreased rapidly within 24 h (**Table 1**; Table S2) during conventional wine production. These findings are supported by the calculated rarefaction curves (Figure S1). Shannon's diversity and Simpson indices are higher

TABLE 1 | Summary of generated reads and OTUs observed.

Duration of fermentation [days]	Organic						Conventional					
	0	2	3	10	16	Total	0	2	6	7	12	Total
Quality filtered reads	5,420	3,569	4,188	1,583	1,382	16,142	16,001	1,531	7,588	2,127	1,243	28,490
OTUs observed	173	165	176	202	146	529	268	201	612	220	160	1099

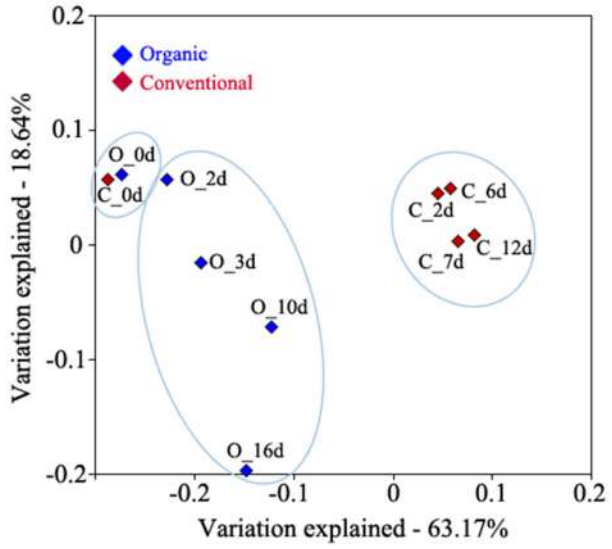

FIGURE 1 | Principal component analysis of 16S rRNA data from microbiomes associated with grape must during the fermentation process. 16S rRNA amplicon data was generated from PDC (O_0d and C_0d) and during organic (O_2d, O_3d, O_10d, and O_16d) and conventional (C_2d, C_6d, C_7d, and C_12d) bulk fermentation. The percentage of variation explained by the plotted principal coordinates is indicated on the axes.

in conventionally fermented wine (Table S2), suggesting that the bacterial community in conventionally produced wine became more diverse than in organically produced wine. Principal component analysis suggests that the wine microbiome profiles associated with grape must during conventional fermentation were distinct from the microbiome profiles associated with grape must from organic fermentation (**Figure 1**).

Phylogenetic Profiles of the Bacterial Communities during the Fermentation Processes

Clustering of the obtained 16S rRNA gene sequences based on a 97% sequence identity cut-off and assigning phylogeny to each of the obtained OTUs suggest that a total of 15 phyla (contributing $\geq$1 of the reads) were present during the fermentation process of the two grape musts under observation (**Figure 2** and Table S3). Nine of the observed 15 phyla were found in musts from both fermentation techniques (i.e., *Proteobacteria*, *Cyanobacteria*, *Bacteroidetes*, *Firmicutes*, *Actinobacteria*, *Acidobacteria*, *Spirochaetes*, *Verrucomicrobia*, and *Fusobacteria*), while the presence of some phyla depended on the applied fermentation technique. Specifically, *Nitrospirae*, *Planctomycetes*, and *Tenericutes* were detected solely in the samples from organically fermented must, whereas *Fibrobacteres* and members of the candidate phylum WYO were detected only in the conventionally produced wine musts (**Figure 2** and Table S3). It is possible that members of these specific phyla might contribute to the distinct chemical characteristics of the produced wines. *Proteobacteria* is the predominant phylum in both wine musts (**Figure 2** and Table S3), which was represented primarily by the *Gammaproteobacteria* within the PDC (0 day). During fermentation the relative abundance of *Gammaproteobacteria* decreased significantly in both wine musts (6–8 fold), which was partially complemented by an increase of other members of the *Proteobacteria*, i.e., *Alphaproteobacteria*, *Betaproteobacteria,* and *Deltaproteobacteria* (**Table 2**). During organic fermentation, the abundance of *Alphaproteobacteria* increased and this phylogenetic group became the dominant class (57% at 15 days). During conventional fermentation, population of *Alphaproteobacteria* increased as well (∼4.5 fold) but did not dominate the community (21.72–27.63%). Abundance of *Betaproteobacteria* increased 250–380 fold to a relative abundance between 18.15 and 27.10% (**Table 2**). Overall population changes suggest a notable reduction of *Proteobacteria* (**Figure 2** and Table S3), which is similar to what has been observed previously during botrytized wine fermentation (Bokulich et al., 2012). This decrease in *Proteobacteria*, specifically of the *Gammaproteobacteria*, was accompanied by an increase of the *Bacteroidetes*, *Firmicutes,* and *Actinobateria*. The increase was in particular notable within the microbiome from the conventionally fermented wine, while the increase was less notable within the microbiome from organically fermented wine (**Table 2** and Table S3). Within the conventionally fermented wine, the increase of abundance of *Bacteroidetes* was caused through a significant increase in *Spingobacteriia* and a moderate increase in *Bacteroidia* (**Figure 2**; **Table 2** and Table S3). The increase of *Firmicutes* was due largely to an increase of the *Bacilli* and a moderate increase of the *Clostridia* (**Table 2**). Further analysis of the bacterial community resulted in the detection of 96 genera across all samples, of which 33 genera were found both in organically and conventionally fermented must. Twenty-one of the 96 genera were detected only within the bacterial communities associated with organically fermented must, whereas 42 genera were found only within the bacterial communities associated with conventionally fermented grapes (**Table 3**). Increased genus diversity was observed for the microbiome from conventionally fermented must (75 genera total) when compared to the microbiome from organically fermented must (54 genera total). Representatives of the genus *Gluconobacter*, an acetic acid bacterium commonly found associated with grape skin (Joyeux et al., 1984), was detected in the microbiome of both wine types, however discrete changes within the *Gluconobacter* population were observed between organically and conventionally fermented wines. Comparison between organically and conventionally produced wines revealed that the population of *Gluconobacter* was highly abundant in organic PDC fermentation (8.67% at 0 day), while it possessed very low abundance in conventional PDC fermentation (0.47% at 0 day; **Table 3**). During the fermentation process, the *Gluconobacter* population increased in both musts and eventually represented the predominant genus from organically produced wine at late stage (49%; 16 day), while it was relatively stable, accounting for 5–7% of population, throughout the conventional fermentation process (5–7%; **Table 3**). Beside the dominant genus *Gluconobacter*, a number of other genera (total sequences detected >1% in data from at least one of the time points) were also detected during both fermentation procedures (i.e., *Clavibacter*, *Propionibacterium*, *Hymenobacter*, *Pedobacter*, *Bacillus*, *Staphylococcus*, *Acetobacter*,

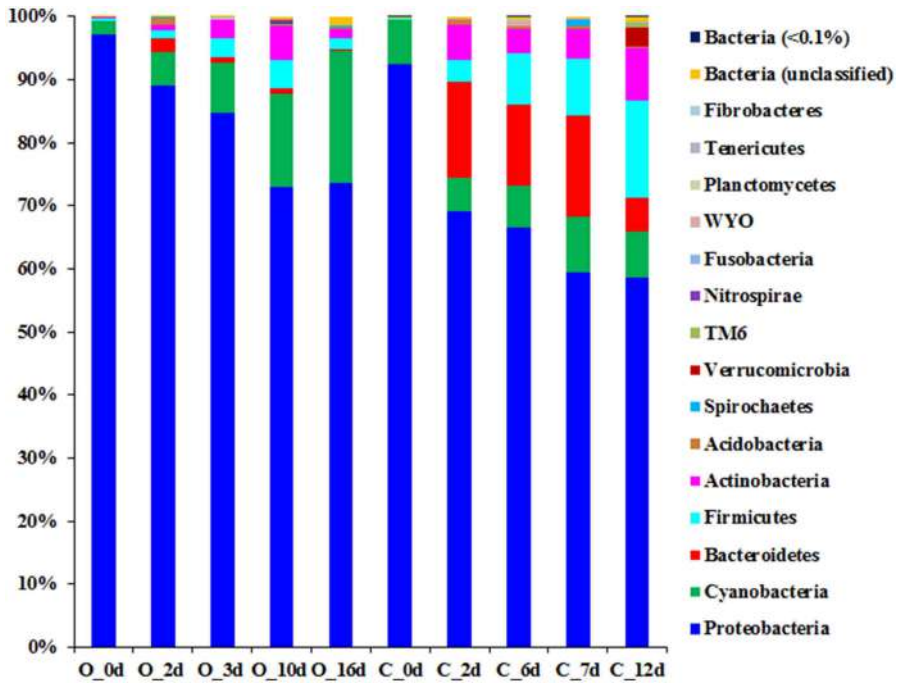

FIGURE 2 | Phylogenetic profile of microbiomes associated with grape must during the fermentation process. Phylogeny was assigned in the phylum level based on the RDP database after quality-filtered reads were clustered using 97% sequence identity cut-off.

Spingomonas, *Diaphorobacter*, *Janthinobacterium*, *Ralstonia*, *Neisseria*, *Acinetobacter, Pseudomonas*, and *Leptospira*), with *Pedobacter*, *Spingomonas*, *Janthinobacterium*, and *Pseudomonas* exhibiting dominance only during the conventional fermentation process (**Table 3**). In addition, other less abundant phylogenetic groups (total sequences detected between 0.1 and 1%) were observed during the two distinct fermentation processes (i.e., *Corynebacterium*, *Micrococcus*, *Sediminibacterium*, *Dyadobacter*, *Exiguobacterium*, *Lactobacillus*, *Clostridium*, *Roseburia*, *Faecalibacterium*, *Fusobacterium*, *Bradyrhizobium*, *Methylobacterium*, *Roseomonas*, *Salinispora*, *Curvibacter*, *Pelomonas*, *Trabulsiella*, and *Haemophilus*) (**Table 3**). Interestingly, *Oenococcus*, a genus containing known lactic acid bacteria, was detected only in the microbiome of conventionally fermented wine (**Table 3**).

Chemical Component Analysis from Organic and Conventional Wine

Several parameters, such as sugar concentration, temperature, pH value, ethanol concentration and a variety of chemical characteristics, of the grape must were monitored during the fermentation process (**Figure 3** and **Table 4**). Sugar concentrations were stable until 3 days into the fermentation process, after this period sugar concentration decreased linearly in both wine fermentations (**Figure 3A**). Overall pH values were slightly lower from organically produced wine than conventionally produced wine, while ethanol reached a higher concentration during the organic fermentation process (**Figures 3C,D**). Lactic acid concentration at the end of the organic PDC fermentation was higher, while it was same in both wine fermentation processes, suggesting that wine fermentation was terminated before secondary fermentation was initiated. Malic acid content increased during both fermentation processes, however overall malic acid content was higher in conventionally fermented wine. Volatile acidity (VA) content changed irregularly, at early stage of fermentation (2–3 days) lower VA contents were measured for both types of wine samples, afterwards it increased to about three-fold in conventionally fermented wine, while it returned to first day level in organically fermented wine. Overall tartaric acid concentration was higher in organically fermented wine compare to conventionally fermented wine. A summary of the chemical characteristics of the grape musts is provided in **Table 4**. Initial nitrogen concentration was similar in both juices at the first day of fermentation and additional nitrogen was provided during the fermentation process to support continuous growth of yeast. Nitrogen concentrations are summarized in **Table 4**. More detailed and controlled studies will help to enhance our understanding of the molecular processes and microbe-microbe and microbe-must interaction would be of great value.

Discussion

Culture-independent NGS is a cost-effective approach to study composition and the spatial and temporal changes of microbial communities and it has been applied to various environment samples (Piao et al., 2014; Nguyen and Landfald, 2015). However, to our knowledge, as of today only a few studies have been

TABLE 2 | Relative abundance of prokaryotes associated with grape musts during organic and conventional fermentation at the class level.

Duration of fermentation [days]	Organic					Conventional				
	0	2	3	10	16	0	2	6	7	12
Acidobacteria;c_Acidobacteria-2	0.00	0.90	0.00	0.38	0.29	0.00	0.72	0.46	0.33	0.00
Actinobacteria;c_Actinobacteria	0.06	0.98	2.96	5.31	1.52	0.05	5.68	3.58	4.84	8.21
Actinobacteria;c_Thermoleophilia	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.13	0.00	0.00
Bacteroidetes;c_Bacteroidia	0.04	0.03	0.05	0.32	0.00	0.01	2.55	1.74	2.77	0.24
Bacteroidetes;c_Flavobacteriia	0.00	0.73	0.00	0.13	0.00	0.00	0.26	0.58	0.47	0.00
Bacteroidetes;c_Sphingobacteriia	0.02	1.37	0.72	0.38	0.14	0.02	12.48	10.46	12.60	5.15
Cyanobacteria;c_4C0d-2	0.04	0.03	0.00	0.00	0.00	0.00	0.13	0.91	0.00	0.00
Cyanobacteria;c_S15B-MN24	0.02	0.11	0.31	0.63	0.43	0.00	0.98	0.61	1.13	2.65
Cyanobacteria;c_Synechococcophycideae	0.00	0.00	0.00	1.01	0.00	0.00	0.00	0.00	0.00	0.00
Fibrobacteres;c_Fibrobacteria	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.18	0.00	0.00
Firmicutes;c_Bacilli	0.44	0.98	2.65	3.79	1.52	0.19	1.44	6.80	5.88	10.62
Firmicutes;c_Clostridia	0.02	0.17	0.43	0.76	0.22	0.06	1.96	1.32	3.10	4.83
Fusobacteria;c_Fusobacteria	0.00	0.00	0.00	0.13	0.00	0.01	0.00	0.00	0.00	0.16
Nitrospirae;c_Nitrospira	0.07	0.00	0.00	0.32	0.00	0.00	0.00	0.00	0.00	0.00
Planctomycetes;c_Planctomycetia	0.00	0.00	0.19	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Proteobacteria;c_Alphaproteobacteria	12.49	9.78	24.52	30.32	57.16	5.82	27.63	22.26	23.93	21.72
Proteobacteria;c_Betaproteobacteria	0.06	4.82	3.15	7.14	2.53	0.07	22.73	27.10	18.15	23.65
Proteobacteria;c_Deltaproteobacteria	0.11	0.31	0.00	0.44	0.43	0.02	0.00	0.29	1.50	1.93
Proteobacteria;c_Gammaproteobacteria	84.59	74.05	57.07	34.87	13.46	86.56	18.68	16.75	15.84	11.34
Spirochaetes;c_Leptospirae	0.00	0.22	0.05	0.19	0.14	0.00	0.07	0.00	1.18	0.00
Tenericute;c_Mollicutes	0.00	0.00	0.14	0.00	0.00	0.00	0.00	0.03	0.00	0.00
TM6;c_SJA-4	0.00	0.00	0.05	0.06	0.07	0.00	0.00	0.00	0.00	0.72
Verrucomicrobia;c_Opitutae	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	3.06
Verrucomicrobia;c_Verruco-5	0.00	0.00	0.00	0.13	0.00	0.00	0.00	0.00	0.00	0.00

published that employed NGS to study the dynamics of the microbial wine ecosystem (Bokulich et al., 2012, 2014, 2015). To enhance our understanding of the microbial dynamics, specifically of bacterial dynamics, during grape fermentation, we employed culture-independent 16S rRNA amplicon sequencing to determine changes in the bacterial population of grape must during the fermentation process. Currently, the most commonly used culture-independent method within the wine industry for comparing microbial populations associated with different grape products is PCR-DGGE (Cocolin et al., 2000; Lopez et al., 2003). PCR-DGGE possesses only a limited ability to provide detailed information about biodiversity within a sample as bands associated with different phylogenetic groups might be visible as a single band resulting in underestimation of microbial community diversity.

In this study we identified 96 genera and discriminated over 30 species that were present during wine fermentation. Importantly, most of the species we detected have not been reported previously during wine fermentation (Table S4), with the exception of a few species (i.e., *Propionibacterium acnes*, *Bacillus thermoamylovorans*, *Pseudomonas stutzeri*) that were isolated from grapevine, palm wine, and wine corks (Combet-Blanc et al., 1995; Bañeras et al., 2013; Yousaf et al., 2014). The genus *Gluconobacter* increased significantly during organic fermentation (from 3.28 to 49.42%), while it exhibited less notable changes during the conventional fermentation (from 5.63 to 7.57%) process (**Table 3**). A major difference of the organic and conventional wine making processes employed in this study was the addition of SO_2 to the conventional wine prior to PDC fermentation (50 mg/L) and bulk fermentation (38.5 mg/L), while no SO_2 added to the organic wine until completion of primary fermentation. The availability of SO_2 during primary fermentation might represent a selective effect on the *Gluconobacter* population. Bokulich and colleagues showed that *Gluconobacter* population was significantly suppressed by SO_2 at concentrations $\geq$25 mg/L (Bokulich et al., 2015). At higher taxonomic resolution the genus *Gluconobacter* was dominated by one distinct OTU (i.e., OTU denovo952) during the fermentation process (Table S5). To further define this specific OTU, its representing nucleotide sequence was compared to sequences deposited in NCBI database. Results revealed a 99.6% sequence identity with *Gluconobacter oxydans*, the main representative of AAB on grapes (Joyeux et al., 1984). *Gluconobacter oxydans* is known as spoilage acetic acid bacterium together with *Acetobacter* during winemaking; *Gluconobacter oxydans* is often detected in grapes, while *Acetobacter* is found in wine (Bartowsky and Henschke, 2008). Although AAB have been identified as wine spoilage bacteria previously, the population of AAB are often underestimated with culture-dependent method due to the lack of appropriate cultivation techniques (Millet and Lonvaud-Funel, 2000). Amplicon sequencing data allowed us to observe significant population changes of *Gluconobacter*

TABLE 3 | Relative abundance of prokaryotes associated with grape musts during organic and conventional fermentation at the genus level.

Duration of fermentation [days]	Organic					Conventional				
	0	2	3	10	16	0	2	6	7	12
Actinobacteria;c_Actinobacteria;o_Actinomycetales;f_Corynebacteriaceae;g_Corynebacterium	0.00	0.00	0.79	0.69	0.29	0.01	0.39	0.18	0.24	0.48
Actinobacteria;c_Actinobacteria;o_Actinomycetales;f_Dermabacteraceae;g_Brachybacterium	0.00	0.00	0.00	0.00	0.00	0.00	0.20	0.00	0.00	0.00
Actinobacteria;c_Actinobacteria;o_Actinomycetales;f_Dietziaceae;g_Dietzia	0.00	0.00	0.00	0.00	0.14	0.01	0.00	0.00	0.00	0.00
Actinobacteria;c_Actinobacteria;o_Actinomycetales;f_Gordoniaceae;g_Gordonia	0.00	0.00	0.00	1.45	0.00	0.00	0.00	0.00	0.00	0.24
Actinobacteria;c_Actinobacteria;o_Actinomycetales;f_Microbacteriaceae;g_Clavibacter	0.00	0.00	0.19	0.32	0.00	0.00	0.20	0.00	0.00	1.53
Actinobacteria;c_Actinobacteria;o_Actinomycetales;f_Microbacteriaceae;g_Curtobacterium	0.00	0.08	0.00	0.00	0.00	0.00	0.20	0.67	0.47	0.00
Actinobacteria;c_Actinobacteria;o_Actinomycetales;f_Microbacteriaceae;g_Frigoribacterium	0.00	0.00	0.00	0.00	0.00	0.00	0.26	0.00	0.00	0.00
Actinobacteria;c_Actinobacteria;o_Actinomycetales;f_Microbacteriaceae;g_Leucobacter	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.03	0.00
Actinobacteria;c_Actinobacteria;o_Actinomycetales;f_Microbacteriaceae;g_Microbacterium	0.00	0.34	0.05	0.00	0.00	0.00	0.00	0.05	0.00	0.00
Actinobacteria;c_Actinobacteria;o_Actinomycetales;f_Microbacteriaceae;g_Rathayibacter	0.00	0.00	0.00	0.00	0.00	0.00	0.26	0.18	0.00	0.00
Actinobacteria;c_Actinobacteria;o_Actinomycetales;f_Microbacteriaceae;g_Salinibacterium	0.00	0.00	0.00	0.00	0.00	0.00	0.46	0.12	0.00	0.00
Actinobacteria;c_Actinobacteria;o_Actinomycetales;f_Micrococcaceae;g_Kocuria	0.00	0.00	0.00	0.00	0.14	0.00	0.00	0.00	0.00	0.00
Actinobacteria;c_Actinobacteria;o_Actinomycetales;f_Micrococcaceae;g_Micrococcus	0.00	0.00	0.00	0.76	0.00	0.00	0.33	0.11	0.28	0.08
Actinobacteria;c_Actinobacteria;o_Actinomycetales;f_Mycobacteriaceae;g_Mycobacterium	0.00	0.08	0.00	0.06	0.00	0.00	0.00	0.03	0.00	0.56
Actinobacteria;c_Actinobacteria;o_Actinomycetales;f_Nocardiaceae;g_Rhodococcus	0.00	0.00	0.26	0.00	0.00	0.00	0.07	0.00	0.00	0.00
Actinobacteria;c_Actinobacteria;o_Actinomycetales;f_Propionibacteriaceae;g_Propionibacterium	0.06	0.48	0.55	1.52	0.43	0.03	0.65	1.75	1.97	4.51
Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Bacteroidaceae;g_Bacteroides	0.04	0.00	0.00	0.00	0.00	0.01	0.07	0.03	0.47	0.00
Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Prevotellaceae;g_Prevotella	0.00	0.03	0.00	0.00	0.00	0.00	0.52	0.37	0.71	0.16
Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_[Paraprevotellaceae];g_[Prevotella]	0.00	0.00	0.00	0.00	0.00	0.00	0.20	0.01	0.05	0.00
Bacteroidetes;c_Flavobacteriia;o_Flavobacteriales;f_Flavobacteriaceae;g_Chryseobacterium	0.00	0.00	0.00	0.06	0.00	0.00	0.26	0.46	0.47	0.00
Bacteroidetes;c_Sphingobacteriia;o_Sphingobacteriales;f_Chitinophagaceae;g_Sediminibacterium	0.00	0.00	0.00	0.13	0.00	0.00	0.00	0.21	0.09	0.80
Bacteroidetes;c_Sphingobacteriia;o_Sphingobacteriales;f_Chitinophagaceae;g_Segetibacter	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.04	0.47	0.00
Bacteroidetes;c_Sphingobacteriia;o_Sphingobacteriales;f_Flexibacteraceae;g_Dyadobacter	0.00	0.28	0.00	0.19	0.00	0.00	0.00	0.17	0.00	0.00
Bacteroidetes;c_Sphingobacteriia;o_Sphingobacteriales;f_Flexibacteraceae;g_Hymenobacter	0.00	0.56	0.10	0.00	0.00	0.00	1.05	1.79	1.74	1.53
Bacteroidetes;c_Sphingobacteriia;o_Sphingobacteriales;f_Flexibacteraceae;g_Spirosoma	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.18	0.00	0.00
Bacteroidetes;c_Sphingobacteriia;o_Sphingobacteriales;f_Sphingobacteriaceae;g_Pedobacter	0.00	0.50	0.62	0.00	0.14	0.00	9.67	7.39	8.56	2.33
Cyanobacteria;c_Synechococcophycideae;o_Synechococcales;f_Synechococcaceae;g_Prochlorococcus	0.00	0.00	0.00	1.01	0.00	0.00	0.00	0.00	0.00	0.00
Fibrobacteres;c_Fibrobacteria;o_Fibrobacterales;f_Fibrobacteraceae;g_Fibrobacter	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.18	0.00	0.00
Firmicutes;c_Bacilli;o_Bacillales;f_Bacillaceae;g_Anoxybacillus	0.00	0.00	0.00	0.06	0.00	0.00	0.00	0.03	0.33	0.00
Firmicutes;c_Bacilli;o_Bacillales;f_Bacillaceae;g_Bacillus	0.02	0.87	1.27	0.95	0.43	0.08	0.33	0.12	0.00	1.77
Firmicutes;c_Bacilli;o_Bacillales;f_Bacillaceae;g_Geobacillus	0.00	0.00	0.00	0.25	0.00	0.00	0.00	0.00	0.00	0.00
Firmicutes;c_Bacilli;o_Bacillales;f_Bacillaceae;g_Terribacillus	0.00	0.00	0.00	0.00	0.51	0.00	0.00	0.00	0.00	0.00
Firmicutes;c_Bacilli;o_Bacillales;f_Paenibacillaceae;g_Paenibacillus	0.00	0.00	0.00	0.06	0.14	0.00	0.00	0.09	0.00	0.00
Firmicutes;c_Bacilli;o_Bacillales;f_Staphylococcaceae;g_Staphylococcus	0.07	0.00	0.91	0.69	0.00	0.04	0.33	1.67	0.52	2.98
Firmicutes;c_Bacilli;o_Bacillales;f_Thermoactinomycetaceae;g_Planifilum	0.00	0.11	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Firmicutes;c_Bacilli;o_Exiguobacterales;f_Exiguobacteraceae;g_Exiguobacterium	0.00	0.00	0.00	0.00	0.22	0.00	0.00	0.00	0.00	0.64

(Continued)

TABLE 3 | Continued

Duration of fermentation [days]	Organic					Conventional				
	0	2	3	10	16	0	2	6	7	12
Firmicutes;c_Bacilli;o_Lactobacillales;f_Aerococcaceae;g_Aerococcus	0.00	0.00	0.00	0.19	0.00	0.00	0.00	0.00	0.00	0.00
Firmicutes;c_Bacilli;o_Lactobacillales;f_Lactobacillaceae;g_Lactobacillus	0.07	0.00	0.24	0.00	0.00	0.00	0.13	0.00	0.00	0.00
Firmicutes;c_Bacilli;o_Lactobacillales;f_Leuconostocaceae;g_Leuconostoc	0.04	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Firmicutes;c_Bacilli;o_Lactobacillales;f_Leuconostocaceae;g_Oenococcus	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.83	4.00	0.88
Firmicutes;c_Bacilli;o_Lactobacillales;f_Leuconostocaceae;g_Weissella	0.00	0.00	0.00	0.13	0.00	0.00	0.00	0.00	0.00	0.00
Firmicutes;c_Bacilli;o_Lactobacillales;f_Streptococcaceae;g_Streptococcus	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.05	0.05	0.16
Firmicutes;c_Clostridia;o_Clostridiales;f_Clostridiaceae;g_Anaerococcus	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	3.30
Firmicutes;c_Clostridia;o_Clostridiales;f_Clostridiaceae;g_Clostridium	0.00	0.00	0.00	0.13	0.00	0.00	0.00	0.28	0.00	0.00
Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_Blautia	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.01	0.28	0.00
Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_Butyrivibrio	0.00	0.00	0.00	0.00	0.00	0.00	0.26	0.00	0.00	0.00
Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_Moryella	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.16
Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_Oribacterium	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.22	0.00	0.00
Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_Roseburia	0.00	0.00	0.00	0.19	0.00	0.00	0.00	0.01	0.00	1.37
Firmicutes;c_Clostridia;o_Clostridiales;f_Peptococcaceae;g_Desulfosporosinus	0.00	0.00	0.00	0.00	0.14	0.00	0.00	0.00	0.00	0.00
Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_Faecalibacterium	0.02	0.11	0.00	0.00	0.07	0.00	0.13	0.11	0.61	0.00
Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_Oscillospira	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.16	0.33	0.00
Firmicutes;c_Clostridia;o_Clostridiales;f_Veillonellaceae;g_Megamonas	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.03	0.14	0.00
Firmicutes;c_Clostridia;o_Clostridiales;f_Veillonellaceae;g_Veillonella	0.00	0.00	0.00	0.00	0.00	0.00	0.33	0.00	0.09	0.00
Fusobacteria;c_Fusobacteria;o_Fusobacteriales;f_Fusobacteriaceae;g_Fusobacterium	0.00	0.00	0.00	0.13	0.00	0.00	0.00	0.00	0.00	0.16
Nitrospirae;c_Nitrospira;o_Nitrospirales;f_Nitrospiraceae;g_Nitrospira	0.07	0.00	0.00	0.32	0.00	0.00	0.00	0.00	0.00	0.00
Proteobacteria;c_Alphaproteobacteria;o_Rhizobiales;f_Bradyrhizobiaceae;g_Balneimonas	0.00	0.00	0.00	0.00	0.14	0.00	0.00	0.00	0.00	0.00
Proteobacteria;c_Alphaproteobacteria;o_Rhizobiales;f_Bradyrhizobiaceae;g_Bosea	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.36	0.00	0.00
Proteobacteria;c_Alphaproteobacteria;o_Rhizobiales;f_Bradyrhizobiaceae;g_Bradyrhizobium	0.00	0.00	0.00	0.88	0.14	0.00	0.00	0.16	0.00	0.00
Proteobacteria;c_Alphaproteobacteria;o_Rhizobiales;f_Methylobacteriaceae;g_Methylobacterium	0.00	0.28	0.00	0.76	0.07	0.00	0.33	0.82	0.00	0.40
Proteobacteria;c_Alphaproteobacteria;o_Rhizobiales;f_Phyllobacteriaceae;g_Phyllobacterium	0.00	0.00	0.29	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Proteobacteria;c_Alphaproteobacteria;o_Rhizobiales;f_Rhizobiaceae;g_Agrobacterium	0.00	0.00	0.00	0.00	0.00	0.00	0.13	0.00	0.00	0.00
Proteobacteria;c_Alphaproteobacteria;o_Rhodospirillales;f_Acetobacteraceae;g_Acetobacter	0.00	0.00	0.29	0.00	0.29	0.00	0.65	0.32	2.07	0.24
Proteobacteria;c_Alphaproteobacteria;o_Rhodospirillales;f_Acetobacteraceae;g_Acidocella	0.00	0.00	0.00	0.00	0.00	0.00	0.26	0.00	0.00	0.00
Proteobacteria;c_Alphaproteobacteria;o_Rhodospirillales;f_Acetobacteraceae;g_Gluconobacter	8.67	3.28	19.56	17.50	49.42	0.47	7.32	6.10	7.57	5.63
Proteobacteria;c_Alphaproteobacteria;o_Rhodospirillales;f_Acetobacteraceae;g_Roseomonas	0.00	0.31	0.19	0.19	0.00	0.00	0.00	0.18	0.47	0.32
Proteobacteria;c_Alphaproteobacteria;o_Rhodospirillales;f_Rhodospirillaceae;g_Telmatospirillum	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.32
Proteobacteria;c_Alphaproteobacteria;o_Rickettsiales;f_Rickettsiaceae;g_Rickettsia	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.13	0.00
Proteobacteria;c_Alphaproteobacteria;o_Rickettsiales;f_Rickettsiaceae;g_Wolbachia	0.02	0.00	0.00	0.00	0.22	0.00	0.07	0.00	0.00	0.00
Proteobacteria;c_Alphaproteobacteria;o_Sphingomonadales;f_Sphingomonadaceae;g_Sphingomonas	0.02	1.01	0.41	0.95	0.80	0.00	8.43	9.83	6.58	6.60
Proteobacteria;c_Betaproteobacteria;o_Burkholderiales;f_Alcaligenaceae;g_Pigmentiphaga	0.00	0.00	0.00	0.00	0.00	0.00	0.07	0.08	0.42	0.00
Proteobacteria;c_Betaproteobacteria;o_Burkholderiales;f_Burkholderiaceae;g_Burkholderia	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.64
Proteobacteria;c_Betaproteobacteria;o_Burkholderiales;f_Burkholderiaceae;g_Salinispora	0.00	0.00	0.00	0.13	0.07	0.00	0.13	0.07	0.00	0.48

(Continued)

TABLE 3 | Continued

Duration of fermentation [days]	Organic					Conventional				
	0	2	3	10	16	0	2	6	7	12
Proteobacteria;c_Betaproteobacteria;o_Burkholderiales;f_Comamonadaceae;g_Curvibacter	0.00	0.00	0.17	0.06	0.36	0.00	0.00	0.24	0.00	0.08
Proteobacteria;c_Betaproteobacteria;o_Burkholderiales;f_Comamonadaceae;g_Diaphorobacter	0.00	0.17	0.00	0.13	0.00	0.00	0.00	0.00	0.00	1.05
Proteobacteria;c_Betaproteobacteria;o_Burkholderiales;f_Comamonadaceae;g_Methylibium	0.00	0.00	0.00	0.00	0.00	0.00	0.07	0.30	0.56	0.00
Proteobacteria;c_Betaproteobacteria;o_Burkholderiales;f_Comamonadaceae;g_Pelomonas	0.00	0.00	0.14	0.00	0.00	0.00	0.59	0.00	0.00	0.00
Proteobacteria;c_Betaproteobacteria;o_Burkholderiales;f_Comamonadaceae;g_Ramlibacter	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.04	0.47	0.00
Proteobacteria;c_Betaproteobacteria;o_Burkholderiales;f_Comamonadaceae;g_Rubrivivax	0.00	0.00	0.00	0.00	0.00	0.00	0.13	0.25	0.00	0.00
Proteobacteria;c_Betaproteobacteria;o_Burkholderiales;f_Comamonadaceae;g_Schlegelella	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.13	0.00	0.00
Proteobacteria;c_Betaproteobacteria;o_Burkholderiales;f_Comamonadaceae;g_Variovorax	0.00	0.03	0.00	0.00	0.00	0.00	0.20	0.16	0.00	0.00
Proteobacteria;c_Betaproteobacteria;o_Burkholderiales;f_Oxalobacteraceae;g_Janthinobacterium	0.00	0.84	0.38	0.63	0.14	0.00	7.12	8.91	5.59	1.37
Proteobacteria;c_Betaproteobacteria;o_Burkholderiales;f_Oxalobacteraceae;g_Ralstonia	0.00	0.00	0.05	1.83	0.36	0.03	0.59	0.18	0.09	2.98
Proteobacteria;c_Betaproteobacteria;o_Neisseriales;f_Neisseriaceae;g_Neisseria	0.00	0.00	0.10	0.25	0.00	0.00	0.13	0.00	0.00	1.05
Proteobacteria;c_Betaproteobacteria;o_Rhodocyclales;f_Rhodocyclaceae;g_KD1-23	0.00	0.00	0.00	0.00	0.00	0.00	0.26	0.00	0.00	0.00
Proteobacteria;c_Gammaproteobacteria;o_Alteromonadales;f_Shewanellaceae;g_Shewanella	0.00	0.00	0.00	0.13	0.00	0.00	0.00	0.00	0.00	0.00
Proteobacteria;c_Gammaproteobacteria;o_Enterobacteriales;f_Enterobacteriaceae;g_Candidatus Hamiltonella	0.00	0.00	0.00	0.00	0.00	0.00	0.26	0.00	0.00	0.00
Proteobacteria;c_Gammaproteobacteria;o_Enterobacteriales;f_Enterobacteriaceae;g_Citrobacter	0.00	0.28	0.00	0.00	0.00	0.00	0.00	0.04	0.05	0.00
Proteobacteria;c_Gammaproteobacteria;o_Enterobacteriales;f_Enterobacteriaceae;g_Erwinia	0.02	0.00	0.00	0.06	0.07	0.01	0.52	0.30	0.33	0.00
Proteobacteria;c_Gammaproteobacteria;o_Enterobacteriales;f_Enterobacteriaceae;g_Escherichia	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.24	0.00
Proteobacteria;c_Gammaproteobacteria;o_Enterobacteriales;f_Enterobacteriaceae;g_Trabulsiella	0.20	0.22	0.17	0.25	0.14	0.36	0.00	0.00	0.00	0.00
Proteobacteria;c_Gammaproteobacteria;o_Pasteurellales;f_Pasteurellaceae;g_Haemophilus	0.00	0.00	0.29	0.32	0.07	0.00	0.07	0.00	0.00	0.40
Proteobacteria;c_Gammaproteobacteria;o_Pseudomonadales;f_Moraxellaceae;g_Acinetobacter	0.00	0.20	0.14	0.00	0.14	0.00	0.33	1.16	3.06	4.99
Proteobacteria;c_Gammaproteobacteria;o_Pseudomonadales;f_Moraxellaceae;g_Enhydrobacter	0.00	0.00	0.00	0.00	0.00	0.00	0.20	0.12	0.00	0.00
Proteobacteria;c_Gammaproteobacteria;o_Pseudomonadales;f_Pseudomonadaceae;g_Pseudomonas	0.02	0.45	1.03	0.63	0.14	0.12	10.39	8.95	6.54	2.57
Spirochaetes;c_[Leptospirae];o_[Leptospirales];f_Leptospiraceae;g_Leptospira	0.00	0.22	0.05	0.19	0.14	0.00	0.07	0.00	1.18	0.00

oxydans during wine fermentation and less abundant changes of *Acetobacter* from both organically and conventionally fermented wine (**Table 3** and Table S5). The increased abundance of *G. oxydans* during the organic fermentation process might explain the increased susceptibility to wine spoilage in wines that are produced using organic fermentation techniques. Overall, these results demonstrate that 16S rRNA gene sequencing technique can be used efficiently to obtain a detailed description of the bacterial population associated with grape juice and must and to discover novel microorganisms that might lead to wine spoilage. This ability will allow wine makers to prevent losing revenues and investing in NGS technologies pose a promising avenue for wine makers, in particular as NGS has become a commodity and software for NGS data analysis is freely available. By comparing community dynamics of organically and conventionally fermented grape musts, we also observed that the population of *Pedobacter*, *Sphingomonas, Janthinobacterium, and Pseudomonas* were significantly higher in musts subjected to conventional than organic fermentation practices. It also appears that the bacterial population associated with the conventionally produced wine, experiences more significant community changes during the vinification process. This finding can be explained by the fact that commonly additives such as DAP have a significant effect on the indigenous bacterial population (**Figure 1**) and affect the community profile almost instantly. On the other hand, the increased community complexity of conventionally fermented must is less expected although it can also be explained by the affect of the additives that are employed in the conventional fermentation process. These additives appear to affect primarily phylogenetic groups that are undesired during the fermentation process and that dominate the prokaryotic community prior to their addition. Additionally, decreased community complexity and diversity in the organically fermented grape juice might be caused by the presence of indigenous yeasts on the skin of grapes that are not subjected to fungicide (i.e., SO_2) treatments during the organic PDC fermentation. This antimicrobial affect by indigenous yeasts in bacteria during the fermentation process was reported previously (Lonvaudfunel et al., 1988; Henick-Kling and Park, 1994) and it is possible that a defined mixture of naturally occurring yeast strains might represent a highly sustainable approach for controlling the composition and temporal succession of the bacterial population during the fermentation process. In order to make such yeast mixtures effective they would need to include additional strains that are efficient against the wine spoilage bacteria (e.g., *Gluconobacter oxydans*) that appear to be little affected by currently known indigenous grape skin yeasts.

Previously it was reported that winery surfaces were dominated by non-fermentation-related bacteria (i.e., *Pseudomonas*, *Comamonadaceae*, *Flavobaterium*, *Enterbacteraceae*, *Brevundimonas*, and *Bacillus*). Accordingly, we detected *Pseudomonas*, *Comamonadaceae, Enterbacteraceae*, and *Bacillus* during both organic and conventional fermentation (Table S6). The population of *Pseudomonas* and *Comamonadaceae* are larger at the early stage of conventional fermentation (2 days), which suggests that *Pseudomonas* and some members of *Comamonadaceae* originated from conventionally vinification process or their growth was not instantly inhibited by addition of SO_2 prior to conventional

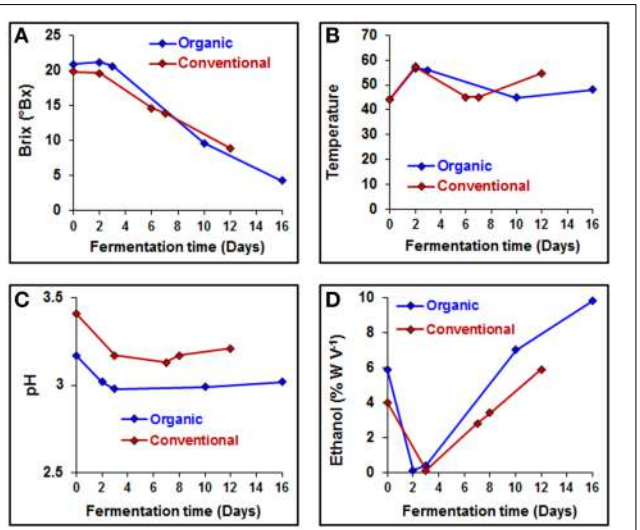

FIGURE 3 | Physicochemical characteristics of the organically and conventionally fermented grape musts. (A) Fermentation rate (Brix), **(B)** fermentation temperature, **(C)** grape musts pH, and **(D)** production of ethanol were measured on each day of fermentation.

TABLE 4 | Chemical profile of grape musts during organic and conventional fermentation.

Duration of fermentation [days]	Organic					Conventional				
	0	2	3	10	16	0	2	6	7	12
Ethanol at 20°C (% Vol)	5.9	0.1	0.4	7	9.8	4	0.1	2.8	3.4	5.9
L-lactic acid (g/L)	0.97	0.05	0.05	0.05	0.05	0.29	0.05	0.05	0.05	0.05
L-malic acid (g/L)	1.97	3.08	3.04	2.45	2.31	2.7	4.56	4.02	4.09	3.79
Volatile acidity(acetic) (g/L)	0.16	0.05	0.09	0.12	0.2	0.14	0.07	0.34	0.42	0.46
Tartaric acid (g/L)	2.1	4.2	4	3.2	3	1.7	2.5	2.1	1.9	1.9
Titratable acidity (g/L)	7.3	7.6	7.3	7.3	7.5	5.8	7.6	7.3	7.5	7.2
Yeast assimilable nitrogen (mg/L)	18	137	103	18	18	18	219	101	155	143
Alpha-amino compounds (as N) (mg/L)	10	91	70	14	12	14	112	54	56	59
Ammonia (mg/L)	10	56	40	10	10	10	130	57	120	102

vinification. The other possibility might be that the growth of *Pseudomonas* and some members of *Comamonadaceae* was suppressed by antimicrobial components produced by indigenous yeasts associated with organically fermented wine. *Enterbacteraceae*, a dominant family from grapevine (Pinto et al., 2014), is extremely abundant during PDC fermentation (about 85% in both samples), with a rapid population decrease during conventional fermentation (5% at day 2), this might be caused by addition of SO_2. A less significant decrease was observed during organic fermentation [73% (day 2), 34% (day 10), 13% (day 13)], which might also be explained by the antimicrobial activity of an indigenous yeast that might have been associated with the grapes.

In this study, we obtained a more detailed understanding of the temporal succession of the bacterial population and associated changes of the wine chemistry during conventionally and organically fermented grapes using NGS technologies, which could not be studied with less sensitive molecular approaches (i.e., PCR-DGGE). The sequences generated during this study were deposited in NCBI's short read archive using the study accession number SRP058864. In summary, these results suggest that there are temporal changes in the bacterial population that is associated with the fermentation process and that these populations might contain microorganisms that have until today not been linked with the fermentation process. Further comprehensive study of how the bacterial species of wine interact and how the microbial community dynamics correlated with grape must and wine components during the fermentation process will be of great value for developing improved methods to control wine quality.

Author Contributions

Conceived and designed the experiments: MH. Performed the experiments: MH, HP, EH, SK, and SS. Generated and analyzed the data: MH, HP, EH, SK, RD, and SS. Wrote the paper: MH, HP, EH, and TH.

Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.00809

References

Andorrá, I., Landi, S., Mas, A., Guillamón, J. M., and Esteve-Zarzoso, B. (2008). Effect of oenological practices on microbial populations using culture-independent techniques. *Food Microbiol.* 25, 849–856. doi: 10.1016/j.fm.2008.05.005

Bae, S., Fleet, G. H., and Heard, G. M. (2006). Lactic acid bacteria associated with wine grapes from several Australian vineyards. *J. Appl. Microbiol.* 100, 712–727. doi: 10.1111/j.1365-2672.2006.02890.x

Bañeras, L., Trias, R., Godayol, A., Cerdán, L., Nawrath, T., Schulz, S., et al. (2013). Mass spectrometry identification of alkyl-substituted pyrazines produced by *Pseudomonas* spp. isolates obtained from wine corks. *Food Chem.* 138, 2382–2389. doi: 10.1016/j.foodchem.2012.12.030

Barata, A., Malfeito-Ferreira, M., and Loureiro, V. (2012). Changes in sour rotten grape berry microbiota during ripening and wine fermentation. *Int. J. Food Microbiol.* 154, 152–161. doi: 10.1016/j.ijfoodmicro.2011.12.029

Bartowsky, E. J., and Henschke, P. A. (2008). Acetic acid bacteria spoilage of bottled red wine - A review. *Int. J. Food Microbiol.* 125, 60–70. doi: 10.1016/j.ijfoodmicro.2007.10.016

Beneduce, L., Spano, G., Vernile, A., Tarantino, D., and Massa, S. (2004). Molecular characterization of lactic acid populations associated with wine spoilage. *J. Basic Microbiol.* 44, 10–16. doi: 10.1002/jobm.200310281

Bokulich, N. A., Joseph, C. M., Allen, G., Benson, A. K., and Mills, D. A. (2012). Next-generation sequencing reveals significant bacterial diversity of botrytized wine. *PLoS ONE* 7:e36357. doi: 10.1371/journal.pone.0036357

Bokulich, N. A., Swadener, M., Sakamoto, K., Mills, D. A., and Bisson, L. F. (2015). Sulfur dioxide treatment alters wine microbial diversity and fermentation progression in a dose-dependent fashion. *Am. J. Enol. Vitic.* 66, 73–79. doi: 10.5344/ajev.2014.14096

Bokulich, N. A., Thorngate, J. H., Richardson, P. M., and Mills, D. A. (2014). Microbial biogeography of wine grapes is conditioned by cultivar, vintage, and climate. *Proc. Natl. Acad. Sci. U.S.A.* 111, E139–E148. doi: 10.1073/pnas.1317377110

Caporaso, J. G., Bittinger, K., Bushman, F. D., Desantis, T. Z., Andersen, G. L., and Knight, R. (2010a). PyNAST: a flexible tool for aligning sequences to a template alignment. *Bioinformatics* 26, 266–267. doi: 10.1093/bioinformatics/btp636

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., et al. (2010b). QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods* 7, 335–336. doi: 10.1038/nmeth.f.303

Capozzi, V., Russo, P., Beneduce, L., Weidmann, S., Grieco, F., Guzzo, J., et al. (2010). Technological properties of *Oenococcus oeni* strains isolated from typical southern Italian wines. *Lett. Appl. Microbiol.* 50, 327–334. doi: 10.1111/j.1472-765X.2010.02795.x

Cappello, M. S., Bleve, G., Grieco, F., Dellaglio, F., and Zacheo, G. (2004). Characterization of *Saccharomyces cerevisiae* strains isolated from must of grape grown in experimental vineyard. *J. Appl. Microbiol.* 97, 1274–1280. doi: 10.1111/j.1365-2672.2004.02412.x

Cocolin, L., Alessandria, V., Dolci, P., Gorra, R., and Rantsiou, K. (2013). Culture independent methods to assess the diversity and dynamics of microbiota during food fermentation. *Int. J. Food Microbiol.* 167, 29–43. doi: 10.1016/j.ijfoodmicro.2013.05.008

Cocolin, L., Bisson, L. F., and Mills, D. A. (2000). Direct profiling of the yeast dynamics in wine fermentations. *FEMS Microbiol. Lett.* 189, 81–87. doi: 10.1111/j.1574-6968.2000.tb09210.x

Combet-Blanc, Y., Ollivier, B., Streicher, C., Patel, B. K., Dwivedi, P. P., Pot, B., et al. (1995). *Bacillus thermoamylovorans* sp. nov., a moderately thermophilic and amylolytic bacterium. *Int. J. Syst. Bacteriol.* 45, 9–16. doi: 10.1099/00207713-45-1-9

Curtin, C. D., Bellon, J. R., Henschke, P. A., Godden, P. W., and Lopes, M. A. D. B. (2007). Genetic diversity of *Dekkera bruxellensis* yeasts isolated from Australian wineries. *FEMS Yeast Res.* 7, 471–481. doi: 10.1111/j.1567-1364.2006.00183.x

Di Toro, M. R., Capozzi, V., Beneduce, L., Alexandre, H., Tristezza, M., Durante, M., et al. (2015). Intraspecific biodiversity and 'spoilage potential' of *Brettanomyces bruxellensis* in Apulian wines. *Lwt-Food Sci. Technol.* 60, 102–108. doi: 10.1016/j.lwt.2014.06.059

Edgar, R. C. (2010). Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* 26, 2460–2461. doi: 10.1093/bioinformatics/btq461

Fleet, G. H. (1993). *Wine Microbiology and Biotechnology*. New York, NY: Taylor & Francis Group.

Fugelsang, K. C., and Edwards, C. G. (2007). *Wine Microbiolog Practical Application and Procedures*. New York, NY: Springer.

Garofalo, C., El Khoury, M., Lucas, P., Bely, M., Russo, P., Spano, G., et al. (2015). Autochthonous starter cultures and indigenous grape variety for regional wine production. *J. Appl. Microbiol.* 118, 1395–1408. doi: 10.1111/jam.12789

González-Barreiro, C., Rial-Otero, R., Cancho-Grande, B., and Simal-Gándara, J. (2015). Wine aroma compounds in grapes: a critical review. *Crit. Rev. Food Sci. Nutr.* 55, 202–218. doi: 10.1080/10408398.2011.650336

Henick-Kling, T., and Park, Y. H. (1994). Considerations for the use of yeast and bacterial starter cultures: SO_2 and timing of inoculation. *Am. J. Enol. Vitic.* 45, 464–469.

Hopfer, H., Nelson, J., Mitchell, A. E., Heymann, H., and Ebeler, S. E. (2013). Profiling the trace metal composition of wine as a function of storage temperature and packaging type. *J. Anal. At. Spectrom.* 28, 1288–1291. doi: 10.1039/c3ja50098e

Joyeux, A., Lafonla-fourcade, S., and Ribéreaugayon, P. (1984). Evolution of acetic-acid bacteria during fermentation and storage of wine. *Appl. Environ. Microbiol.* 48, 153–156.

Laforgue, R., Guérin, L., Pernelle, J. J., Monnet, C., Dupont, J., and Bouix, M. (2009). Evaluation of PCR-DGGE methodology to monitor fungal communities on grapes. *J. Appl. Microbiol.* 107, 1208–1218. doi: 10.1111/j.1365-2672.2009.04309.x

Lonvaudfunel, A., Joyeux, A., and Desens, C. (1988). Inhibition of malolactic fermentation of wines by products of yeast metabolism. *J. Sci. Food Agric.* 44, 183–191. doi: 10.1002/jsfa.2740440209

Lopez, I., Ruiz-Larrea, F., Cocolin, L., Orr, E., Phister, T., Marshall, M., et al. (2003). Design and evaluation of PCR primers for analysis of bacterial populations in wine by denaturing gradient gel electrophoresis. *Appl. Environ. Microbiol.* 69, 6801–6807. doi: 10.1128/AEM.69.11.6801-6807.2003

McDonald, D., Price, M. N., Goodrich, J., Nawrocki, E. P., Desantis, T. Z., Probst, A., et al. (2012). An improved Greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea. *ISME J.* 6, 610–618. doi: 10.1038/ismej.2011.139

Millet, V., and Lonvaud-Funel, A. (2000). The viable but non-culturable state of wine micro-organisms during storage. *Lett. Appl. Microbiol.* 30, 136–141. doi: 10.1046/j.1472-765x.2000.00684.x

Nguyen, T. T., and Landfald, B. (2015). Polar front associated variation in prokaryotic community structure in Arctic shelf seafloor. *Front. Microbiol.* 6:17. doi: 10.3389/fmicb.2015.00017

Oliver, J. D. (2005). The viable but nonculturable state in bacteria. *J. Microbiol.* 43, 93–100.

Pérez-Martín, F., Seseña, S., Fernández-González, M., Arévalo, M., and Palop, M. L. (2014). Microbial communities in air and wine of a winery at two consecutive vintages. *Int. J. Food Microbiol.* 190, 44–53. doi: 10.1016/j.ijfoodmicro.2014.08.020

Pessoa-Filho, M., Barreto, C. C., Dos Reis Junior, F. B., Fragoso, R. R., Costa, F. S., De Carvalho Mendes, I., et al. (2015). Microbiological functioning, diversity, and structure of bacterial communities in ultramafic soils from a tropical savanna. *Antonie Van Leeuwenhoek* 107, 935–949. doi: 10.1007/s10482-015-0386-6

Piao, H., Lachman, M., Malfatti, S., Sczyrba, A., Knierim, B., Auer, M., et al. (2014). Temporal dynamics of fibrolytic and methanogenic rumen microorganisms during *in situ* incubation of switchgrass determined by 16S rRNA gene profiling. *Front. Microbiol.* 5:307. doi: 10.3389/fmicb.2014.00307

Pinto, C., Pinho, D., Sousa, S., Pinheiro, M., Egas, C., and Gomes, A. C. (2014). Unravelling the diversity of grapevine microbiome. *PLoS ONE* 9:e85622. doi: 10.1371/journal.pone.0085622

Renouf, V., Claisse, O., and Lonvaud-Funel, A. (2007). Inventory and monitoring of wine microbial consortia. *Appl. Microbiol. Biotechnol.* 75, 149–164. doi: 10.1007/s00253-006-0798-3

Sinclair, L., Osman, O. A., Bertilsson, S., and Eiler, A. (2015). Microbial community composition and diversity via 16S rRNA gene amplicons: evaluating the illumina platform. *PLoS ONE* 10:e0116955. doi: 10.1371/journal.pone.0116955

Spano, G., Lonvaud-Funel, A., Claisse, O., and Massa, S. (2007). *In vivo* PCR-DGGE analysis of *Lactobacillus plantarum* and *Oenococcus oeni* populations in red wine. *Curr. Microbiol.* 54, 9–13. doi: 10.1007/s00284-006-0136-0

Styger, G., Prior, B., and Bauer, F. F. (2011). Wine flavor and aroma. *J. Ind. Microbiol. Biotechnol.* 38, 1145–1159. doi: 10.1007/s10295-011-1018-4

Trexler, R., Solomon, C., Brislawn, C. J., Wright, J. R., Rosenberger, A., McClure, E. E., et al. (2014). Assessing impacts of unconventional natural gas extraction on microbial communities in headwater stream ecosystems in Northwestern Pennsylvania. *Front. Microbiol.* 5:522. doi: 10.3389/fmicb.2014.00522

Wang, Q., Garrity, G. M., Tiedje, J. M., and Cole, J. R. (2007). Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl. Environ. Microbiol.* 73, 5261–5267. doi: 10.1128/AEM.00062-07

Yousaf, S., Bulgari, D., Bergna, A., Pancher, M., Quaglino, F., Casati, P., et al. (2014). Pyrosequencing detects human and animal pathogenic taxa in the grapevine endosphere. *Front. Microbiol.* 5:327. doi: 10.3389/fmicb.2014.00327

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Fungal diversity in grape must and wine fermentation assessed by massive sequencing, quantitative PCR and DGGE

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The diversity of fungi in grape must and during wine fermentation was investigated in this study by culture-dependent and culture-independent techniques. Carignan and Grenache grapes were harvested from three vineyards in the Priorat region (Spain) in 2012, and nine samples were selected from the grape must after crushing and during wine fermentation. From culture-dependent techniques, 362 isolates were randomly selected and identified by 5.8S-ITS-RFLP and 26S-D1/D2 sequencing. Meanwhile, genomic DNA was extracted directly from the nine samples and analyzed by qPCR, DGGE and massive sequencing. The results indicated that grape must after crushing harbored a high species richness of fungi with *Aspergillus tubingensis*, *Aureobasidium pullulans,* or *Starmerella bacillaris* as the dominant species. As fermentation proceeded, the species richness decreased, and yeasts such as *Hanseniaspora uvarum*, *Starmerella bacillaris* and *Saccharomyces cerevisiae* successively occupied the must samples. The "*terroir*" characteristics of the fungus population are more related to the location of the vineyard than to grape variety. Sulfur dioxide treatment caused a low effect on yeast diversity by similarity analysis. Because of the existence of large population of fungi on grape berries, massive sequencing was more appropriate to understand the fungal community in grape must after crushing than the other techniques used in this study. Suitable target sequences and databases were necessary for accurate evaluation of the community and the identification of species by the 454 pyrosequencing of amplicons.

Keywords: culture-independent techniques, pyrosequencing, SO_2 treatment, community diversity and composition, wine yeast

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INTRODUCTION

Investigating the fungal community in grape must and wine fermentation is relevant for understanding its relationship with the grape sanitary status and the final wine characteristics (Bokulich et al., 2014). Recently, the development of next-generation sequencing provided a useful tool for the description of prokaryotic and eukaryotic microbial communities that exist in grape leaves, berries, must and wineries (Bokulich et al., 2013, 2014; David et al., 2014; Pinto et al., 2014; Taylor et al., 2014; Valera et al., 2015). The common approach used in these studies was targeted metasequencing: generic target sequences were amplified by PCR to establish a library; then amplicons were sequenced; and identification was performed by comparison with known sequences in databases (Huggett et al., 2013; Mayo et al., 2014).

These studies indicated advances relative to the traditional culture-dependent techniques: a greater abundance of bacteria and fungi found in grape leaves and berries and higher sensitivity to minor species due to the possibility of massive sequencing in a short time. Moreover, other culture-independent techniques have played important roles in monitoring the main yeast dynamics during wine fermentation for the last 10 years (Mills et al., 2002; Hierro et al., 2006; Andorrà et al., 2010). Thus, the main aim of this study was to apply these techniques to interpret the fungal communities in grape must and wine fermentation from the Priorat region in Spain.

The Priorat region, the second qualified DOC (Denominación de Origen Calificada) wine region in Spain, is located in southwest Catalonia. This region is characterized by its own "*terroir*" (French word widely use in the wine industry and wine marketing that means specific place character): a topsoil of reddish and black slate with small particles of mica, a hot and dry summer climate with different micro-climates due to the hilly landform (average annual rainfall is 400–600 mm), and vineyards on terraced slopes at altitudes between 100 and 700 m above sea level (Robinson, 2006; Hudin and Serra, 2013). However, few studies have reported on the native microbial ecology of grapes in this region. Torija et al. (2001) investigated the yeast population in spontaneous fermentation from this region over 3 years and reported a unique ecology of yeast species and *Saccharomyces* strains. To investigate the probable fungal "*terroir*" of this region, grapes from slopes at altitudes 400 m above sea level in the villages of Poboleda, Escaladei, and Porrera were crushed into must and fermented in this study. The fungal diversity from grape must and fermentation samples was analyzed by culture-dependent techniques and culture-independent techniques and compared among different samples. The effect of SO_2 treatment on fungal diversity was also evaluated by low-dosage addition to two grape must varieties from Porrera.

MATERIALS AND METHODS

Spontaneous Fermentations

Mature grapes (Carignan and Grenache) were randomly taken from vineyards in three villages (Poboleda, Escaladei, and Porrera) of the Priorat region (Spain) in 2012. The grapes were hand-harvested from the plants with gloves and kept in sterile bags in an ice box for transportation. Approximately 1.8 L of grape must was obtained from each 2 kg of grapes at different locations, which were crushed sterilely in the same plastic bag by hand and put into 2 L bottles for spontaneous fermentation. The fermentations were performed at 24°C with 120 rpm agitation speed, and 30 ppm of SO_2 was added at 24 h in the form of potassium metabisulfite. The fermentation proceeded in semianaerobic conditions as the bottles are not tightly closed and some gas exchange is allowed. All the fermentations were monitored daily using a Densito 30PX Portable Density Meter (Mettler Toledo, Spain), and samples were taken at five different fermentation stages: 0 h (grape must after crush), 24 h (before SO_2 treatment), 48 h (24 h after SO_2 treatment), middle stage (density approximately 1040–1060 g/L) and end stage (stable density less than 1000 g/L). Fresh samples were directly analyzed by culture-dependent techniques; cell pellets from 1 mL of samples at each fermentation stage were collected by centrifugation after washing with sterile water and kept at −20°C for further culture-independent analysis by qPCR, DGGE, and massive sequencing techniques.

Culture-dependent Techniques

One milliliter of sample at each fermentation stage was diluted in series and spread onto YPD med/ium (2% glucose, 2% peptone, 1% yeast extract and 1.7% agar) and Lysine medium (Oxoid, USA) for incubation at 25°C for 2–3 days. For plating, a Whitley Automatic Spiral Plater (AES Laboratoire, France) was used, and the viable yeast quantification was performed using a ProtoColHr automatic colony counter (Microbiology International, USA). For further colony identification, 25 colonies were selected randomly from YPD and Lysine plates of each sample (50 colonies in total for each sample) and identified by 5.8S-ITS-RFLP analysis and 26S rDNA D1/D2 domain sequencing. In 5.8S-ITS-RFLP analysis, colony amplifications were first performed by primer pairs of ITS1/ITS4 as described by Esteve-Zarzoso et al. (1999). The amplification products were digested by five restriction enzymes (*Hinf*I, *Hae*III, *Cfo*I, *Dde*I, and *Mbo*I), and corresponding restriction profiles were identified according to Esteve-Zarzoso et al. (1999) and Csoma and Sipiczki (2008). Then, 26S rDNA D1/D2 domain sequencing was used to confirm the colony identification. Each PCR reaction was performed with primer pairs of NL1/NL4 and the program described by Kurtzman and Robnett (1998). An ABI3730 XL DNA sequencer (Macrogen, Korea) was used for the sequencing process, and corresponding sequence alignment was performed by BLAST from the NCBI database (http://blast.ncbi.nlm.nih.gov/).

DNA Extraction

DNA was extracted from the cell pellets stored at −20°C using the DNeasy Plant minikit (Qiagen, USA) as described in Hierro et al. (2006). The same extraction protocol was used for DGGE, qPCR and massive sequencing analyses.

DGGE Analysis

The PCR reactions were performed using a Gene Amp PCR System 2720 (Applied Biosystems, USA) with Primers $U1^{GC}$ and U2 (Meroth et al., 2003). The DGGE procedures followed the description in Andorrà et al. (2008) with a modified DGGE gel using a denaturing gradient from 35 to 55% urea and formamide.

qPCR Analysis

The qPCR reactions were performed using an Applied Biosystems 7300 Fast Real-Time PCR System (Applied Biosystems, USA) with primers for total yeast, *Saccharomyces*, *Hanseniaspora,* and *Starmerella bacillaris* as described in Andorrà et al. (2010). Standard curves were built for each yeast species in triplicate using 10-fold serial dilutions of fresh cultures.

Massive Sequencing Analysis

A fragment of approximately 600 nt from D1/D2 of 26S rDNA was amplified using modified NL1/NL4 primers, which were

designed with adaptor and molecular identifier (MID) sequences specially for massive sequencing (Invitrogen, USA). The whole sequencing process was performed using a 454 Roche platform with the Genome Sequencing FLX System (LifeSequencing S.L., Spain): DNA libraries with specific MID sequences were built for each sample by target PCR with the improved primers, and then a primer-dimer removal protocol was applied to each PCR product to increase the sequencing throughput. An equimolecular pool was generated by quantification of the clean PCR products using the Quan-IT™ PicoGreen® kit (Invitrogen), and sequencing of the pooled samples was performed using a 454 FLX Roche sequencer (LifeScience, USA).

The bioinformatic analysis of each sample was conducted by LifeSequencing S.L. (Spain). Quality control of all sequences was first performed by removing sequences with low quality or length lower than 300 nt and the PCR primers. An updated database of 26S rDNA sequences obtained from GenBank of NCBI was constructed for local alignment comparison. By local alignment comparison, each read was assigned to the most probable operational taxonomic unit (OTU) at different taxonomical levels (family, genera and species) with a confidence cutoff value of 80% and an *e*-value of 10^{-5}. Sequences with identity value lower than 80% and *e*-value lower than 10^{-5} were assigned as "no hit."

The fungal community in each sample was analyzed by different biodiversity and similarity metrics at the species level using Estimate S v9.1.0 (Colwell, 2013). Both Shannon diversity and Simpson diversity were used to evaluate species diversity because Simpson diversity is less sensitive to richness and more sensitive to evenness than Shannon diversity (Colwell, 2009). The estimated species richness was also calculated by a nonparametric estimator, Chao1, which depends on the observed number of singletons and doubletons in a sample. Similarities were evaluated using Jaccard Classic and Bray-Curtis because we focused on comparing community compositions.

RESULTS

Nine samples were obtained from different stages of fermentations; the details are described in **Table 1**. They were analyzed by culture-dependent techniques (YPD and Lysine plating) and three different culture-independent techniques (qPCR, DGGE and massive sequencing).

Yeast Diversity Analysis by Culture-dependent Techniques

The 183 isolates from YPD plates were identified as five different species by 5.8S-ITS-RFLP analysis and 26S-D1/D2 sequencing (**Table 2**). *Hanseniaspora uvarum* was the most frequently isolated species in all samples except sample IX (the end of fermentation, when *Saccharomyces cerevisiae* dominated). *Starmerella bacillaris* was the second most common species, isolated in samples III, IV, V, VIII, and IX. *Issatchenkia terricola* was mainly isolated from fresh grape must after crushing (sample I, III, V, and VI). *Hanseniaspora valbyensis* and *S. cerevisiae* only appeared in a single sample.

The 179 non-*Saccharomyces* isolates from Lysine medium were identified. Only three species were recovered, with *H. uvarum* as the main species (**Table 2**). *I. terricola* was only isolated from grape must after crushing (sample I and V), and *Starm. bacillaris* was present in grape must after crushing and also at the end of fermentation.

Yeast Population Diversity by qPCR Analysis

The population levels of total yeast, *Hanseniaspora* spp., *Starm. Bacillaris,* and *Saccharomyces* spp., were separately quantified (**Table 2**). The total yeast population in grape must after crushing (sample I, II, and V) was lower than 10^6 cells/mL, but the yeast population then increased to 10^5 to 10^8 cells/mL. *Hanseniaspora* was the main genus detected in almost all samples, ranging from 10^2 to 10^7 cells/mL. *Starm. bacillaris* mainly appeared in grape must from Porrera (10^2 to 10^6 cells/mL), although it was not detected at the end of fermentation. Surprisingly, the *Saccharomyces* population was only detected by this technique at the end of fermentation. The total yeast population size was not affected by the SO_2 treatment; however, the *Starm. bacillaris* population was reduced by approximately tenfold after SO_2 addition. This observation was made in the two samples analyzed before and after SO_2 addition.

TABLE 1 | Details of nine samples from grape must fermentations.

Samples	Fermentation stages	Grape varieties	Locations	Coordinates
I	0 h grape must	Grenache	Poboleda	41.227148, 0.844750
II	0 h grape must	Carignan	Escaladei	41.258156, 0.808214
III	24 h grape must (before SO_2 treatment)	Carignan	Porrera	41.179651, 0.860334
IV	48 h grape must (24 h after SO_2 treatment)			
V	0 h grape must	Grenache	Porrera	41.176748, 0.860619
VI	24 h grape must (before SO_2 adding)			
VII	48 h grape must (24 h after SO_2 treatment)			
VIII	Middle stage of fermentation (day 3)			
IX	Final stage of fermentation (day 11)			

The middle and end stages of fermentation were determined by density analysis.

TABLE 2 | The fungal diversity of nine different grape must and fermentation samples evaluated by culture-dependent and culture-independent techniques.

Techniques	Yeast	I	II	III	IV	V	VI	VII	VIII	IX
Culture-dependent techniques by YPD plating	Total yeast	*	4.80×10^3	*	1.51×10^8	3.58×10^6	1.06×10^7	2.10×10^7	3.00×10^7	9.10×10^5
	Hanseniaspora uvarum	7/9	*	9/25	19/24	14/25	24/25	25/25	22/25	nd
	Hanseniaspora valbyensis	nd	*	6/25	nd	Nd	nd	nd	nd	nd
	Issatchenkia terricola	2/9	*	8/25	nd	3/25	1/25	nd	nd	nd
	Saccharomyces cerevisiae	nd	*	nd	nd	Nd	nd	nd	nd	18/25
	Starmerella bacillaris	nd	*	2/25	5/24	8/25	nd	nd	3/25	7/25
Culture-dependent techniques by Lysine plating	Total yeast	*	2.70×10^3	4.88×10^6	1.41×10^7	1.75×10^6	*	1.33×10^7	3.10×10^8	1.10×10^5
	H. uvarum	13/25	8/9	25/25	25/25	13/20	*	25/25	25/25	nd
	I. terricola	9/25	nd	nd	nd	7/20	*	nd	nd	nd
	Starm. bacillaris	3/25	1/9	nd	nd	Nd	*	nd	nd	25/25
qPCR	Total yeast	7.62×10^2	2.85×10^5	6.07×10^7	1.13×10^8	1.82×10^4	6.06×10^6	2.93×10^6	4.35×10^5	2.31×10^5
	Hanseniaspora	nd	9.95×10^3	2.46×10^7	1.44×10^7	6.70×10^2	2.64×10^6	3.42×10^5	2.45×10^5	1.94×10^4
	Saccharomyces	nd	nd	nd	nd	Nd	nd	nd	nd	5.98×10^4
	Starm. bacillaris	nd	nd	1.85×10^6	2.90×10^5	8.93×10^2	1.49×10^6	3.54×10^4	4.67×10^2	nd
DGGE	*Aureobasidium pullulans*	–	+	nd	nd	+	nd	nd	nd	nd
	Botryosphaeria dothidea	nd	+	nd	nd	Nd	nd	nd	nd	nd
	Hanseniaspora opuntiae	nd	+	nd	nd	+	+	+	+	+
	H. uvarum	nd	+	+	+	+	+	+	+	+
	S. cerevisiae	nd	nd	nd	nd	Nd	nd	nd	nd	+
	Starm. bacillaris	nd	nd	+	+	Nd	+	+	+	+
Massive sequencing	*Aspergillus tubingensis*	55.80%	18.18%	<	<	<	<	–	–	–
	Aureo. pullulans	<	18.63%	<	<	<	<	–	–	–
	B. dothidea	–	<	–	–	–	–	–	–	–
	Hanseniaspora thailandica	–	<	5.25%	5.00%	<	<	<	<	<
	H. opuntiae	–	6.05%	<	<	<	<	<	<	–
	H. uvarum	–	<	60.78%	56.68%	<	13.57%	11.80%	<	<
	uncultured Hanseniaspora	–	<	12.37%	13.44%	<	<	<	<	<
	I. terricola	–	–	<	<	<	<	<	<	–
	Penicillium brevicompactum	<	5.47%	–	–	<	–	–	–	–
	Penicillium crustosum	<	5.56%	–	–	<	–	–	–	–
	Penicillium glabrum	8.64%	–	–	–	<	–	–	–	–
	S. cerevisiae	–	<	–	–	–	<	–	–	25.98%
	Uncultured Saccharomyces	–	<	–	<	–	<	–	–	<
	Starm. bacillaris	–	<	17.19%	20.22%	87.86%	79.61%	80.22%	98.10%	71.19%
	Uncultured soil fungus	14.45%	<	–	<	<	<	<	–	–

The results from culture-dependent techniques are shown as specie colony numbers compared with total colony numbers, with total yeast concentration also shown (cfu/ml). Grape must samples with molds mainly found on plates, resulting in hard quantification or isolation, are labeled with "." The qPCR results are shown as cells concentration (cells/ml), the species detectable by DGGE are represented by "+," and the results from massive sequencing are shown as percentages (%). Because of the rich diversity of the massive sequencing results, only the major species with percentages higher than 5% are shown in the table, and the species with lower percentages in some samples, if listed, are marked with "<." The symbol of "nd" represents the species with lower concentrations below the detection limit (100 cells/ml) by qPCR and species undetectable by the other three techniques.*

DGGE Analysis of Grape Must Samples

The bands obtained in DGGE profiles were assigned to six species by sequencing, as indicated in **Table 2**. No species were observed from sample I, and in the remaining eight samples, *H. uvarum* appeared in each sample, *Hanseniaspora opuntiae* and *Starm. bacillaris* in six samples, *Aureobasidum* (*Aureo.*) *pullulans* in sample II and V, and *Botryosphaeria dothidea* and *S. cerevisiae* in only one sample.

Fungal Diversity Analysis by Massive Sequencing

Species Diversity and Similarity of Grape Must Samples

A total of 120,081 original sequences were obtained from nine samples, of which 106,095 sequences passed the quality control filter. As shown in **Table 3**, approximately 10,000 high quality reads were obtained from each sample, and the average sequence

TABLE 3 | Total sequences obtained from massive sequencing and fungal community metrics of all samples.

Metrics	I	II	III	IV	V	VI	VII	VIII	IX
High quality reads	10033	9301	10255	12798	10503	18162	11529	12559	10955
Average length (nt)	499	471	545	539	505	512	510	511	486
Number of OTUs at species level	86	186	22	21	64	25	15	11	15
Number of no hit reads	30	165	55	85	69	61	29	10	50
Estimated species richness	152	329	32	22	96	32	16	11	20
Confidence intervals	113–248	263–451	24–76	21–33	75–153	26–59	15–30	11–17	16–44
Shannon exponential species diversity	5.57	20.61	3.32	3.49	1.99	2.04	1.90	1.12	2.03
Simpson inverse species diversity	2.88	10.94	2.37	2.57	1.28	1.52	1.43	1.04	1.72

Estimated species richness was calculated using the Chao1 richness estimator, with log-linear 95% confidence intervals. OTU, operational taxonomic unit.

length was approximately 500 nt. The similar level of read numbers from each sample established comparability among samples. The analysis of massive sequencing was performed based on taxonomy-dependent methods, by which query sequences were compared with known sequences deposited in annotated databases. After alignment, 247 OTUs were identified at the species level from the 105,541 hit reads, and 554 reads were not assigned an identity in the current eukaryotic database of NCBI (0.5% of no hit reads).

Rich OTUs were found in the three grape must samples after crushing (I, II, and V). However, the fermentation samples showed a lower OTU richness. The species richness of each sample was estimated by Chao 1, and more OTUs were expected from the three grape must samples after crushing; however, in the other six fermentation samples, the observed OTUs were similar to the estimated species richness. Thus, both observed and estimated species richness decreased as fermentation proceeded, as we expected. The Shannon (exponential form) and Simpson (inverse form) diversity indices were used to evaluate the community diversity, in which both richness and evenness were integrated. The diversity values were no less than 1 due to the corresponding forms used, and higher values meant higher diversity. Thus, sample II presented the highest diversity and the best evenness of the nine samples. Although sample V had a higher value of richness than some fermentation samples (III, IV, VI, IX), its diversity by both indexes was lower, mainly due to its poor evenness.

The community similarity in nine samples was pairwise analyzed using the Jaccard Classic and Bray-Curtis indices (**Table 4**). Values from both indices range from 0 to 1, with 0 representing no similarity between two samples and 1 meaning no differentiation. Samples I and V showed similarities of 0.271 by Jaccard Classic and 0.066 by Bray-Curtis, which were lower values than the similarities between III and VI (0.516 Jaccard Classic and 0.376 Bray-Curtis) or IV and VII (0.565 Jaccard Classic and 0.377 Bray-Curtis). As noted in **Table 1**, samples I and V were from the same grape variety (Grenache) but different locations (Poboleda, Porrera), while III/IV and VI/VII were from the same location (Porrera) but from two different grape varieties (Carignan and Grenache). Therefore, the location seemed to contribute more to the dissimilarities between two samples than the grape variety.

Fungal Community Composition at Different Phylogenetic Levels

The fungal communities of the grape must were mainly characterized by high amounts of OTUs from the *Ascomycota* phylum (more than 95% in each sample). Forty-six of the 247 OTUs were present at 0.1–5% in each sample, and 189 OTUs presented a minor proportion (lower than 0.1%). Only 12 species were higher than 5% in each sample, as shown in **Table 2**. The dominant species were *Aspergillus* (*Asper.*) *tubingensis* in sample I, *Aureo. pullulans* in sample II, *H. uvarum* in samples III and IV, and *Starm. bacillaris* in samples V–IX. Species from the *Eurotiomycetes* and/or *Dothideomycetes* class mainly occupied the grape must after crushing (sample I and II), and most of the species found in grape fermentation must (sample III–IX) were from the *Saccharomycetes* class. At the genus level, the eight most abundant genera in nine samples are listed in **Figure 1**. Their sum accounts for more than 80% in each sample. The fungal community composition at different phylogenetic levels was more obviously affected by region and grape variety than the SO_2 treatment, as the latter only caused small percentage changes in some non-*Saccharomyces* species, mainly in the *Hanseniaspora* yeast genus.

Comparison Among Culture-dependent Techniques and Different Culture-independent Techniques

Comparing the results from different techniques, all the species detected by culture-dependent techniques, qPCR and DGGE were also found by massive sequencing except for sample I; however, the quantity or percentage of some species from the *Hanseniaspora* and *Starmerella* genera varied depending on the techniques used. *Saccharomyces* was found only in sample IX by culture-dependent techniques, qPCR and DGGE, while a minor population was also found in samples II, IV, and VI by massive sequencing. Most of the fungi from the non-*Saccharomycetes* class were detectable by massive sequencing, whereas only dominant species could be found by DGGE. Although they were also observed on YPD or Lysine plates, it was difficult to perform identification and quantification by culture-dependent techniques. Furthermore, non-culturable cells at the end of fermentation, such as *H. uvarum*, were quantifiable or detectable by the three culture-independent techniques.

TABLE 4 | Community similarity metrics (Jaccard Classic and Bray-Curtis) by pairwise multivariate analysis of all samples (I–IX).

		Bray-Curtis								
		I	II	III	IV	V	VI	VII	VIII	IX
Jaccard	I		0.357	0.004	0.002	0.066	0.001	0.000	0.000	0.000
Classic	II	0.242		0.087	0.078	0.118	0.062	0.079	0.040	0.045
	III	0.049	0.072		0.887	0.217	0.376	0.343	0.173	0.174
	IV	0.059	0.078	0.593		0.263	0.400	0.377	0.221	0.226
	V	0.271	0.185	0.284	0.288		0.677	0.880	0.822	0.738
	VI	0.078	0.093	0.516	0.704	0.290		0.777	0.819	0.543
	VII	0.020	0.052	0.423	0.565	0.197	0.538		0.809	0.703
	VIII	0.000	0.037	0.375	0.391	0.154	0.333	0.625		0.672
	IX	0.020	0.052	0.276	0.385	0.145	0.379	0.364	0.444	

DISCUSSION

The nine samples from different locations, grape varieties and corresponding fermentation stages allowed the analysis of yeast diversity and ecology in the Priorat wine region of Spain. However, our study went beyond descriptive analysis and focused on the comparison between culture-dependent techniques and culture-independent techniques to evaluate the fungal diversity based on rDNA-PCR polymorphism. Recent studies have mentioned drawbacks of rDNA-PCR-based methods, especially for culture-independent techniques, such as preferential annealing of the primers, the representativity and quality of DNA, and variable gene copy numbers in different species, and these drawbacks might lead to overestimation/underestimation of the proportion of some species in the overall fungal community (Andorrà et al., 2008; Angly et al., 2014; Valera et al., 2015). Although, it was also observed in this study that massive sequencing, culture-dependent techniques and qPCR detected different percentages of *Starm. bacillaris*, these methods were all necessary for yeast identification and quantification analysis. Culture-dependent techniques and culture-independent techniques such as qPCR, DGGE and massive sequencing were used in this study to weigh the biases introduced by the techniques in an effort to estimate the true fungal community diversity, similarity, and composition.

Fungal Community in Grape Must After Crushing

The main fungi in grape must from the three vineyards of the Priorat region were *Eurotiomycetes*, *Dothideomycetes,* and *Saccharomycetes*, all in the *Ascomycota* phylum. These fungi are commonly found in grape berries or grape must after crushing in various world wine regions (Bokulich et al., 2014; David et al., 2014; Taylor et al., 2014). The dominant species in a single vineyard were *Asper. tubingensis* (Grenache from Poboleda), *Aureo. pullulans* (Carignan from Escaladei), and *Starm. bacillaris* (Grenache from Porrera). None of these three species are plant pathogens. The high population of *Starm. bacillaris* in grape must after crushing is unexpected but understandable: approximately 31% of *Candida* (previous denomination of *Starm. bacillaris*) was found in Chardonnay grapes of Burgundy (France) (David et al., 2014), indicating the possibility of dominance of this yeast over other fungi in grape must. Moreover, some species that are considered common plant pathogens, such as *Alternaria alternate*, *Aspergillus niger*, *B. dothidea*, *Cladosporium cladosporioides,* and *Cytospora sacculus*, were found in low percentages (0.1–5% according to massive sequencing results). Only one sequence of *Botrytis cinerea* was found in Carignan from the Escaladei vineyard and Grenache from Porrera. No other common grape pathogen was detected. As noted by Taylor et al. (2014), the presence of DNA from these species does not necessarily mean that the grapes or plants have an infection. Fungal diseases are rare in the Priorat region because of the high temperature and low level of rainfall in the summer (Robinson, 2006). Some reads of *S. cerevisiae* (1.03%) were found in Carignan from the Escaladei vineyard but did not appear in the other two grape must samples. The low or absent evidence of DNA from *Saccharomyces* was consistent with other reports based on high-throughput sequence analysis, and with the presence of other non-dominant non-*Saccharomyces* yeasts such as *Hanseniaspora*, *Issatchenkia,* or *Pichia* in this study (Bokulich et al., 2014; David et al., 2014; Taylor et al., 2014).

Regional microbial "*terroir*" was proposed by Bokulich et al. (2014) as a probable explanation for the regional characteristics of final wine quality, as the fungal community was more resistant to vintage variation than regional or even vineyard variation. Our results also showed that the fungal community was more affected by geographical location than by grape variety, even though the three vineyards were all located in the Priorat region with similar altitudes and were geographically close (approximately from 5 to 12 km to each other). Interestingly, Torija et al. (2001) found that *Candida stellata* (currently renamed *Starm. bacillaris*) was the only species isolated from grape must at the same location (Porrera) in 1996. Nevertheless, the formation of grape-surface communities by vineyard or region needed more proof to be established. Furthermore, the fungal community analysis in grape must after crushing was more reliable when estimated by massive sequencing than other techniques used in this study because of the "deep community sequencing" due to the larger number of sequences analyzed (Taylor et al., 2014).

Fungal Community in Grape Must During Wine Fermentation

Fungal community dynamics during wine fermentation involve the decline of non-yeast fungi during the first 24 h, the simultaneous increase of *Hanseniaspora* species and the increase of *S. cerevisiae* at the end of fermentation. The non-yeast fungi seemed to be less tolerant of environmental change from grape skin to grape must, as few sequences were detected in grape must at 24 h, and only one sequence of *Aspergillus niger* was found in grape must at 48 h. The massive decline in non-yeast fungi contributed directly to the decreased biodiversity in grape must during fermentation. Although, the lack of detection of non-yeast fungi in grape must after 48 h resulted partly from their reduction in grape must after crushing, the decrease in non-yeast fungi could also be correlated with the dominance of *Hanseniaspora* species. A clear increase in *S. cerevisiae* appeared

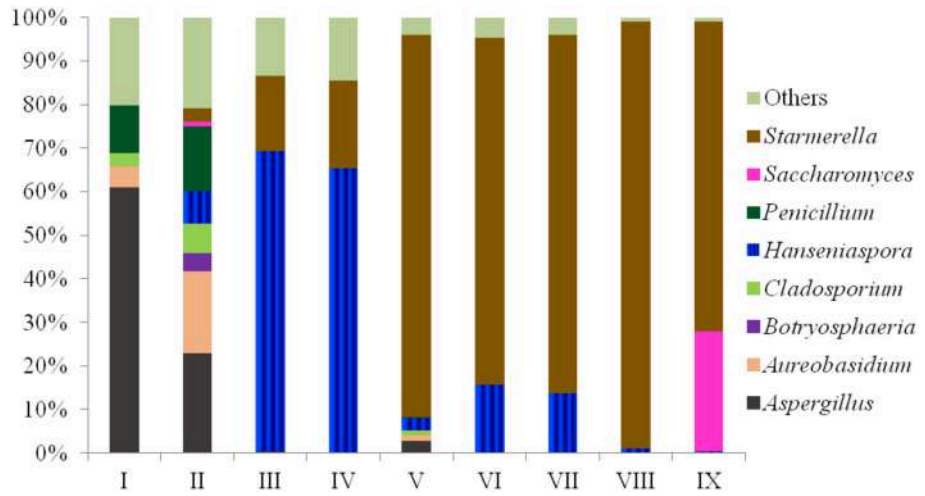

FIGURE 1 | Community distribution of the eight most abundant genera of nine samples (I–IX) using massive sequencing analysis.

at the end of fermentation, which was expected (Ribéreau-Gayon et al., 2006) and was consistent in all the results with all the techniques used in this study. Only one sequence of *S. cerevisiae* was occasionally detected in grape must at 24 h, which is also consistent with the consolidated knowledge. This low percentage of *Saccharomyces* species was also observed by David et al. (2014), and in their studies, when fermentations had reached two-thirds of the process (late stages), *Saccharomyces* species were detected at lower levels. The high representation of non-*Saccharomyces* yeast in grape must (*Starmerella* in this study, and *Candida* in David et al., 2014, which could be equivalent) can account for this late detection of *S. cerevisiae* as a main species during fermentation. This competition between *Starmerella*/*Candida* and *Saccharomyces* needs further investigation.

Regardless of regional and varietal factors, fungal diversity decreased as fermentation proceeded, with the disappearance of non-yeast fungi and the predominance of non-*Saccharomyces* yeast (*Hanseniaspora*). Thus, the grape must changes during wine fermentation also seemed to affect the fungal community. However, the analysis of similarity during wine fermentation showed a high value, likely resulting from the dominance of *Starm. bacillaris* throughout the process. Moreover, the influence of SO_2 did not change the community similarity and composition. This result was consistent with the conclusions from former studies based on culture-dependent and culture-independent techniques (Andorrà et al., 2008; Wang and Liu, 2013). However, more studies are necessary to explain how the fungal community is formed in the vineyard, the changes during wine fermentation, and the relationship between the fungal communities and regional wine characteristics.

The results from different techniques were more comparable during fermentation than in grape must. Massive sequencing was still the most comprehensive technique used in this study, as the detection of fungi is based on few sequences. For these results from massive sequencing analysis, it was important to accurately compare and search for information in the appropriate databases. To analyze the fungal community in this study, primers targeting the D1/D2 region of 26S rDNA were used due to lower differences in the sequence length and more comprehensive reference databases than for the ITS region (Taylor et al., 2014). Some other authors used different approaches based on massive sequencing: Pinto et al. (2014) analyzed sequences from both regions (D1/D2 region of 26S rDNA and ITS) to analyze the whole community, and the results indicated some variations but no significant differences were found. David et al. (2014) used amplicons of 18S rDNA for yeast diversity analysis, and the yeast dynamics trend was basically consistent with our study here. Bokulich and Mills (2013) analyzed very short amplicons from the ITS region to improve the accuracy of high-throughput sequencing, and this approach decreased the bias caused by the differences in length of conventional ITS amplicons. The amplification of different regions might provide results with fewer biases, but databases for corresponding identification are also essential if taxonomy-dependent methods are used. RDP, SILVA, and GenBank were used to assign an identity to all the sequences here (data not shown), and GenBank provided the most complete databases, with which identification at a lower taxonomical level (species) with a high confidence value of identity was achieved (Taylor et al., 2014).

In conclusion, this work indicated different fungal community diversities in grape must after crushing Grenache or Carignan grapes from three vineyards in the Priorat region of Spain. The massive sequencing analysis of grape must could provide information on the presence of plant pathogens and the species able to successfully ferment grape must. The community dynamics during wine fermentation as analyzed by qPCR, DGGE and massive sequencing showed consistent results, especially for detecting non-culturable yeast at the end of fermentation. The population changes from grape skin to grape must are related with the presence of non-*Saccharomyces* yeast on the grapes. The changes during fermentation including ethanol, nutrition, or even some yeast metabolites, introduce the appropriate

conditions for the imposition of *S. cerevisiae*, which conducts the final part of the alcoholic fermentation.

AUTHOR CONTRIBUTIONS

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

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REFERENCES

Andorrà, I., Landi, S., Mas, A., Esteve-Zarzoso, B., and Guillamón, J. M. (2010). Effect of fermentation temperature on microbial population evolution using culture-independent and dependent techniques. *Food Res. Int.* 43, 773–779. doi: 10.1016/j.foodres.2009.11.014

Andorrà, I., Landi, S., Mas, A., Guillamón, J. M., and Esteve-Zarzoso, B. (2008). Effect of oenological practices on microbial populations using culture-independent techniques. *Food Microbiol.* 25, 849–856. doi: 10.1016/j.fm.2008.05.005

Angly, F. E., Dennis, P. G., Skarshewski, A., Vanwonterghem, I., Hugenholtz, P., and Tyson, G. W. (2014). CopyRighter: a rapid tool for improving the accuracy of microbial community profiles through lineage-specific gene copy number correction. *Microbiome* 2, 1–13. doi: 10.1186/2049-2618-2-11

Bokulich, N. A., and Mills, D. A. (2013). Improved selection of internal transcribed spacer-specific primers enables quantitative, ultra-high-throughput profiling of fungal communities. *Appl. Environ. Microb.* 79, 2519–2526. doi: 10.1128/AEM.03870-12

Bokulich, N. A., Ohta, M., Richardson, P. M., and Mills, D. A. (2013). Monitoring seasonal changes in winery-resident mirobiota. *PLoS ONE* 8:e66437. doi: 10.1371/journal.pone.0066437

Bokulich, N. A., Thorngate, J. H., Richardson, P. M., and Mill, D. A. (2014). Microbial biogeography of wine grapes is conditioned by cultivar, vintage, and climate. *Proc. Natl. Acad. Sci. U.S.A.* 111, E139–E148. doi: 10.1073/pnas.1317377110

Colwell, R. K. (2009). "Biodiversity: concepts, patterns, and measurement," in *The Princeton Guide to Ecology*, ed S. A. Levin (Oxfordshire: Princeton University Press), 257–263.

Colwell, R. K. (2013). *EstimateS: Statistical Estimation of Species Richness and Shared Species from Samples*. Available online at: http://purl.oclc.org/estimates

Csoma, H., and Sipiczki, M. (2008). Taxonomic reclassification of *Candida stellata* strains reveals frequent occurrence of *Candida zemplinina* in wine fermentation. *FEMS Yeast Res.* 8, 328–336. doi: 10.1111/j.1567-1364.2007.00339.x

David, V. D., Terrat, S., Herzine, K., Claisse, O., Rousseaux, S., Tourdot-Maréchal, R., et al. (2014). High-throughput sequencing of amplicons for monitoring yeast biodiversity in must and during alcoholic fermentation. *J. Ind. Microbiol. Biot.* 41, 811–821. doi: 10.1007/s10295-014-1427-2

Esteve-Zarzoso, B., Belloch, C., Uruburu, F., and Querol, A. (1999). Identification of yeasts by RFLP analysis of the 5.8S rRNA gene and the two ribosomal internal transcribed spacers. *Int. J. Syst. Bacteriol.* 49, 329–337. doi: 10.1099/00207713-49-1-329

Hierro, N., Esteve-Zarzoso, B., González, Á., Mas, A., and Guillamón, J. M. (2006). Real-time quantitative PCR (QPCR) and resverse transcription-QPCR for detection and enumeration of total yeasts in wine. *Appl. Environ. Microb.* 72, 7148–7155. doi: 10.1128/AEM.00388-06

Hudin, M., and Serra, E. V. (2013). *Vinologue Priorat*. Leavenworth, KS: Leavenworth Press.

Huggett, J. F., Laver, T., Tamisak, S., Nixon, G., O'Sullivan, D. M., Elaswarapu, R., et al. (2013). Considerations for the development and application of control materials to improve metagenomic microbial community profiling. *Accredit. Qual. Assur.* 18, 77–83. doi: 10.1007/s00769-012-0941-z

Kurtzman, C. P., and Robnett, C. J. (1998). Identification and phylogeny of ascomycetous yeasts from analysis of nuclear large subunit (26S) ribosomal DNA partial sequences. *Antonie van Leeuwenhoek* 73, 331–371. doi: 10.1023/A:1001761008817

Mayo, B., Rachid, C. T. C. C., Alegría, Á., Leite, A. M. O., Peixoto, R. S., and Delgado, S. (2014). Impact of next generation sequencing techniques in food microbiology. *Curr. Genomics* 15, 293–309. doi: 10.2174/1389202915666140616233211

Meroth, C. B., Hammes, W. P., and Hertel, C. (2003). Identification and population dynamics of yeasts in sourdough fermentation processes by PCR-denaturing gradient gel electrophoresis. *Appl. Environ. Microb.* 69, 7453–7461. doi: 10.1128/AEM.69.12.7453-7461.2003

Mills, D. A., Johannsen, E. A., and Cocolin, L. (2002). Yeast diversity and persistence in botrytis-affected wine fermentations. *Appl. Environ. Microb.* 68, 4884–4893. doi: 10.1128/AEM.68.10.4884-4893.2002

Pinto, C., Pinho, D., Sousa, S., Pinheiro, M., Egas, C., and Gomes, A. (2014). Unravelling the diversity of grapevine microbiome. *PLoS ONE* 9:e85622. doi: 10.1371/journal.pone.0085622

Ribéreau-Gayon, P., Dubourdieu, D., Donèche, B., and Lonvaud, A. (2006). *Handbook of Enology*. Chichester: John Wiley & Sons, Ltd.

Robinson, J. (2006). *"Priorat" in The Oxford Companion to Wine, 3rd Edn*. New York, NY: Oxford University Press.

Taylor, M. W., Tsai, P., Anfang, N., Ross, H. A., and Goddard, M. R. (2014). Pyrosequencing reveals regional differences in fruit-associated fungal communities. *Environ. Microbiol.* 16, 2848–2858. doi: 10.1111/1462-2920.12456

Torija, M. J., Rozès, N., Poblet, M., Guillamón, J. M., and Mas, A. (2001). Yeast population dynamics in spontaneous fermentations: comparison between two different wine-producing areas over a period of three years. *Antonie van Leeuwenhoek* 79, 345–352. doi: 10.1023/A:1012027718701

Valera, M. J., Torija, M. J., Mas, A., and Mateo, E. (2015). Acetic acid bacteria from biofilm of strawberry vinegar visualized by microscopy and detected by complementing culture-dependent and culture-independent techniques. *Food Microbiol.* 46, 452–462. doi: 10.1016/j.fm.2014.09.006

Wang, C., and Liu, Y. (2013). Dynamic study of yeast species and *Saccharomyces cerevisiae* strains during the spontaneous fermentations of *Muscat blanc* in Jingyang, China. *Food Microbiol.* 33, 172–177. doi: 10.1016/j.fm.2012.09.014

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Overwintering of Vineyard Yeasts: Survival of Interacting Yeast Communities in Grapes Mummified on Vines

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The conversion of grape must into wine involves the development and succession of yeast populations differing in species composition. The initial population is formed by vineyard strains which are washed into the must from the crushed grapes and then completed with yeasts coming from the cellar environment. As the origin and natural habitat of the vineyard yeasts are not fully understood, this study addresses the possibility, that grape yeasts can be preserved in berries left behind on vines at harvest until the spring of the next year. These berries become mummified during the winter on the vines. To investigate whether yeasts can survive in these overwintering grapes, mummified berries were collected in 16 localities in the Tokaj wine region (Hungary-Slovakia) in early March. The collected berries were rehydrated to recover viable yeasts by plating samples onto agar plates. For the detection of minority species which would not be detected by direct plating, an enrichment step repressing the propagation of alcohol-sensitive yeasts was also included in the process. The morphological, physiological, and molecular analysis identified 13 basidiomycetous and 23 ascomycetous species including fermentative yeasts of wine-making relevance among the 3879 isolates. The presence of viable strains of these species demonstrates that the grapes mummified on the vine can serve as a safe reservoir of yeasts, and may contribute to the maintenance of grape-colonizing yeast populations in the vineyard over years, parallel with other vectors and habitats. All basidiomycetous species were known phylloplane yeasts. Three Hanseniaspora species and pigmented *Metschnikowia* strains were the most frequent ascomycetes. Other fermentative yeasts of wine-making relevance were detected only in the enrichment cultures. *Saccharomyces* (*S. paradoxus*, *S. cerevisiae*, and *S. uvarum*) were recovered from 13% of the samples. No *Candida zemplinina* was found. The isolates with *Aureobasidium* morphology turned out to belong to *Aureobasidium subglaciale*, *Kabatiella microsticta*, or *Columnosphaeria fagi*. The ascomyceteous isolates grew at high concentrations of sugars with *Wickerhamomyces anomalus* being the most tolerant species. Complex interactions including antagonism (growth inhibition, contact inhibition, competition for nutrients) and synergism (crossfeeding) among the isolates and with *Botrytis cinerea* shape the composition of the overwintering communities.

Keywords: yeasts, grape, Tokaj, molecular taxonomy, diversity, antagonism, wine

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INTRODUCTION

Wine is the product of the activity of complex microbial communities in which fermentative yeasts and bacteria play the major roles. It has been demonstrated by numerous studies, that the vineyard flora is the primary source of inoculation of the grape must with yeasts (for a review, see Fleet et al., 2002). When the fermentation takes place in wineries regularly used for wine-making, the population of the grape yeasts is supplemented with residential winery yeasts. In spontaneous fermentation, these yeasts drive the fermentation process with successive dominance of less and more alcohol tolerant species. Although fermentative yeasts, including *Saccharomyces*, can occur on grapes (e.g., Combina et al., 2005; Schuller et al., 2005; Valero et al., 2007; Cordero-Bueso et al., 2011; Setati et al., 2012; Bokulich et al., 2014; Taylor et al., 2014), the winery-borne yeasts usually overgrow the grape-borne strains in advanced stages of fermentation performed in cellars and can dominate the process until its completion (e.g., Rosini, 1984; Martini, 1993; Vaughan-Martini and Martini, 1995; Egli et al., 1998; Gutierrez et al., 1999; Ciani et al., 2004; Santamarıa et al., 2005; Mercado et al., 2007; Di Maio et al., 2012).

The grape berries are colonized in the vineyard by both ascomyceteous and basidiomyceteous yeasts, and their communities change over time depending on the stage of ripening (for reviews, see Fleet et al., 2002; Bisson and Joseph, 2009; Barata et al., 2012). In a comprehensive study, Bourret et al. (2013) identified 53 yeast species on grapes in a Washington State vineyard. Other laboratories have found most of these species in many other wine-growing regions of the globe and found numerous additional species (e.g., Yanagida et al., 1992; Zahavi et al., 2002; Antunovics et al., 2003; Prakitchaiwattana et al., 2004; Raspor et al., 2006; Sipiczki, 2006; Renouf et al., 2007; Chavan et al., 2009; Brysch-Herzberg and Seidel, 2015; Nemcová et al., 2015; Setati et al., 2015).

The main factor determining the composition of the yeasts communities on the grape appears to be nutrient availability on the berry surface which increases with ripening. During the growth of the grape, before the unset of ripening, the surface yeast flora is dominated by basidiomyceteous genera (e.g., *Cryptococcus*, *Rhodosporidium*, *Rhodotorula*, *Sporobolomyces*) and the dimorphic ascomyceteous genus *Aureobasidium* (reviewed in Bisson and Joseph, 2009; Barata et al., 2012). These yeasts capable of growth in the nutrient-poor surface of the developing berries are also present on other parts of the grapevine and on the phylloplane of other plants (for a review, see Fonseca and Inacio, 2006). When the fruit begins to ripen, yeasts belonging to ascomyceteous genera (e.g., *Hanseniaspora*, *Metschnikowia*, *Candida*) start proliferating on the grape skin, probably due to nutrients leaking out through the thinning skin. Interestingly, *Saccharomyces*, the major wine yeast is not ubiquitous on the ripening grape and if present, only constitutes very small fractions of the yeast communities (Setati et al., 2012; Bokulich et al., 2014; Taylor et al., 2014). *Saccharomyces* strains were more frequently isolated from heavily damaged grapes (e.g., Mortimer and Polsinelli, 1999), where the juice of the grape became accessible to the yeasts through the skin lesions.

All yeasts present on the grape at harvest are washed into the must at crush. However, not all grape-borne yeasts are equally important for the process of turning the grape must into wine (vinification). The basidiomyceteous species are the least relevant group because they die off very quickly in the must due to their inability to ferment the juice sugars. The ascomyceteous yeast-like *Aureobasidium* does not survive in the wine either (Renouf et al., 2007). Among the fermentative species, *S. cerevisiae* and *S. uvarum* (*S. bayanus* var. *uvarum*) are the most important yeasts because they drive the alcoholic fermentation and release the most important metabolites into the fermenting wine. The non-*Saccharomyces* yeasts usually play secondary roles by fine-tuning the wine character or act as spoilage microorganisms producing off-flavors (for reviews, see Loureiro and Malfeito-Ferreira, 2003; Jolly et al., 2014).

Some important questions are still to be answered about how the fermentative non-phylloplane yeasts show up on the grapes. Insects attracted by damaged berries have been implicated in the dispersal of the yeasts, with honey bees (Goddard et al., 2010), wasps (Stefanini et al., 2012), and the fruit fly *Drosophila* assumed to act as vectors (Lam and Howell, 2015) and perhaps also to preserve the yeasts in their (hibernated or dehydrated) bodies over the winter until the next spring. It is pertinent to note here, that various yeasts species have been isolated from various *Drosophila* species collected in various habitats such as tree exudates, rotting cacti, rain forests, oak-pine forests, etc. (e.g., Dobzhansky et al., 1956; Phaff and Knapp, 1956; Starmer et al., 1976; Gilbert, 1980; Lachance et al., 1995; Morais et al., 2005). These yeasts can easily be vectored onto the ripe grape by the host insects. However, fermentative yeast species are detectable already in early stages of ripening when the berries are still sound (intact). In addition, an important fermentative yeast, *Saccharomyces* does not appear to be regularly associated with the *Drosophila* flies in the nature. In a recent study, *S. cerevisiae* was not detected in 296 flies captured in vineyards, grape waste (marc) piles and wineries of two Australian wine-growing regions during grape harvest (Lam and Howell, 2015). Moreover, it is not the fruit volatiles but the yeasts, that attract *Drosophila* flies (Becher et al., 2012). *Hanseniaspora* has been found to produce aromas that are attractive to *D. melanogaster* (Palanca et al., 2013). So, at least certain berries have to be colonized by yeasts before the flies come, otherwise they would not come.

Vineyard soil is a potential source of the grape yeasts because the berries which fall to the ground during ripening and at harvest harbor large populations of yeasts. The work of Cordero-Bueso et al. (2011) describing different yeast populations in musts produced from grapes of wineyards in which different soil management methods were used indicates, that the condition of the soil can have an impact on the yeast communities of the grape. However, the soil is a rather unfavorable habitat for yeast overwintering because the soil microorganisms decompose the organic materials (for a review, see Treseder and Lennon, 2015) including the berries and their yeast colonists. Parle and Di Menna (1966) found very few fermentative yeasts in summer samples of vineyard soil and only *Kloeckera (Hanseniaspora) apiculata* in winter samples.

The goal of this study was to investigate an alternative possibility, the survival of grape yeasts in berries left-behind on the vine at harvest. These berries turn dry during winter and become mummified. To investigate whether the yeasts colonizing the ripe grape in autumn can survive the winter in these berries, mummified grapes were collected in the Tokaj wine-growing region in March. The peculiarity of this region is the extensive botrytisation leading to noble rotting of the grape on the vine. Noble rot is a benevolent *Botrytis*-generated process associated with dehydration (drastic increase of sugar content) and intense colonization of the ruptured berries by complex microbial consortia (Antunovics et al., 2003; Magyar and Bene, 2004). The collected grape mummies were rehydrated and used for recovering viable yeasts. The taxonomic examination of the recovered yeasts identified high numbers of basidiomyceteous and ascomyceteous yeast species, demonstrating, that the grape mummified on the vine also may contribute to the maintenance of the continuity of the vineyard yeast microflora over consecutive years.

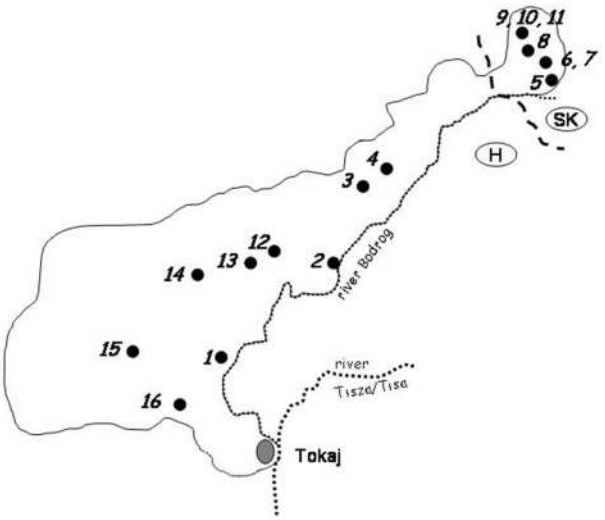

FIGURE 1 | Geographic locations of vineyards in which samples were collected. 1. Szegi; 2. Sárazsadány; 3. Hercegkút; 4. Hercegkút; 5. Viničky; 6. Bara; 7. Bara; 8. Černochov; 9. Malá Tŕňa; 10. Malá Tŕňa; 11. Malá Tŕňa; 12. Tolcsva; 13. Tolcsva; 14. Erdöbénye; 15. Abaujszántó; 16. Mád.

MATERIALS AND METHODS

Sample Collection and Yeast Isolation

Three bunches of mummified grapes were collected from vines in each of the16 vineyards selected for the investigation (**Figure 1**). Five berries were picked aseptically from each bunch and placed in a sterile test-tube. As the berries were completely desiccated, 2.5 ml of YEL (1% yeast extract, 2% glucose) was added to the test-tube to rehydrate them. After 1 h of incubation at room temperature, the soaked berries were macerated with a sterile spatula and homogenized with intense vortexing. 10-μl aliquots of the homogenized sample were spread onto YEA (YEL supplemented with 2% agar) plates. The rest of the sample was incubated at room temperature overnight. Then 10-μl aliquots were plated on YEA again and 0.5-ml volumes were transferred into test-tubes containing 4.5 ml of enrichment medium (0.68% yeast nitrogen base, 1.1% raffinose, and 9% ethanol). This medium selectively supports the growth of the yeasts which tolerate high ethanol concentrations and utilize raffinose as a carbon source (Sampaio and Goncalves, 2008). The tube was incubated at 10°C. After 4 weeks of incubation, 10-μl aliquots of the enrichment culture were plated on YEA (when the cell number was high, the aliquots were diluted before plating). After 7 days of incubation at room temperature, colonies (max 150) were randomly isolated from the plates for each grape sample in order to obtain representative collections of pure isolates. The isolates were stored at 5°C on YEA plates and reinoculated onto fresh plate every month.

Phenotypic Characterization of Isolates

Colony morphology (color, surface ornamentation, production of pigmented zone in the medium) on YEA plates was examined and recorded for each isolate. All isolates were tested for the ability to assimilate 14 compounds as carbon-sources and lysine as a nitrogen source by replica-plating of 5-day old YEA cultures onto assimilation test plates (0.68% DIFCO yeast nitrogen base and 2% agar) supplemented with the carbon sources and onto SMA-lysine plates (2% glucose, 2% agar, 0.5% lysine and vitamins). The carbon sources tested were: cellobiose, ethanol, galactose, glucose, inulin, maltose, melesitose, melibiose, raffinose, rhamnose, ribose, saccharose, trehalose, and xylose. Growth was evaluated after 7 days of incubation at room temperature.

Molecular Taxonomy

For taxonomic identification of the isolates, the D1/D2 domains of their large subunit ribosomal RNA genes and the ITS regions were amplified and sequenced with the primer pairs NL1-NL2 and ITS1-ITS4 as described earlier (Sipiczki, 2003). To assess their taxonomic positions, the resultant sequences were used to identify similar sequences in the GenBank database with the MEGABLAST-querying service of NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi). As the GenBank entries are not checked for the correctness of their taxonomic assignment by the depositors the D1/D2 sequences of the isolates were then aligned with the D1/D2 sequences of the type strains of the species whose sequences were found highly similar in the GenBank search. For this, the sequences of the type strains were downloaded from the CBS database (http://www.cbs.knaw.nl/Collections/). The exact sequence similarity with the type strain sequences (number of identical nucleotides) was determined by pairwise Blast alignment using the bl2seq algorithm available at NCBI.

Osmotolerance

Dense suspensions of cells ($\sim 10^7$ cells ml^{-1}) were prepared from early stationary phase cultures of the isolates cultivated in YEL at room temperature for 2 days. 5-μl aliquots of the suspensions were dropped on YEA plates supplemented with 2, 20, 30, 40, and 50% of a 1:1 mixture of glucose and fructose. Yeast growth

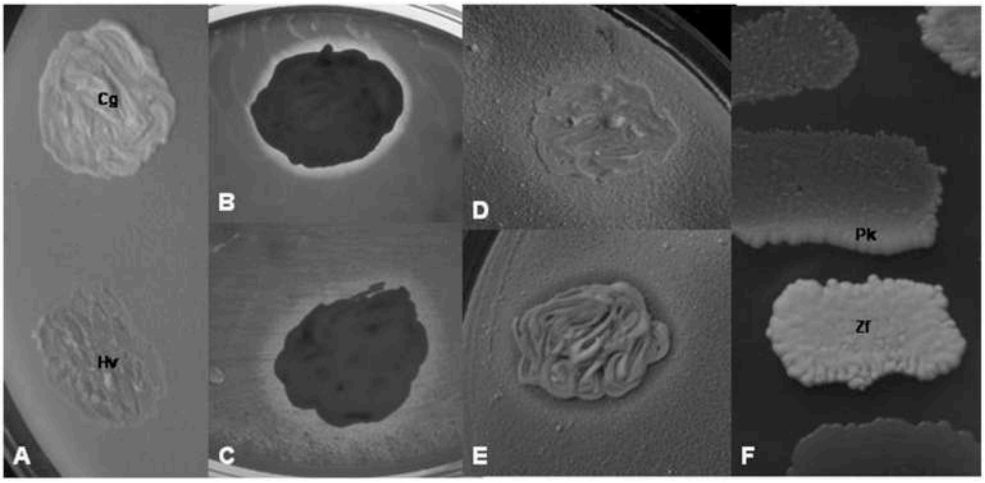

FIGURE 2 | Yeast-yeast interactions. (A) *L. thermotolerans* 8/2z-3 (lawn) shows no interaction with *Ca. glabrata* 14/1z-1 (Cg) but inhibits the growth of *H. vineae* 11/1z-4 (Hv). **(B)** Clear inhibition zone in the *Ca. glabrata* 14/1z-1 lawn around the *W. anomalus* 15/z-7 colony. **(C)** Turbid inhibition zone in the *H. vineae* 11/1z-4 lawn around the *W. anomalus* 15/z-7 colony. **(D)** Synergistic effect of the *P. scaptomyzae* 12/z-4 colony on the *T. delbrueckii* 1/1/z-10 lawn. **(E)** Dual effect: inhibition zone and crossfeeding of the *Kl. dobzhanskii* 9/z-6 lawn by *the P. scaptomyzae* 12z-4 colony. **(F)** Crossfeeding of the melibiose-minus *P. kluyveri* 11/2-104 colony (Pk) by the melibiose-positive *Zt. florentina* 7z-11 colony (Zf) on the medium containing melibiose as carbon source. **(B)** and **(C)** were photographed with transmitted light.

was evaluated after 5 days of incubation at 25°C by comparing the thickness of the spots.

Interaction Tests

(a) Interactions among yeasts. For testing the yeast isolates for interactions with other yeast isolates, 5-day old cultures grown on YEA plates were used. Dense suspensions ($\sim 10^8$ cells ml^{-1}) were prepared from each isolate in 2 ml of sterile water, and YEA plates were flooded with the suspensions to obtain homogeneous lawns of cells. The rests of the suspensions were pored off. After drying the surface of the plates, loopful amounts of other isolates were smeared on the plates to form spots of ∼5 mm in diameter (**Figure 2**). The plates were then incubated at 25°C for 7 days and examined at regular time intervals for the growth intensity of the spots and the lawn around the spots.

(b) Interactions between yeast isolates and *B. cinerea*. The effect of the yeast isolates on fungal growth was examined on YEA and PDA (Potato Dextrose Agar, Scharlab S.L.) plates flooded with suspensions of *Botrytis* conidia. The suspension of conidia was obtained by washing the surface of 2-week old *B. cinerea* 980 (Sipiczki, 2006) cultures grown on PDA at room temperature with sterile water. After removing the rest of the suspension and drying the surface of the plates, yeast isolates were inoculated onto the lawn of conidia as described above. The plates were incubated at 20°C for 2 weeks and examined at regular time intervals.

RESULTS

Yeast Isolation, Characterization, and Taxonomic Identification

Yeast colonies were obtained with both isolation methods (with and without enrichment), even from those enrichment cultures which did not become turbid (no yeast growth). 3879 isolated colonies were examined for morphology, tested for the utilization of 15 compounds as sole carbon or nitrogen sources and then clustered into groups on the basis of their phenotypes. From representatives of the phenotypic groups, D1/D2 domain regions of the rDNA arrays were amplified and sequenced. Based on the similarity of the sequences to those of the type strains of known species, all but one group could be assigned to known species. 13 basidiomyceteous and 23 ascomyceteous species were identified in this way among the isolates.

Three groups of basidiomyceteous isolates could not be assigned to single species because their D1/D2 sequences were equally similar to the D1/D2 sequences of more than one type strain. In one of these groups, the similarity search found three identical type-strain sequences: *Cryptococcus magnus, Filobasidium elegans*, and *Filobasidium floriforme*. These species are indistinguishable when their D1/D2 sequences are compared but can be separated when certain physiological traits are also examined (Fell et al., 2000; Fonseca et al., 2011; Kwon-Chung, 2011). The assimilation tests of the isolates assigned this group to *Cr. magnus* because they grew on galactose (difference from *F. elegans*) and inulin (difference from both *F. elegans* and *F. florioformae*). A different group of isolates differed by one nucleotide from both *Sporobolomyces coprosmae* and *Sp. oryzicola*, a pair of very closely related sibling species which cannot be distinguished by D1/D2 sequencing (Scorzetti et al., 2002). Unfortunately, they cannot be differentiated by their growth reactions on the carbon compounds commonly tested in taxonomical studies, either (Hamamoto et al., 2011). Therefore, the ITS region also was sequenced from one of the isolates. Its sequence differed by 2 substitutions from *Sp. coprosmae* and 3 substitutions from *Sp. oryzicola*. Additional genes should be sequenced to reinforce the somewhat closer relationship to the

former species. The third group showed 100% identity with the *Curvibasidium pallidicorallinum* type strain and differed from the *Rhodotorula nothofagi type* strain only by one substitution. The latter difference was due to an unambiguous nucleotide in the type strain sequence, so the somewhat higher similarity to *Cu. pallidicorallinum* was not relevant. The sugar assimilation tests indicated conspecificity with *Cu. pallidicorallinum:* the isolates grew on maltose, trehalose, and inulin, which are not assimilated by *R. notophagi* (Sampaio, 2011a,b).

The group represented by the isolate 6–13 in **Table 1** could not be assigned to any species because its D1/D2 domain was very different from all type-strain sequences. Its closest relative type strain was *Cr. keelungensis* CBS 10876^T, from which it differed by 24 substitutions (4%). Interestingly, it showed 99 to 100% identity to D1/D2 sequences of taxonomically uncharacterized yeast strains isolated from tree leaves, floral nectar (Alvarez-Perez and Herrera, 2013), and Iberian Pyrite Belt (Gadanho et al., 2006), indicating conspecificity with a hitherto non-described species which seems to live in diverse habitats.

Almost all samples harbored cells, that produced colonies showing the morphology characteristic of the ascomycetous yeast-like fungus *Aureobasidium pullulans*. These rapidly growing colonies contained yeast-like cells and septate hyphae producing lateral blastospores. Since *A. pullulans* plays a very marginal role in wine-making and is quite ubiquitous on the phylloplane, only a few isolates of this morphology were characterized taxonomically in this study. Unexpectedly, their D1/D2 sequences showed closer relationship to *A. glaciale*, *Kabatiella microsticta,* and *Columnosphaeria fagi* than to *A. pullulans*. The examples shown in **Table 1** have D1/D2 sequences identical with those of these species and differ from that of the *A. pullulans* type strain by 17 and 4 substitutions, respectively.

The isolates producing pigmented halos in the medium around their colonies appeared to belong to the *pulcherrima* clade of *Metschnikowia* (Lachance, 2011). However, their exact taxonomic position could not be determined because their D1/D2 sequences were diverse and different from the corresponding sequences of all type strains of the clade. For example, the GenBank database sequences most similar to the example shown in **Table 1** were KM350710 and KM275362 which were cloned from the rDNA arrays of the *M. sinensis* and *M. andauensis* type strains CBS 10359^T and CBS 10357^T, respectively.

Tables S1, S2 show the occurrence and relative abundance of species in the 48 samples. The most frequently encountered species belonged to the genera *Metschnikowia*, *Hanseniaspora*, *Cryptococcus* before enrichment and *Kluyveromyces*, *Lachancea*, and *Pichia* after enrichment. Basidiomyceteous yeasts were detected in 69% of the samples before enrichment and only in 4% of the samples after enrichment (Table S2), indicating that the enrichment conditions were lethal to this group. The yeasts most frequently occurring in the enrichment cultures were strains of the ascomycetous genera *Metschnikowia* (in 33%), *Lachancea thermotolerance* (in 31%), and *Hanseniaspora osmophila* (in 31%; Table S1). Among these species only *L. thermotolerance* can utilize raffinose as carbon source. The other two species must have gained energy from alternative sources released from the dehydrated berry tissues and could overgrow the other yeasts due to their better ability to tolerate the high alcohol concentration and the low incubation temperature. *Saccharomyces* strains were found only in enriched cultures and only in 6 vineyards: *S. paradoxus* in 4, *S. cerevisiae* in 2, *S. uvarum* in 1 vineyard.

Lysine utilization was included in the phenotypic characterization of the isolates because one of the most frequently used methods in wine microbiology to identify *Saccharomyces* versus non-*Saccharomyces* yeasts is plating on lysine agar. *Saccharomyces* does not grow on lysine as a sole nitrogen source, therefore only non-*Saccharomyces* yeasts will grow on these plates (Angelo and Siebert, 1987). Consistent with this, none of the three *Saccharomyces* species identified in this study could utilize lysine as a nitrogen source. However, certain *Hanseniaspora*, *Candida glabrata*, *Zygoascus meyerae*, *Zygosaccharomyces bailii*, *Kl. Dobzhanskii*, and *L. thermotolerans* isolates identified by sequencing were also lysine minus.

Osmotolerance

The results are shown in **Table 2**. Surprisingly, only 7 isolates grew at 50% sugar, but neither the *Saccharomyces* species nor *Zs. bailii* were among them. The most osmotolerant species was *Wickerhamomyces anomalus.*

Antagonistic and *Synergistic* Interactions among *Yeast* Isolates

Negative interaction (growth inhibition) was detected as an inhibition zone in the sensitive lawn around the colony of the antagonist (**Figures 2B,C**) or as the inhibition of the growth of the sensitive colony on the lawn of the antagonistic isolate (**Figure 2A**). Two types of inhibition zones could be distinguished: clear (**Figure 2B**) and turbid (**Figure 2C**) zones. The positive, growth intensifying effect was noticed as a halo of stronger growth of the lawn (lawn thickening) around the colony of the isolate which had such effect (**Figure 2D**). In a few combinations of isolates simultaneous antagonistic and synergistic effects could be seen (**Figure 2E**). The results of the interaction tests carried out with isolates representing all ascomycetous yeasts are shown in **Table 3**.

When the isolates were tested for the assimilation of sugars as carbon sources (see above), an interesting mode of growth stimulation was noticed. On the plates supplemented with saccharose or melibiose, certain isolates able to utilize these disaccharides stimulated the growth of certain other isolates which were unable to assimilate them. An example is shown in **Figure 2F**. The print of the colony of the melibiose-negative isolate 11/2-104 (*Pichia kluyveri*) replica-plated on the melibiose medium grew along its edge facing the colony of the melibiose-positive isolate 7z–11 (*Zygotorulaspora florentina*). Apparently, the positive isolate supplied the negative isolate with utilizable carbon sources (crossfeeding).

The Effect of Yeast Isolates on the Growth of *Botrytis*

The isolates selected for yeast-yeast interactions tests were also tested for effects on the growth of *B. cinerea*. Four types of interactions with the fungus were observed: (1) Clear inhibition

TABLE 1 | D1/D2 sequence differences of selected representatives of the phenotypic groups of isolates from type strains of the most similar species.

Isolate			Most similar type/reference strain		Sequence difference (number of substitutions/indels)
Identification number	**Location of sample collection**	**D1/D2 accession number**	**Taxonomic name**	**D1/D2 accession number**	
ASCOMYCOTA, PEZIZOMYCOTINA					
10–59	Malá Tŕňa	KU254559	*Aureobasidium subglaciale* CBS 123387^T	FJ150913	0
1/2–11	Szegi		*Kabatiella microsticta* CBS 342.66 *Columnosphaeria fagi* CBS 171.93	FJ150952 AY016359	0 0
6/1–5	Bara	KU254558	*Kabatiella microsticta* CBS 342.66 *Columnosphaeria fagi* CBS 171.93	FJ150952 AY016359	0 0
13/1–4	Tolcsva		*Kabatiella microsticta* CBS 342.66 *Columnosphaeria fagi* CBS 171.93	FJ150952 AY016359	0 0
ASCOMYCOTA, SACCHAROMYCOTINA					
14/1z-1	Erdöbénye	KT933331	*Candida glabrata* CBS 138^T	AF399771	1
11/1–54	Malá Tŕňa		*Candida oleophila* CBS 2219^T	U45793	0
11/1-55	Malá Tŕňa	KT122406	*Candida oleophila* CBS 2219^T	U45793	0
7-9	Bara		*Kregervanrija fluxuum* CBS 639^T	U70247	3^z
1/1z-2	Szegi		*Hanseniaspora osmophila* CBS 313^T	U84228	0
2z-22	Sárazsadány		*Hanseniaspora osmophila* CBS 313^T	U84228	0
4–3	Hercegkút		*Hanseniaspora osmophila* CBS 313^T	U84228	0
4z-5	Hercegkút		*Hanseniaspora osmophila* CBS 313^T	U84228	0
5/1z-3	Vinièky		*Hanseniaspora osmophila* CBS 313^T	U84228	0
5/2–6	Viničky		*Hanseniaspora osmophila* CBS 313^T	U84228	1^z
5/2z-6	Viničky		*Hanseniaspora osmophila* CBS 313^T	U84228	0
5/2z-11	Viničky		*Hanseniaspora osmophila* CBS 313^T	U84228	1^z
7/2z-1	Bara		*Hanseniaspora osmophila* CBS 313^T	U84228	0
8–3	Černochov	KT933332	*Hanseniaspora osmophila* CBS 313^T	U84228	0
8z-4	Černochov	KT175536	*Hanseniaspora osmophila* CBS 313^T	U84228	0
9z-3	Malá Tŕňa		*Hanseniaspora osmophila* CBS 313^T	U84228	0
11/2z-2	Malá Tŕňa		*Hanseniaspora osmophila* CBS 313^T	U84228	0
12/2z-3	Tolcsva		*Hanseniaspora osmophila* CBS 313^T	U84228	0
13/2–90	Tolcsva		*Hanseniaspora osmophila* CBS 313^T	U84228	0
13/2z-5	Tolcsva		*Hanseniaspora osmophila* CBS 313^T	U84228	0
15z-4	Abaújszántó		*Hanseniaspora osmophila* CBS 313^T	U84228	0
1–3	Szegi		*Hanseniaspora uvarum* CBS 314^T	U84229	0
1z-2	Szegi		*Hanseniaspora uvarum* CBS 314^T	U84229	0
1/1–32	Szegi		*Hanseniaspora uvarum* CBS 314^T	U84229	0
2–4	Sárazsadány		*Hanseniaspora uvarum* CBS 314^T	U84229	0
4/1–6	Hercegkút		*Hanseniaspora uvarum* CBS 314^T	U84229	0
7–3	Bara		*Hanseniaspora uvarum* CBS 314^T	U84229	0
7/2–17	Bara		*Hanseniaspora uvarum* CBS 314^T	U84229	0
8/2-28	Černochov		*Hanseniaspora uvarum* CBS 314^T	U84229	0
9/1–3	Malá Tŕňa		*Hanseniaspora uvarum* CBS 314^T	U84229	0
9/1–66	Malá Tŕňa	KT156710	*Hanseniaspora uvarum* CBS 314^T	U84229	0
11/1–10	Malá Tŕňa		*Hanseniaspora uvarum* CBS 314^T	U84229	0
13/2–4	Tolcsva		*Hanseniaspora uvarum* CBS 314^T	U84229	0
14/1–7	Erdöbénye		*Hanseniaspora uvarum* CBS 314^T	U84229	0
15/1–10	Abaújszántó		*Hanseniaspora uvarum* CBS 314^T	U84229	0
15/2–1	Abaújszántó		*Hanseniaspora uvarum* CBS 314^T	U84229	0
16–5	Mád		*Hanseniaspora uvarum* CBS 314^T	U84229	0
16/2–2	Mád		*Hanseniaspora uvarum* CBS 314^T	U84229	0
1/1–5	Szegi		*Hanseniaspora vineae* CBS 2171^T	U84224^x	3
11/1z-4	Malá Tŕňa	KT933333	*Hanseniaspora vineae* CBS 2171^T	U84224^x	1

(Continued)

TABLE 1 | Continued

Isolate			Most similar type/reference strain		Sequence difference (number of substitutions/indels)
Identification number	**Location of sample collection**	**D1/D2 accession number**	**Taxonomic name**	**D1/D2 accession number**	
12/1z-2	Tolcsva		*Hanseniaspora vineae* CBS 2171^{T}	U84224^{x}	4
12/1z-5	Tolcsva		*Hanseniaspora vineae* CBS 2171^{T}	U84224^{x}	4
5z-9	Viničky		*Kluyveromyces dobzhanskii* CBS 2104^{T}	U69575	0
5z-17	Viničky		*Kluyveromyces dobzhanskii* CBS 2104^{T}	U69575	0
7/1z-5	Bara		*Kluyveromyces dobzhanskii* CBS 2104^{T}	U69575	0
9/z-1	Malá Tàòa		*Kluyveromyces dobzhanskii* CBS 2104^{T}	U69575	0
9z-6	Malá Tŕňa	KT122408	*Kluyveromyces dobzhanskii* CBS 2104^{T}	U69575	0
10z-4	Malá Tŕňa		*Kluyveromyces dobzhanskii* CBS 2104^{T}	U69575	0
10/1z-1	Malá Tŕňa		*Kluyveromyces dobzhanskii* CBS 2104^{T}	U69575	0
2/2z-8	Sárazsadány		*Lachancea thermotolerans* CBS 6340^{T}	U69581	0
5/1z-7	Viničky		*Lachancea thermotolerans* CBS 6340^{T}	U69581	0
8z-1	Černochov	KT175534	*Lachancea thermotolerans* CBS 6340^{T}	U69581	0
8/2z-3	Černochov	KT933334	*Lachancea thermotolerans* CBS 6340^{T}	U69581	0
9/1-15	Malá Tŕňa		*Lachancea thermotolerans* CBS 6340^{T}	U69581	0
9/1z-4	Malá Tŕňa		*Lachancea thermotolerans* CBS 6340^{T}	U69581	0
10/2z-4	Malá Tŕňa		*Lachancea thermotolerans* CBS 6340^{T}	U69581	0
11–27	Malá Tŕňa		*Lachancea thermotolerans* CBS 6340^{T}	U69581	0
11z-1	Malá Tŕňa		*Lachancea thermotolerans* CBS 6340^{T}	U69581	0
11/2–112	Malá Tŕňa		*Lachancea thermotolerans* CBS 6340^{T}	U69581	0
14/1z-2	Erdöbénye		*Lachancea thermotolerans* CBS 6340^{T}	U69581	0
3z-1	Hercegkút		*Metschnikowia* sp. 11-1090 clone d4	KM350710	11
5z-6	Viničky	KT933337	*Pichia fermentans* CBS 187^{T}	U75726	2
5z-10	Viničky		*Pichia fermentans* CBS 187^{T}	U75726	2^{y}
5z-12	Viničky		*Pichia fermentans* CBS 187^{T}	U75726	1
11/2–104	Malá Tŕňa	KT156709	*Pichia kluyveri* CBS 188^{T}	U75727^{x}	1
4/1–34	Hercegkút	KT933335	*Pichia membranifaciens* CBS 2763^{T}	DQ198963	3
15/1z-9	Abaújszántó		*Pichia membranifaciens* CBS 2763^{T}	DQ198963	3
12z-4	Tolcsva	KT933336	*Pichia scaptomyzae* CBS 8167^{T}	AB045136	0
12z-14	Tolcsva		*Pichia scaptomyzae* CBS 8167^{T}	AB045136	0
3/1z-5	Hercegkút		*Saccharomyces cerevisiae* CBS 1171^{NT}	U44806	0
14/z-1	Erdöbénye	KT933338	*Saccharomyces cerevisiae* CBS 1171^{NT}	U44806	0
3z-28	Hercegkút		*Saccharomyces paradoxus* CBS 432^{NT}	U68555	2
3/2z-5	Hercegkút		*Saccharomyces paradoxus* CBS 432^{NT}	U68555	2
5z-7	Viničky		*Saccharomyces paradoxus* CBS 432^{NT}	U68555	2
7/2z-2	Bara		*Saccharomyces paradoxus* CBS 432^{NT}	U68555	2
7/2z-3	Bara		*Saccharomyces paradoxus* CBS 432^{NT}	U68555	2
10z-2	Malá Tŕňa	KT122407	*Saccharomyces paradoxus* CBS 432^{NT}	U68555	2
4/2z-11	Hercegkút	KT933339	*Saccharomyces uvarum* CBS 395^{T}	AJ279065	0
1/1z-1	Szegi		*Torulaspora delbrueckii* CBS 1146^{T}	U72156	0
1/1z-10	Szegi	KT933340	*Torulaspora delbrueckii* CBS 1146^{T}	U72156	0
15z-7	Abaújszántó	KT933341	*Wickerhamomyces anomalus* CBS 5759^{T}	U74592	0
8z-2	Černochov	KT175535	*Zygoascus meyerae* CBS 7521^{T}	AY447014	0
4–24	Hercegkút	KT933342	*Zygosaccharomyces bailii* CBS 680^{T}	U72161	0
8–29	Černochov		*Zygosaccharomyces bailii* CBS 680^{T}	U72161	0
2z-30	Sárazsadány		*Zygotorulaspora florentina* CBS 746^{T}	U72165	0
3/2–1	Hercegkút		*Zygotorulaspora florentina* CBS 746^{T}	U72165	0
7z-11	Bara	KU254556	*Zygotorulaspora florentina* CBS 746^{T}	U72165	0
9/2z-9	Malá Tŕňa		*Zygotorulaspora florentina* CBS 746^{T}	U72165	0

(Continued)

TABLE 1 | Continued

Isolate			Most similar type/reference strain		Sequence difference (number of substitutions/indels)
Identification number	**Location of sample collection**	**D1/D2 accession number**	**Taxonomic name**	**D1/D2 accession number**	
BASIDIOMYCOTA, AGARIMYCOTINA					
13/1–34	Tolcsva	KT933343	*Bulleromyces albus* CBS 500^{T}	AF416643	0
6/2–10	Bara	KT933344	*Cryptococcus carnescens* CBS 973^{T}	AB035054	0
7/2–10	Bara		*Cryptococcus flavescens* CBS 942^{T}	AB035042	0
10/2–3	Malá Tŕňa		*Cryptococcus flavescens* CBS 942^{T}	AB035042	0
8–30	Černochov	KT933345	*Cryptococcus flavescens* CBS 942^{T}	AB035042	0
12/2–18	Tolcsva		*Cryptococcus flavescens* CBS 942^{T}	AB035042	0
13/1–29	Tolcsva		*Cryptococcus flavescens* CBS 942^{T}	AB035042	0
6–13	Bara	KT001494	*Cryptococcus keelungensis* CBS 10876^{T}	EF621562	24
6–21	Bara	KT933346	*Cryptococcus magnus* CBS 140^{T} *Filobasidium elegans* CBS 7640 *Filobasidium floriforme* CBS 6241	AF181851 AF181548 AF075498	0 0 0
7/1–40	Bara	KT933352	*Cryptococcus magnus* CBS 140^{T} *Filobasidium elegans* CBS 7640 *Filobasidium floriforme* CBS 6241	AF181851 AF181548 AF075498	0 0 0
7/1–55	Bara		*Cryptococcus magnus* CBS 140^{T} *Filobasidium elegans* CBS 7640 *Filobasidium floriforme* CBS 6241	AF181851 AF181548 AF075498	1 1 1
10/2–10	Malá Tŕňa	KT933347	*Cryptococcus stepposus* CBS 10265^{T}	DQ222456	0
11/2–10	Malá Tŕňa	KT933348	*Cryptococcus victoriae* CBS 8685^{T}	AF363647	2
12/2–50	Tolcsva		*Cryptococcus victoriae* CBS 8685^{T}	AF363647	2
13/2–49	Tolcsva	KU254557	*Cryptococcus carnescens* CBS 973^{T} *Cryptococcus victoriae* CBS 8685^{T}	AB035054 AF363647	8 8
13/1–37	Tolcsva	KT933349	*Cryptococcus wieringae* CBS 1937^{T}	AF181541	0
7/1–56	Bara	KT933353	*Holtermanniella festucosa* VKM Y-2930^{T}	AY462119	6
BASIDIOMYCOTA, PUCCINIOMYCOTINA					
8/1–14	Černochov	KT933350	*Curvibasidium cygneicollum* CBS 4551^{T}	AF189928	0
10–10	Malá Tŕňa		*Curvibasidium cygneicollum* CBS 4551^{T}	AF189928	0
10–20	Malá Tŕňa		*Curvibasidium cygneicollum* CBS 4551^{T}	AF189928	0
15–23	Abaújszántó		*Curvibasidium cygneicollum* CBS 4551^{T}	AF189928	0
15–25	Abaújszántó		*Curvibasidium cygneicollum* CBS 4551^{T}	AF189928	1
6–23	Bara		*Curvibasidium pallidicorallinum* CBS 9091^{T} *Rhodotorula nothofagi* CBS 8166^{T}	AF444736 AF189950^{W}	0 1
7/1–1	Bara	KT156708	*Curvibasidium pallidicorallinum* CBS 9091^{T} *Rhodotorula nothofagi* CBS 8166^{T}	AF444736 AF189950^{W}	0 1
7/1–2	Bara		*Curvibasidium pallidicorallinum* CBS 9091^{T} *Rhodotorula nothofagi* CBS 8166^{T}	AF444736 AF189950^{W}	0 1
7/1–50	Bara		*Curvibasidium pallidicorallinum* CBS 9091^{T} *Rhodotorula nothofagi* CBS 8166^{T}	AF444736 AF189950^{W}	0 1
9–25	Malá Tŕňa	KT933351	*Curvibasidium pallidicorallinum* CBS 9091^{T} *Rhodotorula nothofagi* CBS 8166^{T}	AF444736 AF189950^{W}	1 2
9–50	Malá Tŕňa		*Curvibasidium pallidicorallinum* CBS 9091^{T} *Rhodotorula nothofagi* CBS 8166^{T}	AF444736 AF189950^{W}	0 1
11–2	Malá Tŕňa		*Curvibasidium pallidicorallinum* CBS 9091^{T} *Rhodotorula nothofagi* CBS 8166^{T}	AF444736 AF189950^{W}	0 1
13/1z-1	Tolcsva		*Curvibasidium pallidicorallinum* CBS 9091^{T} *Rhodotorula nothofagi* CBS 8166^{T}	AF444736 AF189950^{W}	0 1
13/1–16	Tolcsva	KT933354	*Rhodotorula graminis* CBS 2826^{T}	AF070431	0
6/2–4	Bara	KT933355	*Sporobolomyces coprosmae* CBS 7899^{T} *Sporobolomyces oryzicola* CBS 7228^{T}	AF189980 AF189990	1 1

^{T}, Type strain; ^{NT}, neotype strain; ^{X}, contains one ambiguous nucleotide: N (A, G, C or T); ^{Y}, isolate contains two ambiguous nucleotides: S (G or C) and Y (C or T); ^{Z}, isolate contains one ambiguous nucleotide: Y (C or T); ^{W}, contains one ambiguous nucleotide: Y (C or T); ^{Z}, isolate contains two ambiguous nucleotides: Y (C or T) and R (A or G).

TABLE 2 | Osmotolerance of representative isolates of ascomyceteous yeast species.

Species	Isolate	Growth on media supplemented with 1:1 fructose:glucose			
		2%	30%	40%	50%
Candida glabrata	14/1z-1	+++	+++	++	+
Candida oleophila	11/1–55	+++	+++	++	+
Hanseniaspora osmophila	8-3	+++	+++	++	+
Hanseniaspora uvarum	9/1-66	+++	+++	+	(+)
Hanseniaspora vineae	11/1z-4	+++	+++	+(+)	(+)
Kluyveromyces dobzhanskii	9z-6	+++	+++	++(+)	–
Lachancea thermotolerans	8/2z-3	+++	+++	++(+)	+
Metschnikowia sp.	11/1-3	+++	+++	++	+
Pichia fermentans	5z-6	+++	+++	–	–
Pichia kluyveri	11/2–104	+++	+++	+	–
Pichia membranifaciens	4/1-34	+++	+++	++(+)	–
Pichia scaptomyzae	12z-4	+++	+++	+	–
Saccharomyces cerevisiae	14z-1	+++	+++	++	–
Saccharomyces paradoxus	10z-2	+++	+++	++	–
Saccharomyces uvarum	4/2z-11	+++	+++	++	–
Torulaspora delbrueckei	1/1z-10	+++	+++	++	+
Wickerhamomyces anomalus	15z-7	+++	+++	++(+)	++
Zygoascus meyerae	8z-2	+++	+++	+	–
Zygosaccharomyces bailii	4–24	+++	+++	++	–
Zygotorulaspora florentina	7z-11	+++	+++	++	–

+, growth; (+), weak growth; –, no growth.

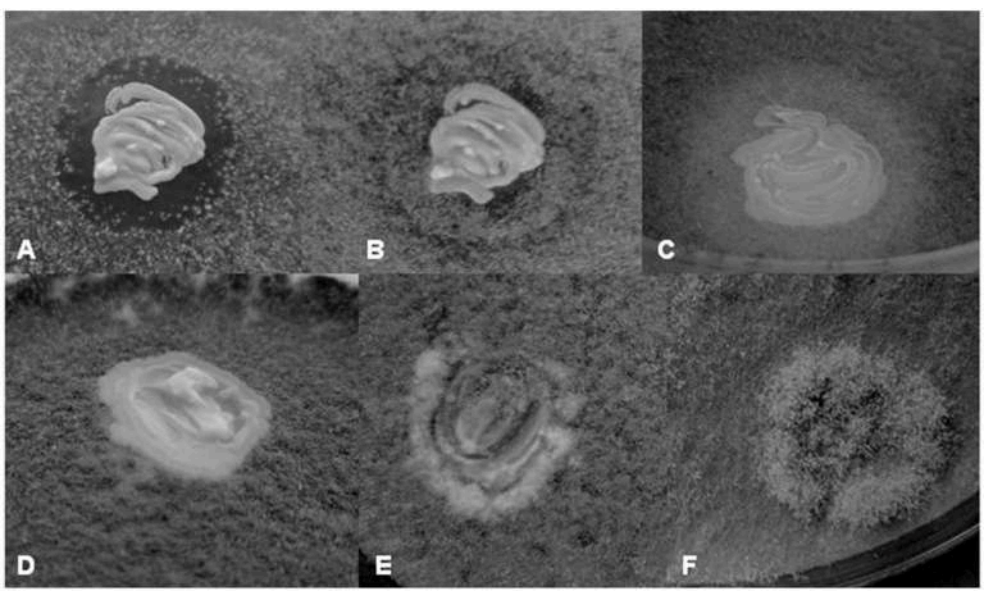

FIGURE 3 | Yeast-*Botrytis cinerea* interactions. (A) Inhibition of the growth of *B. cinerea* around the *Metschnikowia* sp. 11/1–3 colony on YEA after 5 days of incubation. **(B)** Growth of the *Botrytis* mycelium into the inhibition zone after 11 days of incubation. Note that the contact with the yeast colony halts the growth of the mycelium. **(C)** Reduced mycelial growth around the *P. kluyveri* 11/2–104 colony on YEA after 5 days of incubation. **(D)** Contact inhibition: the *Botrytis* mycelium stops growing at the contact with the *P. fermentans* 5z-6 colony. **(E)** Gradual invasion of the *H. osmophila* 5/1z-3 colony by the *Botrytis* mycelium on YEA after 5 days of incubation. **(F)** Growth of the *Botrytis* mycelium on the *L. thermotolerans* 2/2z-8 colony. Note that the mycelium is thicker on the yeast colony.

zone around the yeast colony (**Figure 3A**), (2) turbid inhibition zone around the yeast colony (**Figure 3C**), (3) contact inhibition at the edge of the yeast colony (**Figure 3D**), and (4) overgrowth of the yeast colony by the *Botrytis* mycelium (**Figures 3E,F**). The clear zone can be interpreted as total inhibition of the growth of the fungus, whereas the turbid zone can be attributed

TABLE 3 | Yeast antagonism.

Lawn		Inoculated on the lawn																		
Species	**Isolate**	**14/1z-1**	**11/1–55**	**8-3**	**9/1–66**	**11/1z-4**	**9z-6**	**8/2z-3**	**11/1–3**	**4/1–34**	**12z-4**	**5z-6**	**11/2–104**	**10z-2**	**14z-1**	**4/2z-11**	**1/1z-10**	**15z-7**	**8z-2**	**4–24**	**7z-11**
Candida glabrata	14/1z-1	–	–	–*	–*	–*	1	1	0.5	–	–	–	–	–	–	–	–	2	–	–	–
Candida oleophila	11/1–55	–	–	–	–	–*	0.5	–	–*	–	–	–	–	–	–	–	–	1*	–	–	–
Hanseniaspora osmophila	8–3	±	2^T	–	–	±	–	–*	–*	±	–*+	–*	–*	–*	–*	–*	±	–*+	–	–*	–*
Hanseniaspora uvarum	9/1–66	–	–	+*	–	–*	–	–*	–*	–*	–*	–*	–*	–*	–*	–*	–*	–*	–	–*	–*
Hanseniaspora vineae	11/1z-4	–	–	–*	–*	–	–	–*	–*	–*+	–*+	–*	–*	–*	–*	–*	1^{t*}	3^{t*}	–	–*	–*
Kluyveromyces dobzhanskii	9z-6	–	–	–	–	–	–	–	2^{t*}	2^T	1^t+	–	0.5	1	1^T	0.5	–	1*	–	0.5	1.5
Lachancea thermotolerans	8/2z-3	–	–	–*	–*	–*	1^T	–	–*	1^t+	±	–*	–*	–*	–	–	–	0.5*	–	–	–
Metschnikowia sp.	11/1–3	–	–	–	–	–	2^T	1^T	–	1^T	–	–	–	0.5	–	0.5	1^T	1^T	0.5^T	–	–
Pichia fermentans	5z-6	–	–	–*	–	–	0.5^T	–	–	±	±	–	–	–	–	–	–	–*	–	–	–
Pichia kluyveri	11/2–104	–	–	–	–	–	–	–	–*	1^T	–	–	–	–	–	–	–	–*	–	–	–
Pichia membranifaciens	4/1–34	–	–	±	±	±	2^T	3^T	–	–	–	–	–	–	–	–	–	2^{t*}	–	–	–
Pichia scaptomyzae	12z-4	–	–	–*+	–*+	–*+	1^T	–	–	–*	–	–	–	–	–	–	–	0.5^{t*}	–	–	–
Saccharomyces cerevisiae	14z-1	–	–	–*	–*	–*	0.5^T	0.5	–*	–	–	–*	–*	–	–	–	–	2.5^{t*}	–	–	–
Saccharomyces paradoxus	10z-2	–	–	–*	–*	–*	2	–	1*	–	–	–*	–	–	–	–	–	2*	–	–	–
Saccharomyces uvarum	4/2z-11	–	–	–	–*	–*	1	–	1.5*	–	±	–*	–	–	–	–	–	1*	–	–	–
Torulaspora delbrueckei	1/1z-10	–	–	–*	–*	–*	0.5	–	–*	–	±	–*	–	–	–	–	–	2*	–	–	–
Wickerhamomyces anomalus	15z-7	–	–	–	–	–	–	0.5	2^T	3^T	–	–	–	1	2	0.2	1	–	1^T	–	–
Zygoascus meyerae	8z-2	–	–	–	–	–	0.5^T	–	–*	–	–*+	–	–	–	–	–	–	1^{t*}	–	–	–
Zygosaccharomyces bailii	4–24	–	–	–	–	–	1	0.5^T	–*	–*	–	–*	–*	–	–	–	–	1^{t*}	–	–	–
Zygotorulaspora florentina	7z–11	–	–	–	–	–	2^T	–	–	–	–	–	–	–	–	–	–	–*	–	–	–

*Numerals, width of the inhibition zone in mm; –, no interaction is detected; +, crossfeeding (stronger lawn growth around the yeast colony); *, colony does not grow or grow poorly on the background lawn; ^{T}, the inhibition zone is turbid.*

TABLE 4 | Antagonistic effect of representative isolates of ascomyceteous yeast species on *B. cinerea*.

Yeast		*Botrytis cinerea*					
Species	**Isolate**	**Is inhibited around the yeast colony (inhibition zone in mm)**		**Grows onto the yeast colony**			
		5 day		**7 day**		**11 day**	
		YEA	**PDA**	**YEA**	**PDA**	**YEA**	**PDA**
Candida glabrata	14/1z-1	–	–	–	–	(+)	(+)
Candida oleophila	11/1–55	3^T	–	–	–	(+)	–
Hanseniaspora osmophila	4–3	3^T	–	+	+	+	+
	8–3	–	–	+	+	+	+
Hanseniaspora uvarum	9/1–66	5^T	–	+	+	+	+
Hanseniaspora vineae	1/1–5	–	–	–	–	(+)	(+)
	11/1z-4	3^T	–	+	–	+	+
	12/1z-2	3^T	–	+	–	+	–
Kluyveromyces dobzhanskii	5z-9	1^T	–	–	–	+	(+)
	9z-6	–	–	–	–	+	–
Lachancea thermotolerans	2/2z-8	–	–	+	+	+	+
	8/2z-3	4^T	–	+	+	+	+
	14/1z-2	2^T	–	+	+	+	+
Metschnikowia sp.	11/1–3	4	1	–	–	–	–
	15–8	3	–	–	–	–	–
Pichia fermentans	5z-6	4	4	–	–	(+)	–
Pichia kluyveri	11/2–104	5^T	5^T	–	–	(+)	–
Pichia membranifaciens	4/1–34	2^T	–	(+)	–	+	(+)
	15/1z-9	2^T	–	–	–	+	(+)
Pichia scaptomyzae	12z-4	–	–	(+)	–	+	(+)
Saccharomyces cerevisiae	3/1z-5	–	–	–	–	+	+
	14z-1	–	–	–	–	(+)	(+)
Saccharomyces paradoxus	3z-28	–	–	–	–	(+)	–
	7/2z-2	3^T	–	–	–	+	+
	10z-2	3^T	3^T	–	–	(+)	–
Saccharomyces uvarum	4/2z-11	2^T	2^T	–	–	+	+
Torulaspora delbrueckei	1/1z-10	–	–	–	–	(+)	–
Wickerhamomyces anomalus	15z-7	–	–	–	–	–	(+)
Zygoascus meyerae	8z-2	4^T	4^T	–	–	+	–
Zygosaccharomyces bailii	4–24	3	3	–	–	(+)	(+)
	8–29	2	2	–	–	(+)	–
Zygotorulaspora florentina	2z-30	–	–	–	–	–	–
	3/2–1	–	–	+	(+)	+	+
	7z-11	3	1	–	–	(+)	–
	9/2z-9	3	1	–	–	(+)	(+)

YEA and PDA are media (see Materials and Methods for description); numerals, width of inhibition zone in mm; +, growth; (+), weak growth; –, no growth; ^{T}, inhibition zone is turbid.

to weaker inhibition or to competition for nutrients. The former morphology was observed only around *Metschnikowia*, *P. fermentans*, *Zygosaccharomyces*, and *Zygotorulaspora* isolates. Both types of zones and the contact inhibition (11 isolates) were visible only for 4 to 5 days, then the mycelium gradually grew into the zones (**Figure 3B**) and on the yeast colonies, indicating, that either the inhibitory conditions were changing with time or the hyphae invading the zones and the colonies were fed by cytoplasmic transport from the mycelium growing outside of the inhibition zone (e.g., Sipiczki, 2006). Representative results are shown in **Table 4**. Surprisingly, conspecificity did not always correlate with the intensity of the antagonistic activity. For species showing intraspecific diversity (*H. osmophila, H. vineae, Kl. dobzhanskii, L. thermotolerance, Metschnikowia sp., P. membranifaciens, S. cerevisiae, S. paradoxus, Zs. bailii, Zt. florentina*) more than one strain is listed in the table. Notably,

more isolates produced zones on YEA than on PDA. The difference might be attributed to the much more vigorous growth of *Botrytis* on PDA.

DISCUSSION

Recovery of Viable Yeasts

When the grape is harvested, it is unavoidable, that berries fall to the ground and bunches remain on the vines. The former fall prey to rotting soil fungi and bacteria, which can also decompose the associated yeasts. The bunches on the vines are isolated from the destructive soil microorganisms and will be dehydrated (mummified) by frost and wind on the vines. The results of this study demonstrate, that fermentative yeasts relevant for wine making can tide over winter in these mummies.

This study was focused on the surviving yeasts, therefore the yeast communities of the overwintering grapes were examined by the conventional agar-plate method to obtain viable yeasts able to form colonies on a laboratory medium. The culture-independent strategies (e.g., metagenomics methods, DGGE) based on the analysis of DNA extracted directly from the yeast-containing substrates can identify yeast DNA but cannot distinguish between the DNA sequences of dead and live organisms (Mills et al., 2002; Prakitchaiwattana et al., 2004; Bokulich et al., 2014; Setati et al., 2015).

As certain yeasts of wine-making relevance are usually very sparse on grape berries if present at all (e.g., Mortimer and Polsinelli, 1999), an enrichment step was also included in the procedure. After plating out small aliquots on the agar plates, larger volumes of the samples were added to the enrichment medium restrictive for most non-*Saccharomyces* yeasts. By plating out aliquots before and after enrichment, large number of colonies were randomly isolated from 48 mummified grape bunches collected in 16 locations covering the entire Tokaj wine-growing region, (shared by Hungary and Slovakia; **Figure 1**) at the end of winter, shortly before pruning.

Taxonomy of Isolates

To broaden the spectrum of the phenotypic traits applicable to clustering of the isolates, the colonies were tested for the utilization of 14 compounds as carbon sources and lysine as a nitrogen source. The taxonomic affiliation of the phenotypic groups was then determined by sequencing the chromosomal segments coding for the D1/D2 domains of the LSU (large subunit) rRNA from randomly chosen isolates of the clusters.

Among the isolates, more ascomycetous species and fewer basidiomycetous species were identified but neither group was represented in all samples. All basidiomyceteous species are known phylloplane yeasts (for a review, see Fonseca and Inacio, 2006) belonging to Agaricomycotina or to Pucciniomycotina and have been detected on grape as well (e.g., Yanagida et al., 1992; Sabate et al., 2002; Raspor et al., 2006; Li et al., 2010; Cadez et al., 2010; Bourret et al., 2013; Lederer et al., 2013; Brysch-Herzberg and Seidel, 2015; Nemcová et al., 2015; Setati et al., 2015). The basidiomyceteous yeasts were not considered further in this study because of their marginal significance in winemaking.

The ascomycetevous yeasts most frequently detected in the mummified samples were *Metschnikowia* strains producing pigmented colonies and *Hanseniaspora* strains producing apiculate cells. The *Hanseniaspora* isolates were assigned to three species, *H. osmophila*, *H. uvarum*, and *H. vineae*, with *H. uvarum* being more frequent in samples directly spread on agar plates and *H. osmophila* being more frequent in the enriched cultures. The apiculate yeasts usually predominate the early phase of fermentation and produce compounds, that enrich the aroma profile of the wine (e.g., Zironi et al., 1993; Romano et al., 2003; Moreira et al., 2011). Here, *H. osmophila* was found in the enrichment cultures even if undetected in the non-enriched samples, indicating, that *H. osmophila* can better tolerate high alcohol concentrations than the other two species of the genus. The abundance of the *Hanseniaspora* cells in the samples demonstrates that these yeasts cope well with the harsh microclimatic conditions during overwintering.

Pulcherrimin-producing *Metschnikowia* strains are common on ripe grapes. They are usually assigned to *M. pulcherrima* (*C. pulcherrima*) or less frequently to *M. fructicola* in the oenological literature. However, the pigmented colonies isolated in this study differed from the type strains of all known pulcherrimin-producing species (*pulcherrima* clade) in their D1/D2 sequences and usually contained several ambiguous nucleotides. D1/D2 differences between grape-borne *Metschnikowia* strains and the type strains of the related species were already noticed in a previous study of Tokaj grape yeasts (Sipiczki, 2006). Recently, Brysch-Herzberg and Seidel (2015) encountered a similar problem with *Metschikowia* yeasts isolated from ripe wine grapes in Germany. These difficulties indicate, that the deficiency of the rDNA homogenization process recently discovered in *M. fructicola* and *M. andauensis* (Sipiczki et al., 2013) might characterize all pigmented *Metschnikowia* strains and obscure the species boundaries in the *pulcherrima* clade. As these species cannot be clearly separated by physiological tests either (Lachance, 2011), the taxonomic assignment of the *Metschnikowia* yeasts described in the oenological literature should be treated with prudence. Nevertheless, the isolates examined in this work undoubtedly belong to the *pulcherrima* clade which harbors the pigmented species of the genus (Lachance, 2011). Strains of the clade are usually present in the must during the early phase of fermentation. Their contribution to the quality of the wine is beyond doubt but not yet fully explored (e.g., Gil et al., 1996; Sadineni Naresh et al., 2012; Jolly et al., 2014; Contreras et al., 2015).

The other ascomyceteous yeast species were less abundant than *Metschnikowia* and *Hanseniaspora* in the sampled mummified bunches. Except for a few bunches, ascomyceteous non-*Metschnikowia* and non-*Hanseniaspora* yeasts were found only in the enrichment cultures. Interestingly, even species which are unable to utilize raffinose (*Ca. glabrata. Ca. oleophila, P. kluyvei, P. membranifaciens, T. delbruckei, Za. meyerae, Zs. bailii*) were enriched. In their case, the enriching factor could have been the high alcohol concentration of the medium lethal to *Metschnikowia*, most *Hanseniaspora* species, and the basidiomycetes.

The principal wine yeasts, S. *cerevisiae* and S. *uvarum*, are rarely isolated from grapes by conventional direct agar plating procedures, and there is an ongoing debate about their natural origin in wine fermentation (e.g., Fleet et al., 2002). *S. cerevisiae* was occasionally isolated from mature, overripe, and damaged grapes, but usually enrichment steps had to be applied, that elicit the recovery of minority species which would not be detected by direct plating (e.g., Mortimer and Polsinelli, 1999; Mercado et al., 2007; Sampaio and Goncalves, 2008; Peter et al., 2011). Consistent with these earlier observations, no *Saccharomyces* was found in this study among the colonies when the samples were plated directly on the agar medium. Upon enrichment, *S. paradoxus*, *S. cerevisiae,* and *S. uvarum* could be recovered from certain cultures. Remarkably, *S. paradoxus* was more frequent than the other *Saccharomyces* species. This finding is consistent with the reports on large distribution of *S. paradoxus* in certain grape-growing areas (Redzepovic et al., 2002) but inconsistent with the microbiological analyses which detected only *S. cerevisiae* and *S. uvarum* in fermenting Tokaj wines (Sipiczki et al., 2001; Naumov et al., 2002; Antunovics et al., 2003, 2005). Another interesting finding is the presence of *S. uvarum* in one of the overwintering populations because this yeast has not been reported yet from grape samples. These results unanimously prove, that *S. cerevisiae*, *S. paradoxus* and *S. uvarum* can participate in the colonization of grape berries and can also be transmitted in mummified grape berries over consecutive vegetation periods. Nevertheless, their rather sporadic occurrence indicates, that either they are not regular components of the colonizing yeast communities or they have poor winter tolerance, a property assumed to depend on the sporulation efficiency (Sipiczki, 2010). Further, experiments could reveal to what extent their survival in the mummified grape can contribute to the maintenance of the continuity of the *Saccharomyces* populations in vineyards. It is worth noting that the inability to utilize lysine as a nitrogen source was not an exclusive trait of *Saccharomyces* isolates in this study. Many isolates assigned to 6 other ascomyceteous and 2 basidiomyceteous species could not utilize lysine either. Thus, the widely used method of differentiation of *Saccharomyces* (lys^-) and non-*Saccharomyces* yeasts (lys^+) on the basis of lysine utilization (Angelo and Siebert, 1987) can lead to false results when not combined with other tests.

Surprisingly, no strains of *Ca. zemplinina* were found among the isolates although this osmotolerant and psychrotolerant species (Sipiczki, 2003) is quite regularly encountered on ripe grape (e.g., Li et al., 2010; Brežná et al., 2010; Cadez et al., 2010; Sun et al., 2014; Brysch-Herzberg and Seidel, 2015; Setati et al., 2015) and is the third major wine yeast in the Tokaj region (Csoma and Sipiczki, 2008). As the locations of sample collection covered the entire Tokaj region, the lack of *Ca. zemplinina* among the viable yeasts can be attributed to its inability to survive in the overwintering grapes rather than to its absence on the ripe grapes.

Certain grape samples yielded rapidly extending colonies of yeast-like cells fringed by wide hyphal halos. Their morphology suggested conspecificity with *A. pullulans*, a widespread phylloplane fungus (e.g., Grube et al., 2011) belonging to Pezizomycotina. Surprisingly, the D1/D2 sequences of the isolates indicated closer genetic affinity with *A. subglaciale*, *Ka. Microsticta,* and *Co. fagi* than with *A. pullulans*. *A. subglaciale* was described from subglacial ice (Zalar et al., 2008; Gostinčar et al., 2014) and has not yet been detected in the wine-related environment. As for the isolates showing 100% D1/D2 sequence identity with *Ka. microsticta* and *Co. fagi*, it is worth mentioning, that *Ka. microsticta* ITS sequences were recently amplified from grape must in South Africa (Setati et al., 2015). However, the similarity to database *Ka. microsticta* ITS sequences does not prove conspecificity with *Ka. microsticta* because no *Co. fagi* sequences are available in the databases. In addition, Setati et al. (2015) did not culture the strains from the samples. Thus, this is the first report on the isolation of *A. subglaciale* and *Ka. microsticta/Co. fagi* from grape. As these species are closely related and can easily be confused with *A. pullulans* (Zalar et al., 2008), earlier reports on the occurrence of *A. pullulans* on grapes should be taken with caution if not supported with adequate taxonomic analyses.

Osmotolerance of Isolates

Since, the yeasts residing in the overwintering grape berries have to cope with unfavorable factors, such as the antagonistic effects of other microbes and the osmotic pressure increasing during the dehydration of the berries, representatives of the ascomycetous yeast isolates were tested for response to these challenges. To investigate the ability of the isolates to cope with high osmotic pressure, representatives of the identified ascomyceteous species were tested for growth on agar plates supplemented with various concentrations of sugar. To mimic the real situation, glucose, and fructose were used in 1:1 proportion for supplementation. The *W. anomalus* isolates surpassed all other isolates in osmotolerance. This species has been described before as halophilic (Kagiyama et al., 1988) and a frequent spoilage yeast of fruit juice concentrates (e.g., Combina et al., 2008). As most isolates did not grow or poorly grew at 50% sugar, it can be assumed that the increasing osmotic pressure may also be involved in the preservation of the yeast community in the berries.

Antagonistic and Synergistic Interactions

The interaction tests revealed both antagonistic (growth inhibition) and synergistic (growth promotion) interactions among the isolates. In the test method applied in this study, the lawn of the antagonistic isolate hampered the growth of the colony of the sensitive isolate inoculated on it. In the reversed situation, the colony of the antagonistic isolate elicited an inhibition zone around its colony in the lawn of the sensitive isolate. Turbid zones indicated milder antagonisms (reduction of growth in the sensitive lawn) probably attributable to the depletion of the medium of certain nutrients by the colony of the "antagonist" (competition for nutrients). Clear zones were produced when the antagonist caused total growth inhibition in the lawn of the sensitive isolate. Several mechanisms might account for total inhibition. One possibility is, that the antagonistic isolate killed the cells of the sensitive isolate by secreting a toxic agent into the medium. Numerous yeasts species have been found to have strains harboring extracellular genetic

elements (killer factors) encoding secretable agents referred to as killer toxins (for a review, see Schmitt and Breinig, 2002). The clear zones in the lawn of certain non-*Saccharomyces* isolates around the *Saccharomyces* colonies might be caused by killer toxins. Testing the antagonistic isolates identified in this study for the presence of such killer factors in their cells will be the subject of a different study. Nevertheless, it is rather unlikely that the *Metschnikowia* isolates inhibited the growth of other yeasts by killer toxins. They all formed colonies fringed by maroon-red pigmented zones in the agar media. In a previous study (Sipiczki, 2006), the pigmented zones were found to coincide with the growth inhibition zones around the *Metschnikowia* colonies inoculated onto lawns of sensitive microorganisms. It turned out that the growth inhibition was due to the immobilization of the free iron in the medium by complexing the ferric ions with a compound secreted by the *Metschnikowia* cells (Sipiczki, 2006). The complex referred to as pulcherrimin is water-insoluble and has maroon-red color (Cook and Slater, 1956). The behavior of the *Wickerhamomyces* strain shown in **Table 2** and **Figure 2** demonstrates how complex the interactions within the yeast communities can be. It grew poorly on the lawn of most isolates, but generated clear inhibition zones in the lawn of many of them and its lawn was inhibited by the colonies of certain other isolates. This diversity of interactions is consistent with the diversity of modes by which *W. anomalus* (*P. anomala*) can antagonize other microorganisms. Its strains can produce inhibitory amounts of ethyl acetate (Fredlund et al., 2004), secrete killer toxins (Kagiyama et al., 1988), and cel-wall lytic enzymes (Jijakli and Lepoivre, 1998). Synergistic, growth-facilitating effects of certain isolates on other isolates were observed on all media used in this study and could be attributed to crossfeeding with nutrients. On media containing mellibiose [D-galactose-α(1→6)-D-glucose] as carbon sources, the melibiose-utilizing isolates (*Zs. florentina*) promoted the growth of the melibiose-minus (*P. kluyveri*) isolates. Most probably, the former hydrolyzed the disaccharide in excessive amount and released some of the monosaccharides utilizable by the latter into the medium. On the grapes similar synergistic interactions can take place but with different nutrients.

In the Tokaj region, grape is harvested late in the autumn after a long period of ripening during which high proportions of berries undergo noble rotting generated by the *B. cinerea* infection. The invasion of the berries by the hyphae of the fungus causes ruptures in the skin which are then colonized by yeasts and bacteria. It was found, that at least one type of the colonizing yeasts, the pulcherrimin-producing *Metschnikowia* strains can antagonize the growth of *Botrytis* by inhibiting the germination of its conidia and the extension of its hyphae (Sipiczki, 2006). Consistent with this observation, all *Metschnikowia* isolates investigated in this study showed anti-*Botrytis* antagonism manifested in clear inhibition zones in the mycelium around their colonies. As the inhibition zones and the pigmented halos coincided, it is likely that the growth inhibition by *Metschnikowia* was due to iron immobilization by a secreted compound as described above. The clear zones around *P. fermentans*, *Zs. Bailii,* and *Za. florentina* colonies are most probably due to different mechanisms because these yeasts do not produce pulcherrimin. The mild inhibition of the fungal growth by *S. paradoxus* and *S. uvarum* isolates is an unexpected result. As the zones around their colonies were turbid, the inhibition can be ascribed to competition for nutrients rather than to the secretion of agents having antifungal activities. Contact inhibition observed at the colonies of 11 isolates is a phenomenon which has been noticed in certain yeasts before this study but the mechanisms by which these yeasts exert their influence on the hyphae has not yet been understood (for a review, see Liu et al., 2013).

Potential Contribution of the Mummified Grapes to the Maintenance of the Vineyard Yeast Microflora

Taken together, the findings of this study demonstrate, that the grapes mummified on the vine can serve as a safe reservoir of fermentative yeasts, including *Saccharomyces,* and can transmit these yeasts between consecutive years in the vineyard. It can be reasonably assumed, that these yeasts may contribute to the maintenance of a complex vineyard yeast flora of wine-making relevance over years, together with those dispersed by insects (e.g., wasps, bees, *Drosophila*), and birds (Stevic, 1962; Francesca et al., 2012; Stefanini et al., 2012; Lam and Howell, 2015) visiting the ripening berries. Further, studies are needed to reveal the relative significance of these sources in the maintenance of the autochtonous vineyard yeast communities because soil is a rather unfavorable environment for yeast overwintering (Parle and Di Menna, 1966), the ROS-based antimicrobial defense system kills the ingested yeasts very fast in *Drosophila* (Hoang et al., 2015) the persistence of yeasts in bird cloacae has been shown to be very short (Francesca et al., 2012), the social wasp *Vesta crabro* ("European hornet" originally native only to Europe) recently found to harbor fermentative yeasts in guts (Francesca et al., 2012) is not common (or even absent) in large areas of the globe, where wine is produced. Moreover, certain yeast species were detected in the *V. crabro* guts after grape maturation, suggesting, that the wasps gathered those yeasts from the grapes rather than delivered them there. Mummified grapes can also be rare when modern harvesting technology is used.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2016.00212

REFERENCES

Alvarez-Perez, S., and Herrera, C. M. (2013). Composition, richness and nonrandom assembly of culturable bacterial-microfungal communities in floral nectar of Mediterranean plants. *FEMS Microbiol. Ecol.* 83, 685–699. doi: 10.1111/1574-6941.12027

Angelo, J., and Siebert, K. J. (1987). A new medium for the detection of wild strains in brewing culture yeast. *J. Am. Soc. Brew. Chem.* 45, 135–140.

Antunovics, Z., Csoma, H., and Sipiczki, M. (2003). Molecular and genetic analysis of the yeast flora of botrytized Tokaj wines. *Bull. l'O.I.V.* 76, 380–397.

Antunovics, Z., Irinyi, L., and Sipiczki, M. (2005).Combined application of methods to taxonomic identification of *Saccharomyces* strains in fermenting botrytized grape must. *J. Appl. Microbiol.* 98, 971–979. doi: 10.1111/j.1365-2672.2005.02543.x

Barata, A., Malfeito-Ferreira, M., and Loureiro, V. (2012).The microbial ecology of wine grape berries. *Int. J. Food Microbiol.* 153, 243–259. doi: 10.1016/j.ijfoodmicro.2011.11.025

Becher, P. G., Flick, G., Rozpędowska, E., Schmidt, A., Hagman, A., Lebreton, S., et al. (2012). Yeast, not fruit volatiles mediate *Drosophila melanogaster* attraction, oviposition and development. *Funct. Ecol.* 26, 822–828. doi: 10.1111/j.1365-2435.2012.02006.x

Bisson, L. F., and Joseph, C. M. L. (2009). "Yeasts," in *Biology of Microorganisms on Grapes, in Must and in Wine*, eds H. König, G. Unden, and J. *Fröhlich (Berlin; Heidelberg: Springer-Verlag)*, 45–60.

Bokulich, N. A., Thorngate, J. H., Richardson, P. M., and Mills, D. A. (2014). Microbial biogeography of wine grapes is conditioned by cultivar, vintage, and climate. *Proc. Natl. Acad. Sci. U.S.A.* 111, E139–E148. doi: 10.1073/pnas.1317377110

Bourret, T. B., Grove, G. G., George, J., Vandemark, G. J., Henick-Kling, T., and Glawe, D. A. (2013). Diversity and molecular determination of wild yeasts in a central Washington State vineyard. *N. Am. Fungi* 8, 1–32. doi: 10.2509/naf2013.008.015

Brežná, B., Zenišová, K., Chovanová, K., Chebeňová, V., Kraková, L., Kuchta, T., et al. (2010). Evaluation of fungal and yeast diversity in Slovakian wine-related microbial communities. *Antonie Van Leeuwenhoek* 98, 519–529. doi: 10.1007/s10482-010-9469-6

Brysch-Herzberg, M., and Seidel, M. (2015). Yeast diversity on grapes in two German wine growing regions. *Int. J. Food Microbiol.* 214, 137–144. doi: 10.1016/j.ijfoodmicro.2015.07.034

Cadez, N., Zupan, J., and Raspor, P. (2010). The effect of fungicides on yeast communities associated with grape berries. *FEMS Yeast Res.* 10, 619–630. doi: 10.1111/j.1567-1364.2010.00635.x

Chavan, P., Mane, S., Kulkarni, G., Shaikh, S., Ghormade, V., Nerkar, D. P., et al. (2009). Natural yeast flora of different varieties of grapes used for winemaking in India. *Food Microbiol.* 26, 801–808. doi: 10.1016/j.fm.2009.05.005

Ciani, M., Mannazzu, I., Marinangeli, P., Clementi, F., and Martini, A. (2004). Contribution of winery-resident *Saccharomyces cerevisiae* strains to spontaneous grape must fermentation. *Antonie Van Leeuwenhoek* 85, 159–164. doi: 10.1023/B:ANTO.0000020284.05802.d7

Combina, M., Daguerre, C., Massera, A., Mercado, L., Sturm, M. E., Ganga, A., et al. (2008). Yeast identification in grape juice concentrates from Argentina. *Lett. Appl. Microbiol.* 46, 192–197. doi: 10.1111/j.1472-765X.2007.02291.x

Combina, M., Mercado, L., Borgo, P., Elia, A., Jofré, V., Ganga, A., et al. (2005). Yeasts associated to Malbec grape berries from Mendoza, Argentina. *Int. J. Food Microbiol.* 98, 1055–1061. doi: 10.1111/j.1365-2672.2005.02540.x

Contreras, A., Curtin, C., and Varela, C. (2015). Yeast population dynamics reveal a potential 'collaboration' between *Metschnikowia pulcherrima* and *Saccharomyces uvarum* for the production of reduced alcohol wines during Shiraz fermentation. *Appl. Microbiol. Biotechnol.* 99, 1885–1895. doi: 10.1007/s00253-014-6193-6

Cook, A. H., and Slater, C. A. (1956). The structure of pulcherrimin. *J. Chem. Soc.* 1956, 4133–4135. doi: 10.1039/jr9560004133

Cordero-Bueso, G., Arroyo, T., Serrano, A., Tello, J., Aporta, I., Vélez, M. D., et al. (2011). Influence of the farming system and vine variety on yeast communities associated with grape berries. *Int. J. Food Microbiol.* 145, 132–139. doi: 10.1016/j.ijfoodmicro.2010.11.040

Csoma, H., and Sipiczki, M. (2008). Taxonomic reclassification of *Candida stellata* strains reveals frequent occurrence of *Candida zemplinina* in wine fermentation. *FEMS Yeast Res.* 8, 328–336. doi: 10.1111/j.1567-1364.2007.00339.x

Di Maio, S., Polizzotto, G., Di Gangi, E., Foresta, G., Genna, G., Verzera, A., et al. (2012). Biodiversity of indigenous *Saccharomyces* populations from old wineries of South-Eastern Sicily (Italy): preservation and economic potential. *PLoS ONE* 7:e30428. doi: 10.1371/journal.pone.0030428

Dobzhansky, T., Cooper, D. M., Phaff, H. J., Knapp, E. P., and Carson, H. L. (1956). Differential attraction of species of *Drosophila* to different species of yeasts. *Ecology* 37, 544–550. doi: 10.2307/1930178

Egli, C. M., Edinger, W. D., Mitrakul, C. M., and Henick-Kling, T. (1998). Dynamics of indigenous and inoculated yeast populations and their effect on the sensory character of Riesling and Chardonnay wines. *J. Appl. Microbiol.* 85, 779–789. doi: 10.1046/j.1365-2672.1998.00521.x

Fell, J. W., Boekhout, T., Fonseca, A., Scorzetti, G., and Statzell-Tallman, A. (2000). Biodiversity and systematics of basidiomycetous yeasts as determined by large subunit rDNA D1/D2/domain sequence analysis. *Int. J. Syst. Bacteriol.* 50, 1351–1371. doi: 10.1099/00207713-50-3-1351

Fleet, G. H., Prakitchaiwattana, C., Beh, A. L., and Heard, G. (2002). "The yeast ecology of wine grapes," in *Biodiversity and Biotechnology of Wine Yeast*, ed *M. Ciani (Kerala: Research Signpost)*, 1–17.

Fonseca, A., Boekhout, T., and Fell, J. W. (2011). "*Cryptococcus* Vuillemin (1901)," in *The Yeasts - A Taxonomic Study*, eds *C. P. Kurtzman, J. W. Fell, and T. Boekhout (London: Elsevier)*, 1661–1737.

Fonseca, A., and Inacio, J. (2006). "Phylloplane yeasts," in *Biodiversity and Ecophysiology of Yeasts*, eds *C. Rosa, and G. Peter (Berlin; Heidelberg: Springer-Verlag)*, 263–301.

Francesca, N., Canale, D. E., Settanni, L., and Moschetti, G. (2012). Dissemination of wine-related yeasts by migratory birds. *Envir. Microbiol. Rep.* 4, 105–112. doi: 10.1111/j.1758-2229.2011.00310.x

Fredlund, E., Druvefors, U. A., Olstorpe, M. N., Passoth, V., and Schnürer, J. (2004). Influence of ethyl acetate production and ploidy level on the anti-mould activity of *Pichia anomala*. *FEMS Microbiol. Lett.* 238, 133–137. doi: 10.1016/j.femsle.2004.07.027

Gadanho, M., Libkind, D., and Sampaio, J. P. (2006). Yeast diversity in the extreme acidic environments of the Iberian Pyrite Belt. *Microb. Ecol.* 52, 552–563. doi: 10.1007/s00248-006-9027-y

Gil, J. V., Mateo, J. J., Jimenez, M., Pastor, A., and Huerta, T. (1996). Aroma compounds in wine as influenced by apiculate yeasts. *J. Food Sci.* 61, 1247–1249. doi: 10.1111/j.1365-2621.1996.tb10971.x

Gilbert, D. G. (1980). Dispersal of yeasts and bacteria by *Drosophila* in temperate rain forest. *Oecologia* 46, 135–137. doi: 10.1007/BF00346979

Goddard, M. R., Anfang, N., Tang, R., Gardner, R. C., and Jun, C. (2010). A distinct population of *Saccharomyces cerevisiae* in New Zealand: evidence for local dispersal by insects and human-aided global dispersal in oak barrels. *Environ. Microbiol.* 12, 63–73. doi: 10.1111/j.1462-2920.2009.02035.x

Gostinčar, C., Ohm, R. O., Kogej, T., Sonjak, S., Turk, M., Zajc, J., et al. (2014). Genome sequencing of four *Aureobasidium pullulans* varieties: biotechnological potential, stress tolerance, and description of new species. *BMC Genomics* 15:549. doi: 10.1186/1471-2164-15-549

Grube, M., Schmid, F., and Berg, G. (2011). Black fungi and associated bacterial communities in the phyllosphere of grapevine. *Fungal. Biol.* 115, 978–986. doi: 10.1016/j.funbio.2011.04.004

Gutierrez, A. R., Santamaria, P., Epifanio, S., Garijo, P., and Lopez, R. (1999). Ecology of spontaneous fermentation in one winery during 5 consecutive years. *Letts. Appl. Microbiol.* 29, 411–415. doi: 10.1046/j.1472-765X.1999.00657.x

Hamamoto, M., Boekhout, T., and Nakase, T. (2011). "*Sporobolomyces* Kluyver and van Niel (1924)," in *The Yeasts - A Taxonomic Study*, eds *C. P. Kurtzman, J. W. Fell, and T. Boekhout (London: Elsevier)*, 1929–1990.

Hoang, D., Kopp, A., and Chandler, J. A. (2015). Interactions between *Drosophila* and its natural yeast symbionts - Is *Saccharomyces cerevisiae* a good model for studying the fly-yeast relationship? *PeerJ* 3:e1116. doi: 10.7717/peerj.1116

Jijakli, M. H., and Lepoivre, P. (1998). Characterization of an exo-b-1,3- glucanase produced by *Pichia anomala* strain K, antagonist of *Botrytis cinerea* on apples. *Phytopathology* 88, 335–343. doi: 10.1094/PHYTO.1998.88.4.335

Jolly, N. P., Varela, C., and Pretorius, I. S. (2014). Not your ordinary yeast: non-*Saccharomyces* yeasts in wine production uncovered. *FEMS Yeast Res.* 14, 215–237. doi: 10.1111/1567-1364.12111

Kagiyama, S., Aiba, T., Kadowaki, K., and Mogi, K. (1988). New killer toxins of halophilic *Hansenula anomala*. *Agric. Biol. Chem.* 52, 1–7. doi: 10.1271/bbb1961.52.1

Kwon-Chung, K. J. (2011). "*Filobasidium* Olive (1968)," in *The Yeasts - A Taxonomic Study*, eds *C. P. Kurtzman, J. W. Fell, and T. Boekhout (London: Elsevier)*, 1457–1465.

Lachance, M. A. (2011). "Metschnikowia Kamienski (1899)," *in The Yeasts - A Taxonomic Study, eds C. P. Kurtzman, J. W. Fell, and T. Boekhout (London: Elsevier)*, 575–619.

Lachance, M. A., Gilbert, D. G., and Starmer, W. T. (1995). Yeast communities associated with *Drosophila* species and related flies in an eastern oak-pine forest: a comparison with western communities. *J. Ind. Microbiol.* 14, 484–494. doi: 10.1007/BF01573963

Lam, S. S. T. H., and Howell, K. S. (2015). *Drosophila*-associated yeast species in vineyard ecosystems. *FEMS Microbiol. Lett.* 362:fnv170. doi: 10.1093/femsle/fnv170

Lederer, M. A., Nielsen, D. S., Toldam-Andersen, T. B., Herrmann, J. V., and Arneborg, N. (2013). Yeast species associated with different wine grape varieties in Denmark. *Acta Agric. Scand. B Soil Plant Sci.* 63, 89–96. doi: 10.1080/09064710.2012.723738

Li, S. S., Cheng, C., Li, Z., Chen, J. Y., Yan, B., Han, B. Z., et al. (2010). Yeast species associated with wine grapes in China. *Int. J. Food Microbiol.* 138, 85–90. doi: 10.1016/j.ijfoodmicro.2010.01.009

Liu, J., Sui, Y., Wisniewski, M., Droby, S., and Liu, Y. (2013). Review: utilization of antagonistic yeasts to manage postharvest fungal diseases of fruit. *Int. J. Food Microbiol.* 167, 153–160. doi: 10.1016/j.ijfoodmicro.2013.09.004

Loureiro, V., and Malfeito-Ferreira, M. (2003). Spoilage yeasts in the wine industry. *Int. J. Fodd Microbiol.* 86, 23–50. doi: 10.1016/S0168-1605(03)00246-0

Magyar, I., and Bene, Z. (2004). Characterization of yeast and mould biota of botrytized grapes in Tokaj wine region in the years 2000 and 2001. *Acta Alimentaria* 3, 259–267. doi: 10.1556/AAlim.33.2004.3.6

Martini, A. (1993). The origin and domestication of the wine yeast *Saccharomyes cerevisiae*. *J. Wine Res.* 4, 165–176. doi: 10.1080/09571269308717966

Mercado, L., Dalcero, A., Masuelli, R., and Combina, M. (2007). Diversity of *Saccharomyces* strains on grapes and winery surfaces: analysis of their contribution to fermentative flora of Malbec wine from Mendoza (Argentina) during two consecutive years. *Food Microbiol.* 24, 403–412. doi: 10.1016/j.fm.2006.06.005

Mills, D. A., Eric, J., Eric, A., and Cocolin, L. (2002). Yeast diversity and persistence in *Botrytis*-affected wine fermentation. *Appl. Environ. Microbiol.* 68, 4884–4893. doi: 10.1128/AEM.68.10.4884-4893.2002

Morais, P. B., Lachance, M.-A., and Rosa, C. A. (2005). Saturnispora hagleri sp. *nov., a yeast species isolated from Drosophila flies in Atlantic rainforest in Brazil. Int. J. System. Evol. Microbiol.* 55, 1725–1727. doi: 10.1099/ijs.0.63673-0

Moreira, N., Pina, C., Mendes, F., Couto, J. A., Hogg, T., and Vasconcelos, I. (2011). Volatile compounds contribution of *Hanseniaspora guilliermondii* and *Hanseniaspora uvarum* during red wine vinifications. *Food Control* 22, 662–667. doi: 10.1016/j.foodcont.2010.07.025

Mortimer, R., and Polsinelli, M. (1999). On the origins of wine yeast. *Res. Microbiol.* 150, 199–204. doi: 10.1016/S0923-2508(99)80036-9

Naumov, G. I., Naumova, E. S., Antunovics, Z., and Sipiczki, M. (2002). *Saccharomyces bayanus* var. *uvarum in Tokaj wine-making of Slovakia and Hungary. Appl. Microbiol. Biotechnol.* 59, 727–730. doi: 10.1007/s00253-002-1077-6

Nemcová, K., Breierová, E., Vadkertiová, R., and Molnárová, J. (2015). The diversity of yeasts associated with grapes and musts of the Strekov winegrowing region, Slovakia. *Folia Microbiol.* 60, 103–109. doi: 10.1007/s12223-014-0347-x

Palanca, L., Gaskett, A. C., Günther, C. S., Newcomb, R. D., and Goddard, M. R. (2013). Quantifying variation in the ability of yeasts to attract *Drosophila melanogaster*. *PLoS ONE* 8:e75332. doi: 10.1371/journal.pone.0075332

Parle, J. N., and Di Menna, M. E. (1966). The source of yeasts in New Zealand wines. *N.Z. J. Agric. Res.* 9, 98–107. doi: 10.1080/00288233.1966.10418122

Peter, G., Dlauchy, D., Szucs, E., and Tornai-Lehoczki, J. (2011). Enrichment in methanol containing broth – a simple method for the isolation of *Saccharomyces* from grapes. *Acta Alimentaria* 40, 376–384. doi: 10.1556/AAlim.40.2011.3.8

Phaff, H. J., and Knapp, E. P. (1956). The taxonomy of yeasts found in exudates of certain trees and other natural breeding sites of some species of *Drosophila*. *Antonie Van Leeuwenhoek* 22, 117–130. doi: 10.1007/BF02538319

Prakitchaiwattana, C. J., Fleet, G. H., and Heard, G. M. (2004). Application and evaluation of denaturing gradient gel electrophoresis to analyse the yeast ecology of wine grapes. *FEMS Yeast Res.* 4, 865–877. doi: 10.1016/j.femsyr.2004.05.004

Raspor, P., Milek, D. M., Polanc, J., Mozina, S. S., and Cadez, N. (2006). Yeasts isolated from three varieties of grapes cultivated in different locations of the Dolenjska vine-growing region, Slovenia. *Int. J. Food Microbiol.* 109, 97–102. doi: 10.1016/j.ijfoodmicro.2006.01.017

Redzepovic, S., Orlic, S., Sikora, S., Majdak, A., and Pretorius, I. S. (2002). Identification and characterization of *Saccharomyces cerevisiae* and *Saccharomyces paradoxus* strains isolated from Croatian vineyards. *Lett. Appl. Microbiol.* 35, 305–310. doi: 10.1046/j.1472-765X.2002.01181.x

Renouf, V., Claisse, O., and Lonvaud-Funel, A. (2007). Inventory and monitoring of wine microbial consortia. *Appl. Microbiol. Biotechnol.* 75, 149–164. doi: 10.1007/s00253-006-0798-3

Romano, P., Fiore, C., Paraggio, M., Caruso, M., and Capece, A. (2003). Function of yeast species and strains in wine flavour. *Int. J. Food Microbiol.* 86, 169–180. doi: 10.1016/S0168-1605(03)00290-3

Rosini, G. (1984). Assessment of dominance of added yeast with fermentation and origin of *Saccharomyces cerevisiae* in wine making. *J. Gen. Appl. Microbiol.* 30, 249–256. doi: 10.2323/jgam.30.249

Sabate, J., Cano, J., Esteve-Zarzoso, B., and Guillamón, J. M. (2002). Isolation and identification of yeasts associated with vineyard and winery by RFLP analysis of ribosomal genes and mitochondrial DNA. *Microbiol. Res.* 157, 267–274. doi: 10.1078/0944-5013-00163

Sadineni Naresh, V., Kondapalli, N., and Obulam, V. (2012). Effect of co-fermentation with *Saccharomyces cerevisiae* and *Torulaspora delbrueckii* or *Metschnikowia pulcherrima* on the aroma and sensory properties of mango wine. *Ann. Microbiol.* 62, 1353–1360. doi: 10.1007/s13213-011-0383-6

Sampaio, J. P. (2011a). "*Curvibasidium* Sampaio and Golubev (2004)," in *The Yeasts - A Taxonomic Study*, eds C. P. Kurtzman, J. W. Fell, and T. *Boekhout (London: Elsevier)*, 1413–1418.

Sampaio, J. P. (2011b). "*Rhodotorula* Harrison (1928)," in *The Yeasts - A Taxonomic Study*, eds C. P. Kurtzman, J. W. Fell, and T. *Boekhout (London: Elsevier)*, 1873–1927.

Sampaio, J. P., and Goncalves, P. (2008). Natural populations of *Saccharomyces kudriavzevii* in Portugal are associated with oak bark and are sympatric with *S. cerevisiae and S. paradoxus. Appl. Environ. Microbiol.* 74, 2144–2152. doi: 10.1128/AEM.02396-07

Santamarıa, P., Garijo, P., Lopez, R., Tenorio, C., and Rosa Gutierrez, A. (2005). Analysis of yeast population during spontaneous alcoholic fermentation: effect of the age of the cellar and the practice of inoculation. *Int. J. Food Microbiol.* 103, 49–56. doi: 10.1016/j.ijfoodmicro.2004.11.024

Schmitt, M. J., and Breinig, F. (2002). The viral killer system in yeast: from molecular biology to application. *FEMS Microbiol. Rev.* 748, 1–20. doi: 10.1111/j.1574-6976.2002.tb00614.x

Schuller, D., Alves, H., Dequin, S., and Casal, M. (2005). Ecological survey of *Saccharomyces cerevisiae* strains from vineyards in the Vinho Verde region of Portugal. *FEMS Microbiol. Ecol.* 51, 167–177. doi: 10.1016/j.femsec.2004.08.003

Scorzetti, G., Fell, J. W., Fonseca, A., and Statzell-Tallman, A. (2002). Systematics of basidiomycetous yeasts: a comparison of large subunit D1/D2 and internal transcribed spacer rDNA regions. *FEMS Yeast Res.* 2, 495–517. doi: 10.1111/j.1567-1364.2002.tb00117.x

Setati, M. E., Jacobson, D., Andong, U.-C., and Bauer, F. (2012). The vineyard yeast microbiome, a mixed model microbial map. *PLoS ONE* 7:e52609. doi: 10.1371/journal.pone.0052609

Setati, M. E., Jacobson, D., and Bauer, F.F. (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

Sipiczki, M. (2003). Candida zemplinina sp. *nov., an osmotolerant and psychrotolerant yeast that ferments sweet botrytised wines. Int. J. Syst. Evol. Microbiol.* 53, 2079–2083. doi: 10.1099/ijs.0.02649-0

Sipiczki, M. (2006). *Metschnikowia* strains isolated from botrytised grapes antagonize fungal and bacterial growth by iron depletion. *Appl. Environ. Microbiol.* 72, 6716–6724. doi: 10.1128/AEM.01275-06

Sipiczki, M. (2010). Diversity, variability and fast adaptive evolution of the wine yeast (*Saccharomyces cerevisiae*) genome. *Ann. Microbiol.* 61, 85–93. doi: 10.1007/s13213-010-0086-4

Sipiczki, M., Pfliegler, W. P., and Holb, I. J. (2013). *Metschnikowia* species share a pool of diverse rRNA genes differing in regions that determine hairpin-loop structures and evolve by reticulation. *PLoS ONE* 8:e67384. doi: 10.1371/journal.pone.0067384

Sipiczki, M., Romano, P., Lipani, G., Miklos, I., and Antunovics, Z. (2001). Analysis of yeasts derived from natural fermentation in a Tokaj winery. *Antonie Van Leeuwenhoek* 79, 97–105. doi: 10.1023/A:1010249408975

Starmer, W. T., Heed, W. B., Miranda, M., Miller, M. W., and Phaff, H. J. (1976). The ecology of yeast flora associated with cactiphilic *Drosophila* and their host plants in the Sonoran desert. *Microb. Ecol.* 3, 11–30. doi: 10.1007/BF02011450

Stefanini, I., Dapporto, L., Legras, J.-L., Antonio Calabretta, A., Di Paola, M., De Filippo, C., et al. (2012). Role of social wasps in *Saccharomyces cerevisiae* ecology and evolution. *Proc. Natl. Acad. Sci. U.S.A.* 109, 13398–13403. doi: 10.1073/pnas.1208362109

Stevic, B. (1962). The importance of bees (*Apis* sp) and wasps (*Vespa* sp) as carrier of yeasts for the microflora of grapes and the quality of wines. *Archiv Poljoprivredre Nauke Beograd* 15, 80–91.

Sun, Y., Guo, J., Liu, F., and Liu, Y. (2014). Identification of indigenous yeast flora isolated from the five winegrape varieties harvested in Xiangning, China. *Antonie Van Leeuwenhoek* 105, 533–540. doi: 10.1007/s10482-013-0105-0

Taylor, M. W., Tsai, P., Anfang, N., Ross, H. A., and Goddard, M. R. (2014). Pyrosequencing reveals regional differences in fruit-associated fungal communities. *Environ. Microbiol.* 16, 2848–2858. doi: 10.1111/1462-2920.12456

Treseder, K. K., and Lennon, J. T. (2015). Fungal traits that drive ecosystem dynamics on land. *Microbiol. Mol. Biol. Rev.* 79, 243–262. doi: 10.1128/MMBR.00001-15

Valero, E., Cambon, B., Schuller, D., Casal, M., and Dequin, S. (2007). Biodiversity of *Saccharomyces* yeast strains from grape berries of wine producing areas using starter commercial yeasts. *FEMS Yeast Res.* 7, 317–329. doi: 10.1111/j.1567-1364.2006.00161.x

Vaughan-Martini, A., and Martini, A. (1995). Facts, myths and legends on the prime industrial microorganisms. *J. Indus. Microbiol.* 14, 514–522. doi: 10.1007/BF01573967

Yanagida, F., Ichinose, F., Shinohara, T., and Goto, S. (1992). Distribution of wild yeasts in the white grape varieties at Central Japan. *J. Gen. Appl. Microbiol.* 38, 505–509. doi: 10.2323/jgam.38.501

Zahavi, T., Droby, S., Cohen, L., Weiss, B., and Ben-Arie, R. (2002). Characterisation of the yeast flora on the surface of grape berries in Israel. *Vitis* 41, 203–308.

Zalar, P., Gostinčar, C., de Hoog, G.S., Uršiè, V., Sudhadham, M., and Gunde-Cimerman, N. (2008). Redefinition of *Aureobasidium pullulans* and its varieties. *Stud. Mycol.* 61, 21–38. doi: 10.3114/sim.2008.61.02

Zironi, R., Romano, P., Suzzi, G., Battistutta, F., and Comi, G. (1993). Volatile metabolites produced in wine by mixed and sequential cultures of *Hanseniaspora guilliermondii* or *Kloeckera apiculata* and *Saccharomyces cerevisiae*. *Biotechnol. Lett.* 15, 235–238. doi: 10.1007/BF00128311

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Indigenous Georgian Wine-Associated Yeasts and Grape Cultivars to Edit the Wine Quality in a Precision Oenology Perspective

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In Georgia, one of the most ancient vine-growing environment, the homemade production of wine is still very popular in every rural family and spontaneous fermentation of must, without addition of chemical preservatives, is the norm. The present work investigated the yeast biodiversity in five Georgian areas (Guria, Imereti, Kakheti, Kartli, Ratcha-Lechkhumi) sampling grapes and wines from 22 different native cultivars, in 26 vineyards and 19 family cellars. One hundred and eighty-two isolates were ascribed to 15 different species by PCR-ITS and RFLP, and partial sequencing of D1/D2 domain 26S rDNA gene. *Metschnikowia pulcherrima* ($F' = 0.56$, $I' = 0.32$), *Hanseniaspora guilliermondii* ($F' = 0.49$, $I' = 0.27$), and *Cryptococcus flavescens* ($F' = 0.31$, $I' = 0.11$) were the dominant yeasts found on grapes, whereas *Saccharomyces cerevisiae* showed the highest prevalence into wine samples. Seventy four isolates with fermentative potential were screened for oenological traits such as ethanol production, resistance to SO_2, and acetic acid, glycerol and H_2S production. Three yeast strains (*Kluyveromyces marxianus* UMY207, *S. cerevisiae* UMY255, *Torulaspora delbrueckii* UMY196) were selected and separately inoculated in vinifications experiments at a Georgian cellar. Musts were prepared from healthy grapes of local varieties, Goruli Mtsvane (white berry cultivar) and Saperavi (black berry cultivar). Physical (°Brix) and microbial analyses (plate counts) were performed to monitor the fermentative process. The isolation of indigenous *S. cerevisiae* yeasts beyond the inoculated strains indicated that a co-presence occurred during the vinification tests. Results from quantitative GC-FID analysis of volatile compounds revealed that the highest amount of fermentation flavors, such as 4-ethoxy-4-oxobutanoic acid (monoethyl succinate), 2-methylpropan-1-ol, ethyl 2-hydroxypropanoate, and 2-phenylethanol, were significantly more produced in fermentation conducted in Saperavi variety inoculated with *K. marxianus*, whereas other aromatic compounds like 3-methylbutyl acetate, ethyl hexanoate and dihydrofuran-2(3H)-one (γ- butyrolactone)

showed a higher content in Goruli Mtsvane variety samples fermented by *S. cerevisiae*. The selected yeast strains have proved to be promising for enhancing the flavor potential in low aromatic Georgian cultivars. This work intends to be a knowledge contribution for a precision oenology toward the strategic concept of "one grape variety-one yeast".

Keywords: Georgian grapevine cultivar, wine volatile compounds, yeast biodiversity, GC-FID analysis, Goruli Mtsvane, Saperavi, *Torulaspora delbrueckii*, *Kluyveromyces marxianus*

INTRODUCTION

The domestication of grapevine (*Vitis vinifera* L.) occurred somewhere in the geographic region including Eastern Anatolia, South Caucasus and Western Asia in the VI millennium B.C. and it was likely consecutive of the development of the wine-making technologies originally based on wild grapes and other juicy fruits (McGovern, 2003; Forni, 2012; Batiuk, 2013). Georgian people, a proud population tied to their traditions, has always cultivated grapes and produced wines in every village and family, as an ancient local proverb says, "a good father makes a good wine". Indeed, this country is one of the homeland of the wild species *Vitis vinifera* ssp. *silvestris*, the ancestor of the cultivated grapevine *Vitis vinifera* ssp. *sativa.* The presence of numerous native varieties in Georgia evidences a high degree of intraspecific diversity as a consequence of the heterogeneity of the environments that passing by a Mediterranean climate near the Black Sea, to a subtropical one in the South or continental in the mountainous Northern territories (This et al., 2006; Ghlonti, 2010; Chkhartishvili and Maghradze, 2012).

According to the long Georgian tradition in winemaking, which is still practiced in Kakheti area, oenologists make wine in the traditional "*qvevri*", a big-size clay vessel put underground and inside coated of beeswax and with long time (until 6 month) of maceration. It is worth mentioning that neither commercial cultures nor sulfur dioxide are used in any of the familiar wineries. Sometimes winemakers use fumigation to sanitize the clay vessel. Nowadays, different types of wine are made in *qvevri*: (i) the "Kakhetian style", where the must is fermented by adding up to 100% of pomace named "*chacha*" (skins, pips, and stalks); ii) the "Imeretian style", where the must is fermented in *qvevri* with partial (2.5–3.0%) addition of *chacha*; (iii) the so called "European style" without addition of *chacha*; (iv) the "Naturally semi-sweet wines" as well as sparkling wines that were also made in *qvevri* in the past (Ghlonti, 2010; NWA – National Wine Agency of Georgia, 2016). Because of the widespread of the "*qvevri* winemaking tradition" in Georgia, as proof of its cultural significance and in accordance with principles of Convention on Protection promoted by UNESCO, the status of National Monument of Intangible Cultural Heritage has been assigned to "The ancient Georgian tradition of *qvevri* winemaking" in 2013 (NWA – National Wine Agency of Georgia, 2016).

In recent years changes in the wine market have led to minor consumption in European countries, but with a strong demand toward health requirements and sensorial satisfaction. Currently the majority of wine production around the world is based on the use of starter cultures consisting of selected strains of yeasts (active dried yeast, ADY) and bacteria, that ensure quick and safe must transformation, reducing the risk of slow or stuck fermentation or spoilage due to microbial contamination. The practice of ADY inoculation, along with other technological innovations, has helped to improve wine quality by increasing the capability of winemakers to control the fermentation process and sensory profile. However, the low number of really different commercial strains often referred with different names (Fernandez-Espinar et al., 2001; Vigentini et al., 2015), has likely led a standardization of the product resulting in a taste leveling. This phenomenon requires the isolation and selection of new yeasts and bacteria showing technological, quality and safety features useful to obtain innovative products.

The work has aimed to explore the microbial biodiversity of a pristine environment that still represents a fascinating source for the isolation of new potential interesting strains, since it is a vine-growing area that has rarely been investigated before (Capece et al., 2013). Throughout the characterization and selection of indigenous yeasts isolated from oenological environments, our study has been addressed to improve quality of Georgian wines made from low aromatic local cultivars by exploiting the volatile compounds developed during fermentations. To obtain this goal, the dominant yeast populations present in 78 samples of grape and wine, from vineyards and traditional cellars located in five regions of Georgia, were analyzed during the 2014 vintage. In a perspective of precision oenology, three strains were chosen, on the basis on their oenological traits to perform vinifications experiments for the valorization of two widespread autochthonous grape varieties.

MATERIALS AND METHODS

Yeast Sampling

Grape samples from 22 different native varieties were collected in 26 vineyards located in five regions, while wine samples were derived from 19 cellars of four regions (**Table 1**). Approximately 100 g of ripe bunches or 50 mL of wine at different stage of aging were taken, maintained at 4°C and transported in sterile bags to the laboratory. After crushing and homogenization by peristaltic apparatus (Stomacher 400, Colworth, UK) the obtained juice from the grape or the wine samples were decimally diluted in Peptoned Water (Merck, Germany); then, 100 μL of the appropriate dilutions were spread onto WL plates (Merck, Germany) that were incubated at 25°C for 3 days. Different type of colonies collected from the plates at the highest dilutions were streaked and purified twice on WL agar. The purified isolates were stored at –80°C in YPD broth (10 g/L yeast extract,

20 g/L peptone, 20 g/L glucose, pH 5.6) added with 20% (v/v) glycerol.

Yeast Identification

Yeast DNA was extracted according to Querol et al. (1992) protocol. The presumptive identification was attained by PCR amplification of the internal transcribed spacers between the 18S and 26S rDNA genes (ITS1-5.8S-ITS2) and subsequent restriction analysis according to Esteve-Zarzoso et al. (1999). The PCR mixture contained 1X *Taq* polymerase buffer with 1.5 mM $MgCl_2$ (5 Prime, Hamburg, Germany), 1 mM $MgCl_2$, 200 μM dNTPs (Fermentas, Vilnius, Lithuania), 0.1 μM of each primer ITSY1, and ITSY4 (Kurtzman and Robnett, 1998), 2 U *Taq*-DNA Polymerase (5 Prime) and 80–100 ng of DNA. The reaction was carried out in a T Gradient Biometra Thermocycler (Biometra, Göttingen, Germany) and the amplification was performed as follows: initial denaturation at 94°C for 5 min, then 35 cycles at 94°C for 1 min, annealing at 55°C for 1 min, and extension

TABLE 1 | Identification and distribution of yeast isolates in grape and wine samples for Georgian grape cultivars and geographic areas.

				Aur. pullulans	*Cand. gotoi*	*Cand. intermedia*	*Cry. carnescens*	*Cry. flavescens*	*Hans. guilliermondii*	*Hans. vineae*	*Kluy. marxianus*	*Met.a pulcherrima*	*Mey. guilliermondii*	*Pic. kluyveri*	*Pic. terricola*	*Sac. cerevisiae*	*Sta. bacillaris*	*Tor. delbrueckii*
Grape cultivar	**Area**	**Source**	**Vineyard/cellar**															
Aladasturi (b)	Guria	Grape	29,30				1	1	1			1						
Alexandreuli (b)	Ratcha-L.	Grape	20,36				1		1			2						
Asuretuli shavi (b)	Kartli	Grape	1,6						3						1			
	Kartli	Wine	24,25													4		
Chinuri (w)	Kartli	Grape	12,13					1	3		1	1						
		Wine	1,3,11,12,13, 26,27						2							11		
Chkapa (w)	Kartli	Grape	14	1								1						
		Wine	14													1	1	
Chkhaveri (r)	Guria	Grape	31				1	1								1		
Dzvelshavi (b)	Ratcha-L.	Grape	21						2			1		1				
Gorula (w)	Kartli	Grape	15					2				2						
Goruli Mtsvane (w)	Kartli	Grape	16	2	1													
Jani (b)	Guria	Grape	33					1				1				1		
Krakhuna (w)	Imereti	Grape	22					2	1					1				
		Wine	5						1							1	1	
Mtsvane Kakhuri (w)	Kakheti	Grape	2,3									4						1
		Wine	8													2		
Mtsvane Rachuli (w)	Ratcha-L.	Grape	23						2			2						
Mujuretuli (b)	Ratcha-L.	Grape	24					1			1	1						
Orbeluri Ojal. (b)	Ratcha-L.	Grape	25					1	1			1		1				
Otskhanuri Sap. (b)	Imereti	Grape	26,35			1			2			3						
Rkatsiteli (w)	Kakheti	Grape	5,7,8						3			3						2
		Wine	6,10,15,16,17,18, 20,21,28,29,30,32						1			1	1			21		
	Kartli	Grape	4						2			1						
Saperavi (b)	Kakheti	Grape	9,10,11,17			1			2			6	1					2
		Wine	7,19,22,23,31,33,34						1							10		
Tavkveri (b)	Kartli	Grape	1,18,19	1		2		1	2			2						
		Wine	2,4						1							2		
Tsitka (w)	Imereti	Grape	27,34						3	1		2						
Tsolikouri (w)	Guria	Grape	37,38,39				2	1					1			1		
		Wine	9,35,36,37,38,39						1							9		
	Imereti	Grape	32				1									1		
Tsulukidzis (w)	Ratcha-L.	Grape	28						2			1						

Berry color of the vine cultivar: b = black, r = rosé, w = white.

step at 72°C for 1 min, followed by final extension at 72°C for 7 min. PCR products were resolved by electrophoresis in 1.0% (w/v) agarose gels in TAE buffer (40 mM Tris–acetate, pH 8.2; 1 mM EDTA) at 100 V for 1 h, stained with 0.5 μg/mL ethidium bromide and photographed under UV illumination (GelDoc XR, BioRad, USA). A 100-bp XL DNA ladder marker (Roche Molecular Biochemicals, Mannheim, Germany) served as the size standard. Then, the amplified products were subjected to endonuclease restriction using 3U of *Hin*6I (Fermentas) according to the supplier's instructions. Restriction fragments were resolved by electrophoresis in 2.5% (w/v) agarose gels in TAE buffer at 100 V for 2 h and detected as described above. Isolates showing the same restriction pattern were grouped and one or two samples per cluster was submitted to the partial amplification and sequencing of the 26S rDNA D1/D2 domain. The PCR mixture was prepared as mentioned above but with primer pairs NL1 and NL4 (Kurtzman and Robnett, 1998). The temperature profile consisted of initial denaturation at 94°C for 5 min then 35 cycles at 94°C for 1 min, annealing at 52°C for 1 min and extension step at 72°C for 2 min, followed by final extension at 72°C for 7 min. Amplification products were resolved by agarose gel electrophoresis as already described and then they were subjected to sequencing by an outdoor provider (Eurofins, Milan, Italy). The obtained sequences were identified through BLAST algorithm by comparison with the sequences listed in databases (www.ncbi.nlm.nih.gov).

Evaluation of Oenological Traits

In order to select strains with oenological potential some phenotypic properties were investigated. Ethanol production was evaluated monitoring the weight loss for 2 weeks at 25°C in YPD modified adding 250 g/L glucose. As for the inoculum, 200 mL flasks containing 100 mL of cultural broth and sealed with a Müller trap, were inoculated at 0.25 $OD_{600\ nm}$. Acetic acid and glycerol production was determined by using specific enzymatic kits based on spectrophotometric UV-method according to the supplier's recommendations (Megazyme International, Bray, Ireland). The resistance against sulfur dioxide was verified by observing the cellular growth of a fresh culture streaked onto YPD, supplied with 15 g/L agar and acidified at pH 3.6 with tartaric acid, after incubation at 25°C for 5 days. A stock sterile solution of potassium metabisulfite was added in the medium at the final concentration of 100, 200, and 300 mg/L. The production of hydrogen sulfide was phenotypically estimated streaking on BIGGY agar plates (Oxoid limited, Basingstoke, UK) a fresh culture and observing the color of the colonies after incubation at 25°C for 4 days.

Vinification Experiments

On the basis of phenotypic results, *K. marxianus* (UMY207), *S. cerevisiae* (UMY255), *T. delbrueckii* (UMY196) were chosen for fermentation tests. Healthy and ripe grapes of Georgian local varieties Goruli Mtsvane (white cultivar) and Saperavi (black cultivar) were picked and manually selected to prepare the musts by moderate crushing of the berries. In case of Goruli Mtsvane must, the juice was clarified by cold settling at +4°C for 16 h, while for Saperavi must the juice was fermented together with skins and seeds. Diammonium phosphate (150 mg/L) and potassium metabisulphite (100 mg/L) were added. Vinification trials were carried out in a Georgian experimental cellar where the room temperature was recorded and set at approximately 20°C. As fermentation containers, 20 L high density polyethylene plastic carboys with stopper and airlock system were used. The inoculum was prepared in YPD broth at 25°C for 2 days with shaking. Fresh cells were collected by centrifugation at 4000 × *g* for 20 min, washed in sterile water and re-suspended in YPD. An aliquot of the cell suspension was added to the must in order to reach an initial concentration of 5×10^6 CFU/mL. Fermentations were daily monitored by measurement of the sugar content with a refractometer (PR32 α digital refractometer, 3405 Palette Series, Atago, Tokyo, Japan) expressed as Brix degree. Samples were weekly collected for microbial and chemical analyses. pH value was measured by a pHmeter (UB-5 model, Denver Instruments Company, Bohemia, NY, USA); titratable acidity (expressed as g/L of tartaric acid) was determined by titration of the juice with 0.1 N NaOH with Bromothymol blue as the indicator. Yeast count, isolation and identification of some colonies at the highest dilutions were done as described in the previous paragraphs in order to control the trend of inoculated yeasts.

Chemical Analysis of Volatile Compounds

Volatile compounds present in must and wine samples during the vinification tests were quantified using the method proposed by Ortega et al. (2001), modified as follows. 2.5 grams of ammonium sulfate and 2 mL of wine were mixed in a 15 mL centrifuge tube. Five mL of ultrapure water and 20 μL of internal standards were added to the solution. The standard mixture consisted of a working solution (50% ethanol) containing 2-butanol (0.948 mg/L), 4-methyl-2-pentanol (0.940 mg/L), ethyl heptanoate (1.077 mg/L), heptanoic acid (1.102 mg/L), 4-hydroxy-4-methyl-2-pentanone (1.145 mg/L), 2-octanol (0.945 mg/L). After salt dissolution, 250 μL of dichloromethane were added to the samples. Tubes were placed on a horizontal stirrer with a speed of approximately 60 rpm/min for 90 min; at the end of the extraction, samples were centrifuged (4000 × *g*, 10 min, 10°C). The supernatants were discarded, while the dichloromethane containing the analytes was removed with a 250 μL syringe, dehydrated with sodium sulfate and placed in 2 mL vials with 250 μL inserts. The analyses were performed with a Hewlett Packard 5890 II series GC-FID in splitless mode using a polar capillary column (HP Innovax, 30m × 0.25mm ID 0,25 μm JW Scientific, Folsom, CA, USA); the carrier gas was helium with a column flow of 1 mL/min and a splitless time of 2 min. The program was as follows: 45°C for 2 min, then the temperature was increased to 80°C at 30°C/min, from 80°C to 230°C at 5°C/min and held at 230°C for 17 min. All compounds were identified by comparison with the retention time of pure standards injected in the same chromatographic conditions.

Samples were analyzed after 6 months of storage. Twenty-four aromatic molecules were quantified, belonging to the following five groups: higher alcohols, ethyl esters, short and medium chain

fatty acids, ester acetates of higher alcohols and a miscellaneous group, comprising other volatiles such as (3-hydroxybutan-2-one or acetoine, (3Z)-hex-3-en-1-ol, 1-hexanol, dihydrofuran-2(3H)-one, or γ-butyrolactone and phenylmethanol or benzyl alcohol. Duplicate analyses were performed for all samples.

Data Analysis

The frequency (*F*') and the incidence (*I*') of the yeast species in the grape samples were calculated according to Tristezza et al. (2013). Significant differences among wine samples analyzed at the same sampling point during fermentations were assessed by one-way ANOVA, for each groups of odorants described before. Differences among means were evidenced by using the Tukey's test and were considered significant at $p < 0.05$. Differences were represented by different letters on the graph. In order to compare yeast strain and grape must effect treatments on the final wines, two-way ANOVA was performed for each analyzed volatile compounds. Statistical analyses were performed with the software package SPSS (SPSS 15.0 for Windows 2004; SPSS, Chicago, IL, USA).

RESULTS

Evaluation of Yeast Biodiversity

The mean value of the yeast concentration on grape samples was 2.946 log CFU/g (±1.240, SD), whereas that found in wine samples was 6.026 log CFU/mL (±1.712). One hundred and eighty two isolates, 110 from 39 grape samples recovered from 22 Georgian autochthonous *Vitis vinifera* cultivars and 72 from 39 wine samples collected from 19 cellars were ascribed to 15 different taxa by RFLP analysis of ITS region and the partial sequencing of 26S rDNA gene (**Table 1**). In particular, the yeasts most frequently isolated on grapes and with the highest incidence were *Metschnikowia pulcherrima* ($F' = 0.56$, $I' = 0.32$), *Hanseniaspora guilliermondii* ($F' = 0.49$, $I' = 0.27$), *Cryptococcus flavescens* ($F' = 0.31$, $I' = 0.11$), and *Cryptococcus carnescens* ($F' = 0.13$, $I' = 0.05$). Other taxa found with a lower frequency and incidence were *Torulaspora delbrueckii* ($F' = 0.10$ $I' = 0.05$), *Aureobasidium pullulans* ($F' = 0.10$, $I' = 0.04$), *Candida intermedia* ($F' = 0.08$, $I' = 0.04$), *Saccharomyces cerevisiae* ($F' = 0.08$, $I' = 0.04$), *Pichia kluyveri* ($F' = 0.08$, $I' = 0.03$), *Meyerozyma guilliermondii* ($F' = 0.05$, $I' = 0.02$), *Kluyveromyces marxianus* ($F' = 0.05$, $I' = 0.02$), *Candida gotoi* ($F' = 0.03$, $I' = 0.01$), *Hanseniaspora vineae* ($F' = 0.03$, $I' = 0.01$), and *Pichia terricola* ($F' = 0.05$, $I' = 0.01$). As expected, *Saccharomyces cerevisiae* was dominant in wine samples since its presence was determined in 97% of cases, confirming the key role of this species in Georgian spontaneous fermentations. However, given that the samples were collected at different stage of aging, a certain biodiversity has been observed including few other species, such as *H. guilliermondii* which was present in seven samples, *Starmerella bacillaris* in two samples, *M. pulcherrima* and *M. guilliermondii* in one sample. These results are generally in accordance with those recently reported by some authors in similar survey activities (Zott et al., 2010; Barata et al., 2012; Vigentini et al., 2015).

Strain Selection by Oenological Traits

Seventy-four out of 182 isolates were sorted on the supposed ability of a fermentative metabolism. In particular, 65 clones of *S. cerevisiae*, 5 of *T. delbrueckii*, 2 of *S. bacillaris*, and 2 of *K. marxianus* were investigated for some oenological traits. As expected, *S. cerevisiae* has proved to be the species with the highest ethanol production (**Figure 1A**). Four *T. delbrueckii* isolates grew up to 10–11% v/v, while one strain reached an alcohol content of 11.5% v/v. Only one isolate of both *K. marxianus* and *S. bacillaris* species was able to produce alcohol between 10 and 11% v/v. Growth tests for sulfur dioxide resistance revealed that most *S. cerevisiae* isolates (69%) withstood up to 200 mg/L of total SO_2, whereas *K. marxianus* and *S. bacillaris* isolates did not (**Figure 1B**). The determination of acetic acid concentration has evinced that *S. cerevisiae* exhibited a heterogeneous behavior, with 17% isolates that produced high amount of this compound (>0.5 g/L). The two S. *bacillaris* strains displayed an acetic acid production lower than 0.4 g/L, while *K. marxianus* and *T. delbrueckii* isolates revealed dissimilar capabilities (**Figure 1C**). Additionally, it was found that 91% of *S. cerevisiae* isolates yielded lesser than 3 g/L of glycerol. *T. delbrueckii* showed a good performance since three isolates out of five (60%) produced a glycerol concentration higher than 4 g/L. On the other hand, *K. marxianus* and *S. bacillaris* strains exposed a lesser capacity to synthesize this compound (**Figure 1D**). Results of the qualitative test on the hydrogen sulfide development showed that 53 *S. cerevisiae* isolates (82%) were high producers (brown colonies), 9 isolates (14%) were low producers (light brown colonies) and only 3 isolates (5%) did not produce it (white colonies). *T. delbrueckii* isolates proved to be low producers, as well as one strain of *K. marxianus* and of *S. bacillaris*; the remaining ones for both species were high producers of hydrogen sulfide. These findings are mostly in agreement with those reported by some authors for non-*Saccharomyces* yeasts (Lambrechts and Pretorius, 2000; Cordero-Bueso et al., 2013; Englezos et al., 2015). The comparison of results obtained from the previous tests allowed to select three yeast strains, *K. marxianus* UMY207, *S. cerevisiae* UMY255, and *T. delbrueckii* UMY196 with oenological potential for the vinification experiments.

Monitoring of the Vinification Experiments

Fermentations trials were carried out for each selected strain that were separately inoculated in 20 L volume musts of two Georgian grape cultivars (Goruli Mtsvane and Saperavi). All operations were rigorously done in strictly hygienic conditions to avoid cross-contamination between the strains. The temperature of the cellar was comprised between 17.5 and 21.0°C, with a mean value of 19°C. Chemical analysis and microbial counts were weekly scheduled and performed to monitor the fermentative process. The initial pH value and the titratable acidity of Goruli Mtsvane must were 3.1 and 7.6 g/L, respectively; the initial pH

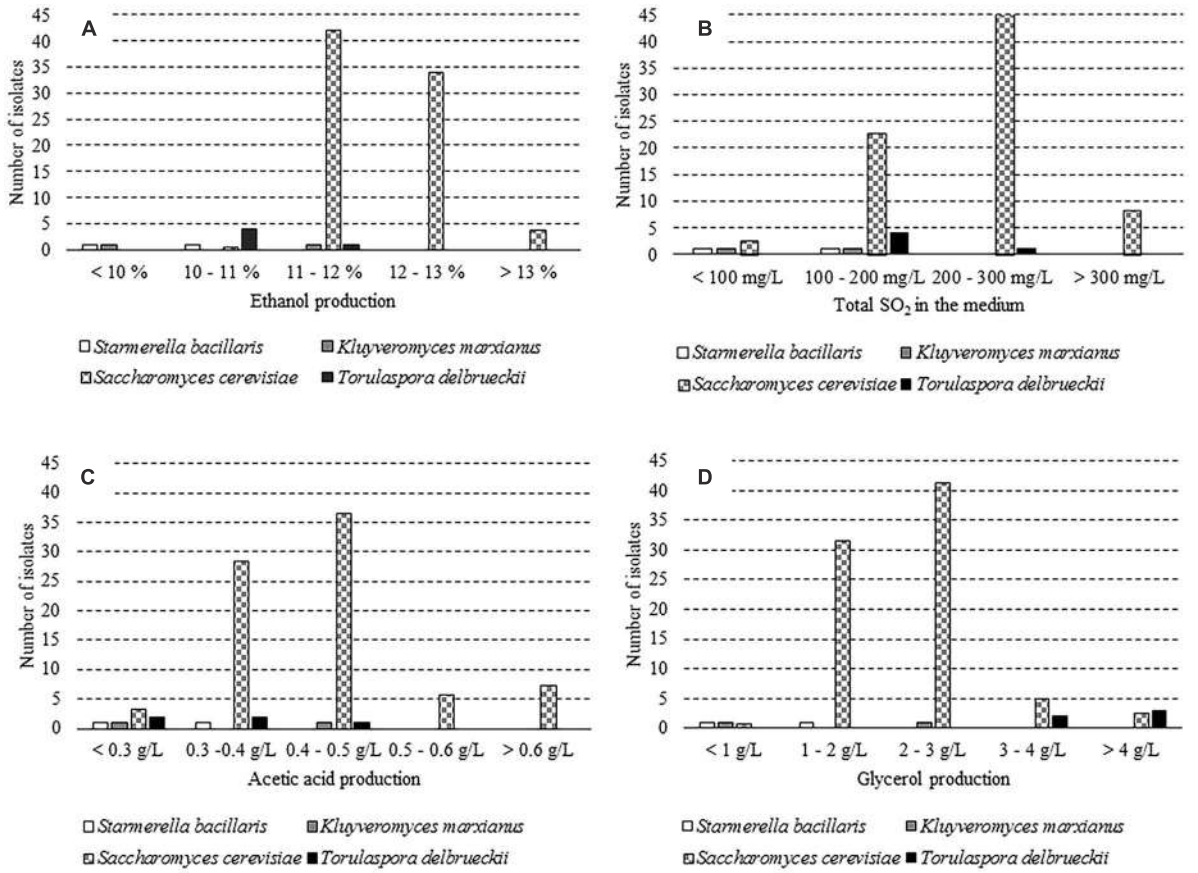

FIGURE 1 | Distribution of the isolates ascribed to four yeast species for some oenological traits. (A) Differences in ethanol production, **(B)** Differences in sulphur dioxide resistance, **(C)** Differences in acetic acid production, and **(D)** Differences in glycerol production.

value and the titratable acidity of Saperavi must were 3.2 and 6.9 g/L. The yeast count of the non-inoculated white grape must was 1.1×10^4 CFU/mL, whereas that of the non-inoculated black grape must was 1.3×10^5 CFU/mL. For all the tested strains, in Saperavi must the fermentation started and ended earlier than in Goruli Mtsvane must (**Figure 2**). The trend of sugars consumption (Brix) revealed that the lag phase in Saperavi must inoculated with *S. cerevisiae* UMY255 was 24 h, while those with *T. delbrueckii* UMY196 and *K. marxianus* UMY207 lasted until 2 and 3 days, respectively. Then, the sugar depletion went on with different rates depending on the tested strain, so that one inoculated with *S. cerevisiae* UMY255 completed the fermentation within 8 days, while the trial inoculated with *T. delbrueckii* UMY196 took 10 days and finally that one inoculated with *K. marxianus* UMY207 needed 14 days. After a week, yeast counts varied from 3.7×10^7 CFU/mL, in Saperavi must inoculated with *T. delbrueckii* UMY196, to 1.8×10^7 CFU/mL, in that inoculated with *K. marxianus* UMY207. At that time, the identification of the dominant populations revealed that 100% *S. cerevisiae*, 100% *T. delbrueckii*, and 60% *K. marxianus* were present, as expected, into the relative vessels. After two weeks the yeast cell concentrations decreased, ranging from 3.1×10^6 CFU/mL in the trial inoculated with *T. delbrueckii* UMY196, to 1.1×10^6 CFU/mL in that inoculated with *S. cerevisiae* UMY255. At this point, in the vessels inoculated with *T. delbrueckii* and *K. marxianus* only 25 and 10%, respectively, of the colonies isolated at the highest dilutions corresponded to the predictable species; conversely, for the trial inoculated with *S. cerevisiae*, 100% was ascribed to the expected species. Then, yeast counts dropped $<10^4$ CFU/mL after twenty days. The fermentation kinetics in Goruli Mtsvane must appeared otherwise, although the cell concentrations after the inocula were very similar to those obtained in Saperavi must and constantly $>10^6$ CFU/mL. The lag phase of the sample inoculated with *S. cerevisiae* UMY255 lasted 2 days, whereas for those with *T. delbrueckii* UMY196 and *K. marxianus* UMY207 it persisted until 3 and 4 days, respectively. Then, sugars consumption rates were much lower than those previously observed: the fermentation of Goruli Mtsvane must inoculated with *S. cerevisiae* UMY255 finished in three weeks, while the trials inoculated with the other strains did not complete the transformation even after a month, leaving some residual sugars (1–3 °Brix). In particular, after a week, yeast cell concentrations varied from 1.9×10^7 CFU/mL (in must inoculated with *S. cerevisiae* UMY255) to 4.2×10^6 CFU/mL (in that one with *K. marxianus* UMY207). The dominant populations were

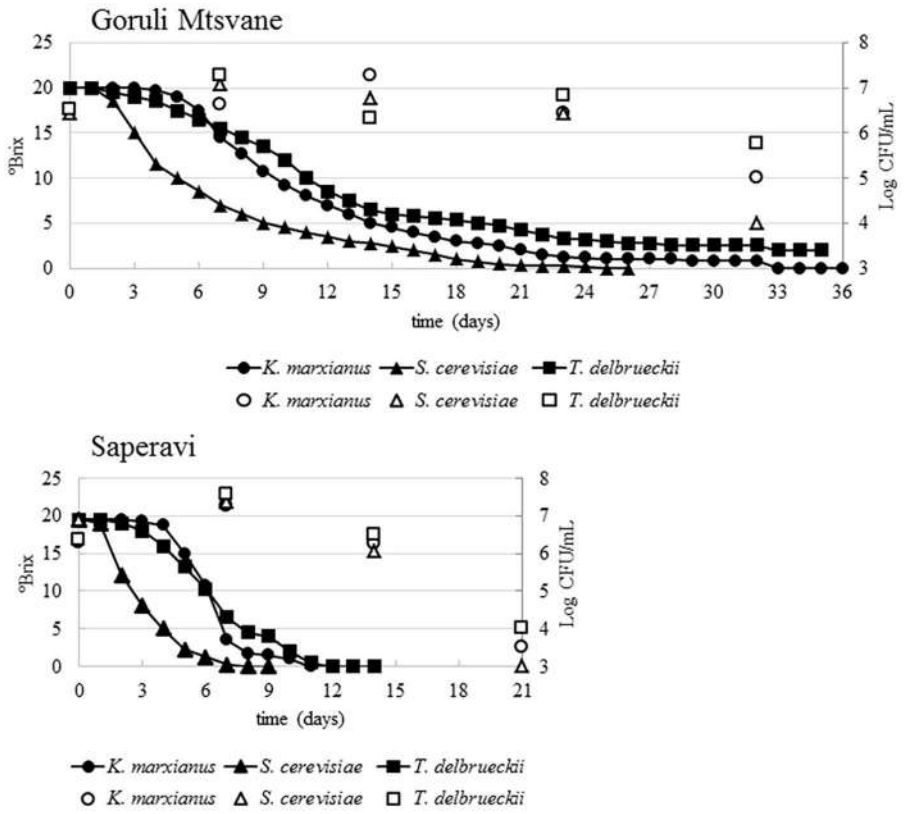

FIGURE 2 | Trends of sugar concentrations (lines and filled dots) and viable counts (unfilled dots) during the fermentation of Goruli Mtsvane (Georgian white berry cultivar) and Saperavi (Georgian black berry cultivar) musts inoculated with *Kluyveromyces marxianus* UMY207, *Saccharomyces cerevisiae* UMY255, and *Torulaspora delbrueckii* UMY196 strains.

attributed to 100% *S. cerevisiae*, 86% *T. delbrueckii* and 100% *K. marxianus*, matching to the inoculated species. After 2 weeks, yeast counts passed from 1.8×10^7 CFU/mL (in must inoculated with *K. marxianus* UMY207) to 2.1×10^6 CFU/mL (in that one with *T. delbrueckii* UMY196); but, from this point on, *K. marxianus* was not found, while *T. delbrueckii* and *S. cerevisiae* represented 50 and 100% of the fermentative biomass in the relative vessels, respectively. Yeast counts approximately decreased to 10^5 CFU/mL after one month. The identification of the isolates at the end of the fermentation showed the dominance of other yeasts, mainly ascribed to *S. cerevisiae* species, which naturally contaminated the musts.

Samples weekly collected were also subjected to analysis GC-FID analysis in order to quantify the most relevant volatile compounds. Some of them, in particular aromas resulting from yeast fermentation (i.e., ethyl esters of short and medium fatty acids) are responsible for the pleasant fresh fruity notes perceived in wines.

Table 2 reports the mean concentrations of the volatile compounds found in wines inoculated with different strains (*S. cerevisiae* UMY255, *T. delbrueckii* UMY196, *K. marxianus* UMY207) or in wines obtained from diverse grape varieties (Saperavi and Goruli Mtsvane). The concentrations of the main higher alcohols, 2-methylpropan-1-ol, 3-methylbutan-1-ol, and 2-phenylethanol, were comparable with those reported by Swiegers et al. (2005), since the sum of these ranged from 130 to 300 mg/L. In particular, *S. cerevisiae* UMY255 was the strain by revealing the greatest yield (241 mg/L). On the contrary the mean concentration of the higher alcohols in wines fermented by *T. delbrueckii* UMY196 (191.5 mg/L) was significantly ($p < 0.001$) lower than the others, showing small contents of 2-methylpropan-1-ol and 2-phenylethanol. These results are in agreement with Renault et al. (2009), who observed a low quantity of higher alcohols in wines fermented with *T. delbrueckii*. However, the composition of the musts can significantly influence the synthesis of aromatic compounds by different yeasts, as significant interactions were pointed out (**Table 2**). High levels of 2-methylpropan-1-ol, 3-methylbutyl acetate, and 2-phenylethanol were produced by *K. marxianus* UMY207 and *S. cerevisiae* UMY255 in Saperavi wine samples (**Figure 3**). This trend was not noticed in that fermented with *T. delbrueckii*. On the other hand, the concentration of these compounds in Goruli Mtsvane wines were higher for those inoculated with *T. delbrueckii* rather than the samples obtained using the other yeast strains. Some remarkable differences regarding the wine composition are related to the ethyl esters production. Actually, the production of ethylhexanoate, ethyloctanoate, and ethyldecanoate proved to be significantly greater in wines

TABLE 2 | Volatile composition of experimental wines and perception threshold of main odorants.

Volatile compound	Odorant (1, 2)	Perception threshold	Yeast strain (Y)			Grape cultivar (Cv)			Interaction
			S. cerevisiae UMY-255	*T. delbrueckii UMY-196*	*K. marxianus* UMY-207		Goruli mtsvane	Saperavi	Y x Cv
Higher alcohols									
2-Methylpropan-1-ol (isobutanol)	Ethereal	40(3)	38^{a}	27^{b}	38^{a}	***	21.6	47	***	***
3-Methylbutan-1-ol (isoamyl alcohol)	Fusel oil	30(3)	182^{a}	149^{b}	152^{b}	***	100	222	***	***
2-Phenylethanol	Rose	14(4)	21.3^{b}	15.5^{c}	25.0^{a}	***	9.4	31.7	***	***
3-(Methylsulfanyl) propan-1-ol (methionol)	Onion-like	1(3)	2.0^{a}	1.3^{b}	1.4^{b}	**	0.3	2.8	***	**
Ethyl esters									
Ethyl hexanoate	Strawberry	0.014(4)	1.3^{a}	0.7^{b}	0.9^{b}	***	1.1	0.8	**	**
Ethyl 2-hydroxypropanoate (ethyl lactate)	Fruity	154(5)	11.8^{c}	27.4^{b}	29.2^{a}	***	7.4	38.2	***	***
Ethyl octanoate	Soap	0.005(4)	0.5^{a}	0.3^{b}	0.4^{ab}	*	0.5	0.2	***	n.s.
Ethyl 3-hydroxybutanoate (ethyl-3-hydroxybutyrate)	Fruity	20(3)	0.9^{a}	0.3^{b}	0.3^{b}	***	0.3	0.7	***	***
Ethyl 4-hydroxybutanoate (ethyl-4-hydroxybutyrate)	Apple	-	2.3^{a}	1.5^{ab}	1.1^{b}	*	1.6	1.6	n.s.	n.s.
Ethyl decanoate	Grape	0.2(4)	0.3^{a}	0.3^{a}	0.1^{a}	n.s.	0.3	0.2	n.s.	n.s.
Diethyl butanedioate (diethyl succinate)	Fruity	200(5)	0.9^{a}	0.6^{b}	0.8^{a}	***	0.8	0.8	n.s.	**
4-Ethoxy-4-oxobutanoic acid (monoethyl succinate)	Cooked apple	-	27^{b}	58^{a}	59^{a}	***	17.4	78.7	***	***
Short and medium chain fatty acids									
Hexanoic acid	cheesy	0.42(4)	1.8^{a}	1.5^{b}	1.5^{b}	*	2.2	1.0	***	***
Octanoic acid	rancid	0.5(4)	1.7^{a}	1.3^{a}	1.5^{a}	n.s.	2.4	0.5	***	*
Decanoic acid	rancid	1(4)	0.4^{a}	0.3^{a}	0.3^{a}	n.s.	0.3	0.3	n.s.	n.s.
3-Methylbutanoic acid (isovaleric acid)	cheesy	0.03(4)	1.2^{b}	3.5^{a}	3.8^{a}	***	0.3	5.3	***	***
Ester acetates of higher alcohols									
Hexyl acetate	green apple	1.5(5)	0.2^{a}	0.1^{b}	0.1^{b}	**	0.1	0.1	n.s.	*
3-Methylbutyl acetate (isoamyl acetate)	banana	0.03(4)	0.8^{a}	0.2^{b}	0.5^{b}	***	0.9	0.2	***	**
2-Phenylethyl acetate	rose, honey	0.25(4)	0.01^{b}	0.01^{b}	0.06^{a}	***	0.05	0.0	***	***
Miscellaneous group									
3-Hydroxybutan-2-one (acetoine)	buttery	150(5)	0.6^{c}	1.2^{b}	1.8^{a}	***	1.1	1.3	***	***
(3Z)-Hex-3-en-1-ol (cis-3-Hexenol)	grassy	0.1(4)	0.04^{a}	0.03^{b}	0.03^{b}	***	0.03	0.03	n.s.	*
Hexan-1-ol	herbal	8(3)	1.5^{a}	1.2^{a}	1.3^{a}	n.s.	0.7	2.0	***	n.s.
Dihydrofuran-2(3H)-one (γ-butyrolactone)	creamy	100	7.2^{a}	4.1^{b}	3.8^{b}	***	5.6	3.9	***	**
Phenylmethanol (benzyl alcohol)	floral	200(3)	0.12^{b}	0.14^{a}	0.13^{ab}	*	0.05	0.2	***	***

*All the values are expressed in mg/L; n.s. = not significant; * = ANOVA, $p \leq 0.05$; ** = ANOVA, $p \leq 0.01$; *** = ANOVA, $p \leq 0.001$. (1) Cordero-Bueso et al. (2013); (2) Vilanova et al. (2013); (3) Guth (1997); (4) Ferreira et al. (2000); (5) Etiévant (1991). Values are grouped by yeast strain (Y) and grape cultivar (Cv). Values on the same line with different letter superscripts are significantly different.*

inoculated with S. *cerevisiae* UMY255 than in those inoculated with *T. delbrueckii* UMY196 and *K. marxianus* UMY207 ones (**Table 2**). These findings confirm the results reported by Renault et al. (2009) where cultures of *T. delbrueckii* have been shown to produce low levels of ethyl esters. Nevertheless, high concentrations of both 4-ethoxy-4-oxobutanoic acid and ethyl 2-hydroxypropanoate were detected in wine samples performed by inoculating *T. delbrueckii* UMY196 and *K. marxianus* UMY207 (about two folds higher than in musts inoculated with *S. cerevisiae* UMY255). 4-ethoxy-4-oxobutanoic acid concentration in white wines appeared significantly lower than that found in red wines (78.7 mg/L vs. 17.4 mg/L, $p < 0.001$). As concerns the corresponding diethylester, the diethyl butanedioate, is normally generated during wine storage, therefore its content is usually low in young wines and the differences between experimental tests limited. Interactions between yeast and must may affect the formation of these esters, namely the 4-ethoxy-4-oxobutanoic acid and ethyl 2-hydroxypropanoate. In Saperavi musts, inoculated with *T. delbrueckii* UMY196 and *K. marxianus* UMY207, a greater accumulation of ethyl esters was found (51.5 and 49.2 mg/L, respectively) than that observed in trials inoculated with *S. cerevisiae* (14.0 mg/L). As regards the short and medium chain fatty acids, the concentrations of hexanoic and octanoic acids were significantly higher ($p < 0.001$) in white

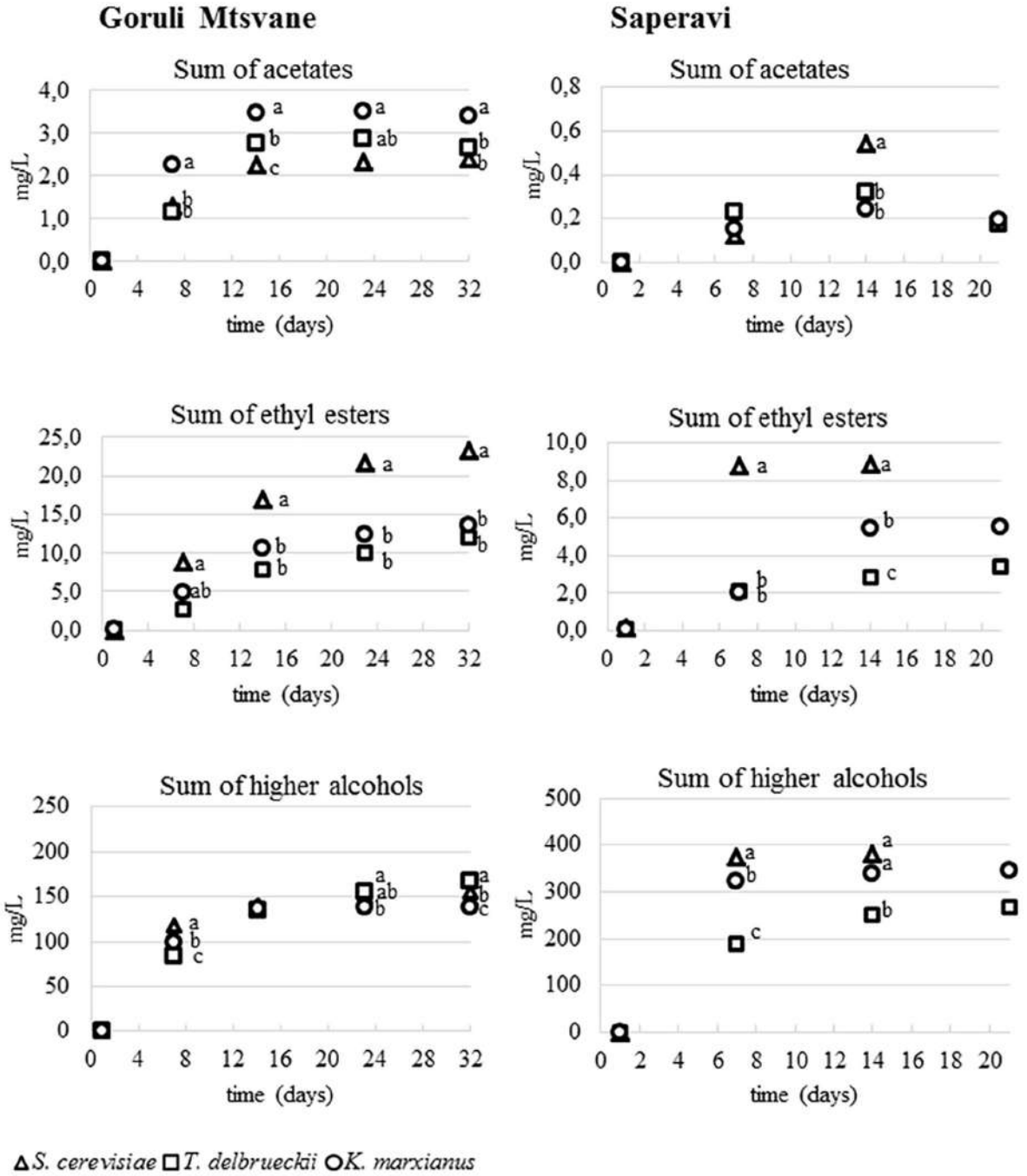

FIGURE 3 | Monitoring of the concentrations of three classes of volatile compounds during the fermentation of Goruli Mtsvane (Georgian white berry cultivar) and Saperavi (Georgian black berry cultivar) musts inoculated with *K. marxianus* UMY207, *S. cerevisiae* UMY255 and *T. delbrueckii* UMY196 strains. Dots with different letters at the same time indicate mean values significantly different ($p < 0.01$).

wines rather than red wines, while no statistical differences were detected for decanoic acid. Yeast related differences were modest and concerned only the 1-hexanoic acid ($p < 0.05$, **Table 2**). On average, the concentration of these compounds was higher in the wines obtained from *S. cerevisiae* fermentations. Finally, it is noteworthy the higher concentration of acid 3-methylbutanoic acid in Saperavi in wines than the corresponding Goruli Mtsvane wines (**Table 2**). The concentrations of the ester acetates of higher alcohols determined in samples fermented with different yeasts, appeared similar. A greater production of these compounds was noticed in musts inoculated with *S. cerevisiae* UMY255, and significant differences ($p < 0.001$) were shown between white

and red wines for the 3-methylbutyl acetate (0.88 mg/L in Goruli Mstvane vs. 0.17 mg/L in Saperavi) and the phenylethylacetate contents. Concerning the others compounds, dihydrofuran-2(3H)-one presented high concentrations in wines fermented with *S. cerevisiae*. 1-Hexanol and (3Z)-hex-3-en-1-ol showed minor yeast related differences, while statistically significant changes were pointed out for 1-hexanol between Saperavi and Goruli Mtsvane wines (**Table 2**). This outcome confirms that the presence in wines of alcohols with six carbon atoms are mainly due both to the grape variety and oxygen uptake during pre-fermentative operations (Oliveira et al., 2006).

Volatile Compounds Development during Fermentations

Figure 3 shows the accumulation kinetics of three different classes of compounds during the trials (acetates of higher alcohols, ethyl esters, and higher alcohols), for both white and red musts inoculated with the different yeast strains. The average concentration of acetates in white samples was significantly higher than that found in the red ones. As reported in literature (Plata et al., 2003), the content of acetates of higher alcohols in wine depends substantially on the yeast strain and the interactions between yeast and grape must as well as the fermentation conditions (Molina et al., 2007). In Goruli Mtsvane samples the total concentrations of acetates increased until 2 weeks, then values remained constant; the highest content was found in that inoculated with *K. marxianus* UMY207. In general, the levels of ethyl esters increased steadily during all fermentations. According with some authors (Renault et al., 2009; Sumby et al., 2010) musts added with *S. cerevisiae* cultures produced the greatest amounts of these compounds respect those in which other yeasts were used. As regards the most odorant ethyl esters (ethyl hexanoate, ethyl octanoate, ethyl decanoate), the differences between the yeast strains during the fermentation were less evident (data not reported). However, *T. delbrueckii* UMY196 showed a small accumulation of these compounds when compared to *S. cerevisiae* and *K. marxianus*. These results are in agreement with those obtained recently by Hernandez-Orte et al. (2008) and Viana et al. (2008). Also in this case, the grape variety affected the ethyl esters production, since the white wine samples presented a double amounts of these fruity compounds compared to red ones. Higher alcohols increased rapidly during the fermentation; these molecules reached their maximum level after three weeks for the Goruli Mtsvane samples and at about the 14th day for the Saperavi ones, then a slight decrease was observed in both trials. The final concentration in wines strictly depended on the interaction between grape must and yeast strain. Indeed, at the end of the fermentation, the greatest amount of the higher alcohols was observed in red wine samples inoculated with *S. cerevisiae* UMY255 and *K. marxianus* UMY207.

DISCUSSION

While the precision viticulture is currently applied to optimize the vineyards performance in maximizing grape yield and quality, the precision oenology, that might exploit the technological potential of wild strains, still remains a matter of research activities. However, new styles of wine products and innovative ways of fermentation management have intensified the interest in search for new strains hidden in the microbial diversity (Pretorius and Bauer, 2002; Romano et al., 2003; Fleet, 2008; Jolly et al., 2014). Indeed, the best expression of the varietal character of a wine may depend on the metabolic activities of microorganisms taking part in the transformation of must and in the aging of wine (Lambrechts and Pretorius, 2000; Swiegers et al., 2005; Hernandez-Orte et al., 2008; Furdikova et al., 2014). Moreover, several research groups have recently addressed their efforts to collect and characterize "autochthonous" yeast strains as strategic activity for the promotion and protection of local wines, since these findings would confirm the link among territory, environment production and final product, with a remarkable commercial impact (Mannazzu et al., 2002; Di Maio et al., 2012; Settanni et al., 2012; Tristezza et al., 2012; Rodriguez-Palero et al., 2013). So, this study have given the opportunity of isolating novel wine-associated yeasts from Georgia, an ancient vine-growing area where the use of starter cultures has not yet spread, in order to select non-conventional yeast strains and species for wine-making.

The results obtained from grape samples have revealed a high level of biodiversity with rates of isolation and yeast species similar to those already described by different authors (Cordero-Bueso et al., 2011; Barata et al., 2012; Bokulich et al., 2013; Milanovic et al., 2013; Vigentini et al., 2015). No evident relationship has appeared between the yeast species and the grape cultivars or the geographic region of isolation, even if the number of isolates per sample was too small for drawing definitive conclusions. The high rate of isolation (10%) of *S. cerevisiae* species from the grape berries and the presence of *K. marxianus* have been interesting outcomes. The occurrence of the yeast species observed in Georgian wine samples are different from those reported by Capece et al. (2013), who only found *S. cerevisiae* species. However, these authors analyzed wines of a unique grape variety, from only one winery, after 1 year maturation in *qvevri* vessels, while we have sampled wines at different stages of aging, not only aged in clay amphorae, from 19 cellars and made with different grape cultivars. This may explains the isolation of other yeast species, such as *H. guilliermondii* and *S. bacillaris*; as well, the presence of other taxa besides *S. cerevisiae* has already been observed in wines from spontaneous fermentations (Torija et al., 2001; Combina et al., 2005; Zott et al., 2010; Vigentini et al., 2014). With regard to the screening activity for the strain selection with oenological potential *S. cerevisiae* has shown an intraspecific variability in phenotypic traits and, as expected, it has revealed the highest ethanol production and sulfur dioxide tolerance (Ribéreau-Gayon et al., 2006). *T. delbrueckii* strains have proved to be low producers of volatile acidity and good producers of glycerol, confirming the results reported by some authors (Bely et al., 2008; Renault et al., 2009); indeed, this species is currently the most applied in commercial starter cultures as non-*Saccharomyces* yeast for mixed fermentation or sequential inoculation technique

(Jolly et al., 2014; Loira et al., 2014). *S. bacillaris* isolates (synonym of *Candida zemplinina*) have shown low rate of isolation and they have not demonstrated high performances in terms of glycerol production or alcohol resistance, respect to previous outcomes (Sadoudi et al., 2012; Englezos et al., 2015); for this reason, they were not taken into consideration for the vinification experiments. Conversely, although in a very limited number, *K. marxianus* strains have been considered appealing since they exhibited promising phenotypic traits for the application in wine-making. Due to its inherent ability to produce abundant quantities of esters, this species is emerging as a model organism to produce flavor compounds (Morrissey et al., 2015). However, in our experimental conditions, *K. marxianus* UMY207 has pointed out a scarce fermentation power and its presence has detected only in the first days of fermentation, and then overcome by the wild yeasts.

The choice of Goruli Mstvane and Saperavi varieties to be tested in the fermentation trials has been determined because they are two of the most cultivated in Georgia and they are low aromatic grapes cultivars, suitable to better show the ability of producing fermentative aromas by the selected strains (Viana et al., 2008; Romano et al., 2014). Indeed, higher alcohols, ethyl esters of short and medium fatty acids and acetates of higher alcohols, have been considered and monitored during the experimental fermentations, being responsible for the pleasant fresh fruity notes perceived in wines. The quantities of higher alcohols obtained in our trials are remarkable and encouraging since they show significant differences in the interactions between inoculated strain and grape variety. Instead, the findings about formation of ethyl esters and acetates of higher alcohols, have been less satisfactory, because most of these volatile compounds were quantified under their perception threshold.

A first reached goal of this study is the microbial collection that represents a contribution to the preservation and valuation of Georgian viti-oenological resources, found in a territory dedicated for thousands years to the wine production through traditional practices. As second target, our findings have highlighted that the production of volatile compounds significantly depends from the interaction between the grape cultivar and the yeast strain or species inoculated. Further vinification experiments in larger volumes with mixed cultures (by co-inoculation or sequential inoculation) should be performed to confirm the positive role of the selected Georgian yeast strains. In general, the outcomes of this work can be regarded as an advancement in the field of wine-making in order to edit the wine quality in a perspective of precision oenology for which the suitable grape cultivar is associated with the skillful yeast.

AUTHOR CONTRIBUTIONS

IV contributed to the design of the work, to the molecular identification of yeasts and the selection of candidates for vinification experiments, to the interpretation of data for the work, to draft the work and revising it; DM contributed to the design of the work, to collect grape and wine samples, to isolate yeasts and to manage vinifications; MP contributed to the chemical analysis of wine samples, to the interpretation of data for the work and to draft the work; FB contributed to the chemical analysis of wine samples, to the interpretation of data for the work and to draft the work; VM contributed to the preparation of musts for winemaking and to monitor chemical and microbiological analysis of vinifications; FV contributed to the yeast isolation and molecular identification of yeasts; OF contributed to the organization of the group and to draft the work; RF contributed to the design of the work, to the acquisition, the analysis of data for the work, to draft the work and revising it, and ensured that that questions related to the accuracy or integrity of any part of the work were appropriately investigated and resolved.

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REFERENCES

Barata, A., Malfeito-Ferreira, M., and Loureiro, V. (2012). The microbial ecology of wine grape berries. *Int. J. Food Microbiol.* 153, 243–259. doi: 10.1016/j.ijfoodmicro.2011.11.025

Batiuk, D. (2013). The fruits of migration: understanding the 'longue dureé' and the socio-economic relations of the Early Transcaucasian Culture. *J. Anthropol. Archaeol.* 32, 449–477. doi: 10.1016/j.jaa.2013.08.002

Bely, M., Stoeckle, P., Masneuf Pomarede, I., and Dubourdieu, D. (2008). Impact of mixed *Torulaspora delbrueckii* and *Saccharomyces cerevisiae* culture on high sugar fermentation. *Int. J. Food Microbiol.* 122, 312–320. doi: 10.1016/j.ijfoodmicro.2007.12.023

Bokulich, N. A., Ohta, M., Richardson, P. M., and Mills, D. A. (2013). Monitoring seasonal changes in winery-resident microbiota. *PLoS ONE* 8:e66437. doi: 10.1371/journal.pone.0066437

Capece, A., Siesto, G., Poeta, C., Pietrafesa, R., and Romano, P. (2013). Indigenous yeast population from Georgian aged wines produced by traditional "Kakhetian" method. *Food Microbiol.* 36, 447–455. doi: 10.1016/j.fm.2013.07.008

Chkhartishvili, N., and Maghradze, D. (2012). "Viticulture and winemaking in Georgia," in *Caucasus and Northern Black Sea Region Ampelography*, eds D. Maghradze, L. Rustioni, J. Turok, A. Scienza, and O. Failla (Quedlinburg: JKI - Julius Kühn-Institut), 169–176.

Combina, M., Elìa, A., Mercado, L., Catania, C., Ganga, A., and Martinez, C. (2005). Dynamics of indigenous yeast populations during spontaneous fermentation of wines from Mendoza. *Argentina. Int. J. Food Microbiol.* 99, 237–243. doi: 10.1016/j.ijfoodmicro.2004.08.017

Cordero-Bueso, G., Arroyo, T., Serrano, A., Tello, J., Aporta, I., Velez, M. D., et al. (2011). Influence of the farming system and vine variety on yeast

communities associated with grape berries. *Int. J. Food Microbiol.* 145, 132–139. doi: 10.1016/j.ijfoodmicro.2010.11.040

Cordero-Bueso, G., Esteve-Zarzoso, B., Cabellos, J. M., Gil-Díaz, M., and Arroyo, T. (2013). Biotechnological potential of non-Saccharomyces yeasts isolated during spontaneous fermentations of Malvar (*Vitis vinifera* cv. L.). *Eur. Food Res. Technol.* 236, 193–207. doi: 10.1007/s00217-012-1874-9

Di Maio, S., Polizzotto, G., Di Gangi, E., Foresta, G., Genna, G., Verzera, A., et al. (2012). Biodiversity of indigenous Saccharomyces populations from old wineries of south-eastern Sicily (Italy): preservation and economic potential. *PLoS ONE* 7:e30428. doi: 10.1371/journal.pone.0030428

Englezos, V., Rantsiou, K., Torchio, F., Rolle, L., Gerbi, V., and Cocolin, L. (2015). Exploitation of the non-Saccharomyces yeast *Starmerella bacillaris* (synonym *Candida zemplinina*) in wine fermentation: physiological and molecular characterizations. *Int. J. Food Microbiol.* 199, 33–40. doi: 10.1016/j.ijfoodmicro.2015.01.009

Esteve-Zarzoso, B., Belloch, C., Uruburu, F., and Querol, A. (1999). Identification of yeasts by RFLP analysis of the rRNA gene and the two ribosomial internal transcribed spacers. *Int. J. Syst. Bacteriol.* 49, 329–337. doi: 10.1099/00207713-49-1-329

Etiévant, P. X. (1991). "H.TNO-CIVO, Food Analysis Institute" in *Volatile Compound in Food and Beverages*, eds H. Wine and Maarse (Zeist: CRC Press), 454–483.

Fernandez-Espinar, M. T., Lopez, V., Ramon, D., Bartra, E., and Querol, A. (2001). Study of the authenticity of commercial wine strains by molecular techniques. *Int. J. Food Microbiol.* 70, 1–10. doi: 10.1016/S0168-1605(01)00502-5

Ferreira, V., López, R., and Cacho, J. F. (2000). Quantitative determination of the odorants of young red wines from different grape varieties. *J. Sci. Food Agric.* 80, 1659–1667. doi: 10.1111/j.1567-1364.2008.00427.x

Fleet, G. H. (2008). Wine yeasts for the future. *FEMS Yeast Res.* 8, 979–995. doi: 10.1111/j.1567-1364.2008.00427.x

Forni, G. (2012). "The origin of 'old world' viticulture," in *Caucasus and Northern Black Sea Region Ampelography*, eds D. Maghradze, L. Rustioni, J. Turok, A. Scienza, and O. Failla (Quedlinburg: JKI – Julius Kühn-Institut), 27–38.

Furdikova, K., Makysova, K., and Durcanska, K. (2014). Influence of yeast strain on aromatic profile of Gewurztraminer wine. *LWT Food Sci. Technol.* 59, 256–262. doi: 10.1016/j.lwt.2014.05.057

Ghlonti, T. (2010). "Traditional technologies and history of Georgian wine," in *Bulletin de l'OIV Proceeding of the 33rd World Congress of Vine and Wine*, Tbilisi, 83, 335–343.

Guth, H. (1997). Quantitation and sensory studies of character impact odorants of different white wine varieties. *J. Agric. Food Chem.* 45, 3027–3032. doi: 10.1021/jf9608433

Hernandez-Orte, P., Cersosimo, M., Loscos, N., Cacho, J., Garcia-Moruno, E., and Ferreira, V. (2008). The development of varietal aroma from non-floral grapes by yeasts of different genera. *Food Chem.* 107, 1064–1077. doi: 10.1016/j.foodchem.2007.09.032

Jolly, N. P., Varela, C., and Pretorius, I. S. (2014). Not your ordinary yeast: non-Saccharomyces yeasts in wine production uncovered. *FEMS Yeast Res.* 14, 215–237. doi: 10.1111/1567-1364.12111

Kurtzman, C. P., and Robnett, C. J. (1998). Identification and phylogeny of ascomycetous yeasts from analysis of nuclear large subunit (26S) ribosomal DNA partial sequences. *Ant. van Leeuw.* 73, 331–371. doi: 10.1023/A:1001761008817

Lambrechts, M. G., and Pretorius, I. S. (2000). Yeast and its importance to wine aroma – a review. *South Afr. J. Enol. Vitic.* 21, 97–129.

Loira, I., Vejarano, R., Banuelos, M. A., Morata, A., Tesfaye, W., Uthurry, C., et al. (2014). Influence of sequential fermentation with *Torulaspora delbrueckii* and *Saccharomyces cerevisiae* on wine quality. *LWT Food Sci. Technol.* 59, 915–922. doi: 10.1016/j.lwt.2014.06.019

Mannazzu, I., Clementi, F., and Ciani, M. (2002). "Strategies and criteria for the isolation and selection of autochthonous starters," in *Biodiversity and Biotechnology of Wine Yeasts*, ed. M. Ciani (Kerala: Research Signpost), 19–33.

McGovern, P. E. (2003). *Ancient Wine: The Search of Origin of Viticulture*. Princeton: Princeton University Press.

Milanovic, V., Comitini, F., and Ciani, M. (2013). Grape berry yeast communities: influence of fungicide treatments. *Int. J. Food Microbiol.* 161, 240–246. doi: 10.1016/j.ijfoodmicro.2012.12.019

Molina, A. M., Swiegers, J. H., Varela, C., Pretorius, I. S., and Agosin, E. (2007). Influence of wine fermentation temperature on the synthesis of yeast-derived volatile aroma compounds. *Appl. Microbiol. Biotechnol.* 77, 675–687. doi: 10.1007/s00253-007-1194-3

Morrissey, J. P., Etschmann, M. M. W., Schrader, J., and de Billerbeck, G. M. (2015). Cell factory applications of the yeast *Kluyveromyces marxianus* for the biotechnological production of natural flavour and fragrance molecules. *Yeast* 32, 3–16. doi: 10.1002/yea.3054

NWA – National Wine Agency of Georgia (2016). Available at: http://georgianwine.gov.ge/eng [accessed December 12, 2015].

Oliveira, J. M., Faria, M., Sá, F., Barros, F., and Araújo, I. M. (2006). C6-alcohols as varietal markers for assessment of wine origin. *Anal. Chim. Acta* 563, 300–309. doi: 10.1016/j.aca.2005.12.029

Ortega, C., López, R., Cacho, J., and Ferreira, V. (2001). Fast analysis of important wine volatile compounds: development and validation of a new method based on gas chromatographic–flame ionisation detection analysis of dichloromethane microextracts. *J. Chromat. A* 923, 205–214. doi: 10.1016/S0021-9673(01)00972-4

Plata, C., Millan, C., Mauricio, J. C., and Ortega, J. M. (2003). Formation of ethyl acetate and isoamyl acetate by various species of wine yeasts. *Food Microbiol.* 20, 217–224. doi: 10.1016/S0740-0020(02)00101-6

Pretorius, I. S., and Bauer, F. F. (2002). Meeting the consumer challenge through genetically customised wine yeast strains. *Trends Biotechnol.* 20, 426–432. doi: 10.1016/S0167-7799(02)02049-8

Querol, A., Barrio, E., Huerta, T., and Ramòn, D. (1992). Molecular monitoring of wine fermentations conducted by active dry yeast strains. *Appl. Eviron. Microbiol.* 58, 2948–2953.

Renault, P., Miot-Sertier, C., Marullo, P., Hernández-Orte, P., Lagarrigue, L., Lonvaud-Funel, A., et al. (2009). Genetic characterization and phenotypic variability in *Torulaspora delbrueckii* species: potential applications in the wine industry. *Int. J. Food Microbiol.* 134, 201–210. doi: 10.1016/j.ijfoodmicro.2009.06.008

Ribéreau-Gayon, P., Dubourdieu, D., Doneche, B., and Lonvaud-Funel, A. (2006). *Handbook of Enology: Volume 1. The Microbiology of Wine and Vinifications*. Chichester: John Wiley & Sons Ltd.

Rodriguez-Palero, M. J., Fierro-Risco, J., Codon, A. C., Benìtez, T., and Valcarcel, M. J. (2013). Selection of an autochthonous *Saccharomyces strain* starter for alcoholic fermentation of Sherry base wines. *J. Ind. Microbiol. Biotechnol.* 40, 613–623. doi: 10.1007/s10295-013-1251-0

Romano, P., Fiore, C., Paraggio, M., Caruso, M., and Capece, A. (2003). Function of yeast species and strains in wine flavor. *Int. J. Food Microbiol.* 86, 169–180. doi: 10.1016/S0168-1605(03)00290-3

Romano, P., Pietrafesa, R., Romaniello, R., Zambuto, M., Calabretti, A., and Capece, A. (2014). Impact of yeast starter formulations on the production of volatile compounds during wine fermentation. *Yeast* 32, 245–256. doi: 10.1002/yea.3034

Sadoudi, M., Tourdot-Marechal, R., Rousseaux, S., Steyer, D., Gallardo-Chacon, J. J., Ballester, J., et al. (2012). Yeast-yeast interactions revealed by aromatic profile analysis of Sauvignon Blanc wine fermented by single or co-culture of non-Saccharomyces and Saccharomyces yeasts. *Food Microbiol.* 32, 243–253. doi: 10.1016/j.fm.2012.06.006

Settanni, L., Sannino, C., Francesca, N., Guarcello, R., and Moschetti, G. (2012). Yeast ecology of vineyards within Marsala wine area (western Sicily) in two consecutive vintages and selection of autochthonous *Saccharomyces cerevisiae* strains. *J. Biosci. Bioengin.* 114, 606–614. doi: 10.1016/j.jbiosc.2012.07.010

Sumby, K. M., Grbin, P. R., and Jiranek, V. (2010). Microbial modulation of aromatic esters in wine: current knowledge and future prospects. *Food Chem.* 121, 1–16. doi: 10.1016/j.foodchem.2009.12.004

Swiegers, J., Bartowsky, E., Henschke, P., and Pretorius, I. S. (2005). Yeast and bacterial modulation of wine aroma and flavour. *Austr. J. Grape Wine Res.* 11, 139–173. doi: 10.1111/j.1755-0238.2005.tb00285.x

This, P., Lacombe, T., and Thomas, M. R. (2006). Historical origins and genetic diversity of wine grapes. *Trends Genet.* 22, 511–519. doi: 10.1016/j.tig.2006.07.008

Torija, M. J., Rozes, N., Poblet, M., Guillamon, J. M., and Mas, A. (2001). Yeast population dynamics in spontaneous fermentations: comparison between two different wine-producing areas over a period of three years. *Ant. van Leeuw.* 79, 345–352. doi: 10.1023/A:1012027718701

Tristezza, M., Vetrano, C., Bleve, G., Spano, G., Capozzi, V., Logrieco, A., et al. (2013). Biodiversity and safety aspects of yeast strains characterized from vineyards and spontaneous fermentations in the Apulia Region. *Italy. Food Microbiol.* 36, 335–342. doi: 10.1016/j.fm.2013.07.001

Tristezza, M., Vetrano, C., Bleve, G., Tufariello, M., Quarta, A., Mita, G., et al. (2012). Autochthonous fermentation starters for the industrial production of Negroamaro wines. *J. Ind. Microbiol. Biotechnol.* 39, 81–92. doi: 10.1007/s10295-011-1002-z

Viana, F., Gil, J. V., Genovés, S., Vallés, S., and Manzanares, P. (2008). Rational selection of non-Saccharomyces wine yeasts for mixed starters based on ester formation and enological traits. *Food Microbiol.* 25, 778–785. doi: 10.1016/j.fm.2008.04.015

Vigentini, I., De Lorenzis, G., Fabrizio, V., Valdetara, F., Faccincani, M., Panont, C. A., et al. (2015). The vintage effect overcomes the terroir effect: a three year survey on the wine yeast biodiversity in Franciacorta and Oltrepò Pavese, two northern Italian vine-growing areas. *Microbiology* 161, 362–373. doi: 10.1099/mic.0.000004

Vigentini, I., Fabrizio, V., Faccincani, M., Picozzi, C., Comasio, A., and Foschino, R. (2014). Dynamics of *Saccharomyces cerevisiae* populations in controlled and spontaneous fermentations for Franciacorta D.O.C.G. base wine production. *Ann. Microbiol.* 64, 639–651. doi: 10.1007/s13213-013-0697-7

Vilanova, M., Genisheva, Z., Graña, M., and Oliveira, J. M. (2013). Determination of odorants in varietal wines from international grape cultivars (*Vitis vinifera*) grown in NW Spain. *South. Afr. J. Enol. Vitic.* 34, 212–222.

Zott, K., Claisse, O., Lucas, P., Coulon, J., Lonvaud-Funel, A., and Masneuf-Pomarede, I. (2010). Characterization of the yeast ecosystem in grape must and wine using real-time PCR. *Food Microbiol.* 27, 559–567. doi: 10.1016/j.fm.2010.01.006

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Yeast Biodiversity from DOQ Priorat Uninoculated Fermentations

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Climate, soil, and grape varieties are the primary characteristics of *terroir* and lead to the definition of various appellations of origin. However, the microbiota associated with grapes are also affected by these conditions and can leave a footprint in a wine that will be part of the characteristics of *terroir*. Thus, a description of the yeast microbiota within a vineyard is of interest not only to provide a better understanding of the winemaking process, but also to understand the source of microorganisms that maintain a microbial footprint in wine from the examined vineyard. In this study, two typical grape varieties, Grenache and Carignan, have been sampled from four different vineyards in the DOQ Priorat winegrowing region. Afterward, eight spontaneous alcoholic fermentations containing only grapes from one sampling point and of one variety were conducted at laboratory scale. The fermentation kinetics and yeast population dynamics within each fermentation experiment were evaluated. Yeast identification was performed by RFLP-PCR of the 5.8S-ITS region and by sequencing D1/D2 of the 26S rRNA gene of the isolates. The fermentation kinetics did not indicate clear differences between the two varieties of grapes or among vineyards. Approximately 1,400 isolates were identified, exhibiting high species richness in some fermentations. Of all the isolates studied, approximately 60% belong to the genus *Hanseniaspora*, 16% to *Saccharomyces*, and 11% to *Candida*. Other minor genera, such as *Hansenula*, *Issatchenkia*, *Kluyveromyces*, *Saccharomycodes*, and *Zygosaccharomyces*, were also found. The distribution of the identified yeast throughout the fermentation process was studied, and *Saccharomyces cerevisiae* was found to be present mainly at the end of the fermentation process, while *Aureobasidium pullulans* was isolated primarily during the first days of fermentation in three of the eight spontaneous fermentations. This work highlights the complexity and diversity of the vineyard ecosystem, which contains yeasts from different species. The description of this yeast diversity will lead to the selection of native microbiota that can be used to produce quality wines with the characteristics of the Priorat.

Keywords: wine, Grenache, Carignan, *Saccharomyces cerevisiae, Hanseniaspora, Candida*

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INTRODUCTION

Wine producers have recently grown concerns about the importance of introducing high quality wines to the market that exhibit geographical characteristics and complexity (Harvey et al., 2014). *Terroir* has been defined as the concept that links the sensory features of wine to the environmental conditions of vineyards. Climate, soil, and grape variety, among other factors, represent the

main characteristics of a *terroir* (Van Leeuwen and Seguin, 2006). Moreover, these elements may condition what has been defined as the microbial biogeography of grapes (Bokulich et al., 2014), as unique microbial strains have been associated with specific geographical locations (Tofalo et al., 2013).

Different microorganisms, and particularly yeasts, are involved and play a key role in the production of wine. Grapes represent one of the main sources of the yeast populations found in wine (Mortimer and Polsinelli, 1999) and contain mainly non-*Saccharomyces* species; however, these species are gradually replaced by *Saccharomyces cerevisiae* throughout the process of alcoholic fermentation (Fleet, 1993). Recently, several non-*Saccharomyces* species have been related to positive attributes such as the production of interesting aroma compounds or the reduction of the final ethanol content of wine (Gonzalez et al., 2013; Jolly et al., 2014). Moreover, it has been reported that these species reach populations of up to 10^8 CFU/mL during the alcoholic fermentation of wines (Combina et al., 2005). Therefore, the presence of non-*Saccharomyces* yeasts during vinification is likely to affect physico-chemical characteristics, leaving behind identifiable characteristics in the resulting wine.

To obtain wines that reflect a certain *terroir*, it is essential to reproduce industrially the microbial fingerprint of the spontaneous fermentations that occur during vinification while avoiding the microbiological and technological risks associated with uncontrolled fermentations. In this sense, the use of native yeasts is a feasible option (Carrascosa et al., 2012; Scacco et al., 2012), but the prior step of isolating and characterizing multiple yeast strains is essential to properly select strains (Tofalo et al., 2013). For this reason, ecological studies of vineyard yeast microbiota are of interest not only to better understand the winemaking process but also to determine the source of microorganisms that produce a particular microbial footprint. Many ecological studies of indigenous yeast microbiota from different vineyards have been published, and have been recently reviewed by Barata et al. (2012). Additional studies complement this information with microbial analyses of spontaneous alcoholic fermentations occurring in different winemaking regions (Torija et al., 2001; Combina et al., 2005; Díaz et al., 2013).

The Priorat Qualified Appellation of Origin (DOQ in Catalan) is a traditional area of wine production located in the south of Catalonia, Spain, where Carignan (CA) and Grenache (GR) are typical and characteristic red grape varieties. Although limited data exist concerning the microbial biogeography of grapes in DOQ Priorat, Torija et al. (2001) studied the yeast population dynamics of GR spontaneous fermentations in a cellar from Priorat, and determined that *Candida stellata* was the primary dominant non-*Saccharomyces* species. However, as different vineyards may broaden the microbial biodiversity of the region, the yeast population should be studied at different geographical points, and its dynamics should be observed under spontaneous conditions.

The aim of this study is to provide a detailed inventory of the yeast populations on GR and CA grapes and that could be developed in oenological conditions from DOQ Priorat. Berries from both varieties were collected at four different vineyards upon the ripening of the 2012 vintage, and spontaneous alcoholic fermentations were performed to characterize yeast population dynamics through the isolation and molecular identification of the yeasts present.

MATERIALS AND METHODS

Grape Sampling and Spontaneous Fermentation

Four different vineyards (V1, V2, V3, and V4) were selected for the collection of both GR and CA grapes. All wine terraces belong to the Priorat DOQ and are between 300 and 800 m above sea level. In the Priorat region, most of the vineyards follow integrated approaches that attempt to minimize the use of pesticides and other chemicals. For each variety and each vineyard 2 kg of grapes were manually collected during vintage 2012 and transported refrigerated into sterile bags to the laboratory.

Grape juice was obtained after sterile manual selection, destemming and squeeze of 1.8 kg of berries. Must was placed at once with seeds and skin into 2 L sterile flasks. The spontaneous fermentation was conducted under agitation at 120 rpm and 24°C. The must was pumped up each 24 h, and after the first day 30 ppm of sulfur dioxide were added as potassium metabisulfite. Daily samples were withdrawn to monitor sugar concentration by measuring must density using an electronic densitometer (Mettler-Toledo S.A.E., Barcelona, Spain). In addition, samples of the grape juice (Day 0), before the addition of SO_2 (Day 1), 24 h after the addition SO_2 (Day 2) at a mid-fermentation point (M; density 1040–1060 g/L) and at the end of the fermentation (F) (density $<$ 1000 g/L) were also aseptically taken for yeast counting and isolation.

Yeast Content and Isolation

Aliquots of different serial decimal dilutions of samples were spread in duplicate on solid YPD (glucose 2%, peptone 0.5%, yeast extract 0.5%, and agar 2%) and agar-Lysine (LYS) plates (6.6% Oxoid lysine medium, 0.5% potassium lactate, 0.2% lactic acid). Plates were incubated at 28°C for 3 days. To identify the yeast present, approximately 25 colonies from each medium and each sampling point were picked randomly.

Yeast Identification: RFLPs of the 5.8S-ITS rRNA Region and Sequencing of the D1/D2 Region from 26S rRNA Gene

Yeast isolates were identified by PCR-RFLP analysis of 5.8S-ITS rDNA according to Esteve-Zarzoso et al. (1999), using primers ITS1 and ITS4 (White et al., 1990). PCR products were digested without further purification by the restriction enzymes *Cfo*I, *Hae*III, *Dde*I, *Hinf*I, and *Mbo*I. The PCR products and their restriction fragments were separated by gel electrophoresis on 1.5% and 3% agarose gels, respectively. The sizes of the DNA fragments were

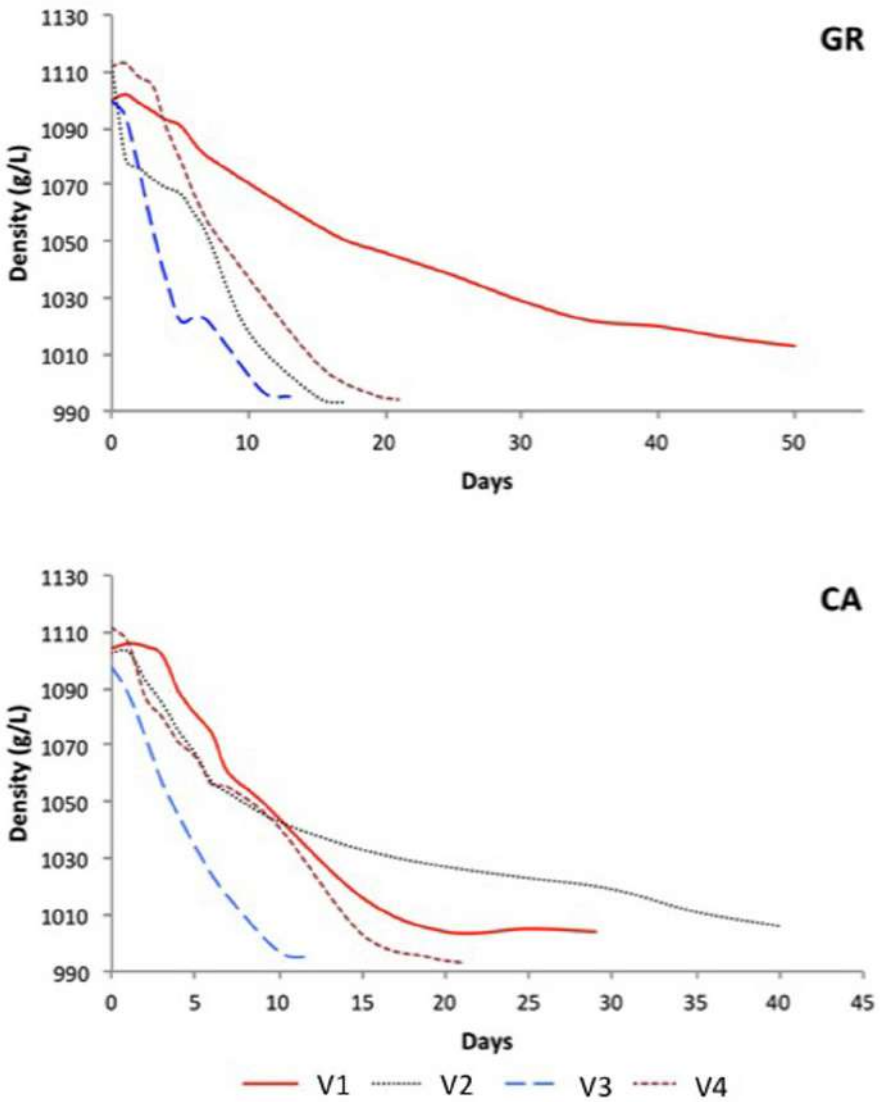

FIGURE 1 | Evolution of the different spontaneous fermentations. GR: Grenache, CA: Carignan.

estimated by comparison against a DNA ladder (100 bp Roche Diagnostics GmBh, Germany). The obtained restriction profiles were compared with previously reported profiles (Esteve-Zarzoso et al., 1999; Sipiczki, 2004; Baffi et al., 2010). One isolate was sent for sequencing of the D1/D2 domains of 26S rRNA was conducted using primers NL1 and NL4 to confirm yeast identification (Kurtzman and Robnett, 1998). The PCR products were purified and sequenced by Macrogen Inc. (Seoul, South Korea) using an ABI3730XL automated capillary DNA sequencer. The sequences were compared with those in GenBank and with those of the Type Strains using the BLASTN tool (NCBI). Identification at species level was achieved with homologies with type strains between 99.2% (*S. cerevisiae*) to 100% (*Hanseniaspora uvarum*). The sequences were deposited in the GeneBank NCBI database with the accession numbers KX272958 (*Aureobasidium pullulans*), KX272959 (*H. uvarum*), KX272960 (*Issatchenkia terricola*), KX272961 (*Lachancea thermotolerans*), KX272962 (*Starmerella bacillaris* synonim *Candida zemplinina*), KX272963 (*S. cerevisiae*), and KX272964 (*Saccharomycodes ludwigii*).

Biodiversity indexes were determined as in McDonald and Dimmick (2003).

Yeast Typing

Isolates from *S. cerevisiae* were genetically characterized by the analysis of inter-delta regions, as described by Legras and Karst (2003) using the primers delta12 and delta21. *H. uvarum* and *C. zemplinina* isolates were typified according to Barquet et al. (2012) with two different combination of primers. Set A included primers 5CAG and TtRNASc while set B was composed of the primers ISSR-MB and TtRNASc. PCR products were

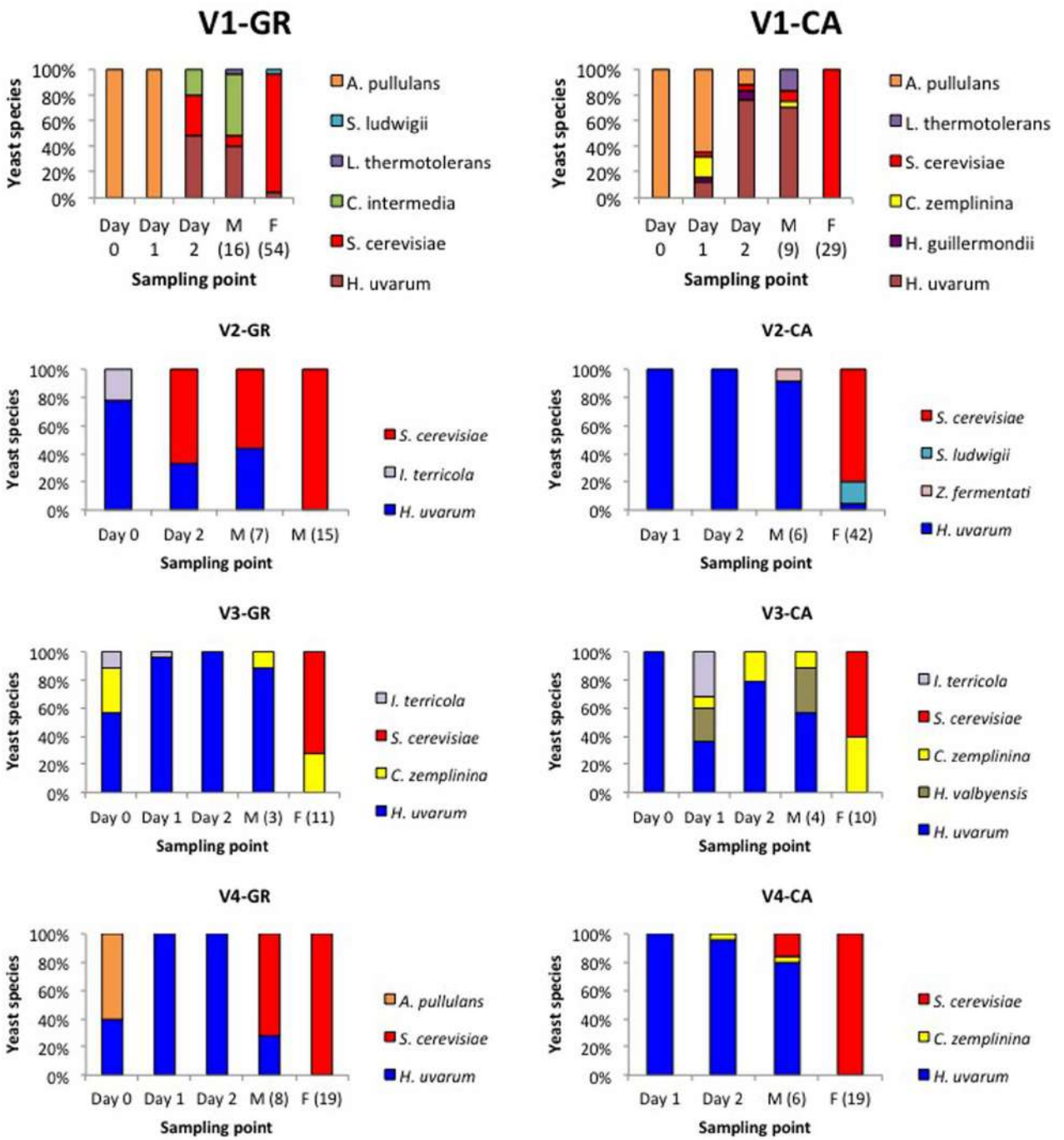

FIGURE 2 | Yeast population dynamics established by RFLP-ITS-PCR of YPD-cultured isolates.

separated by electrophoresis on 2% agarose gels. *H. uvarum* isolates were further characterized by RAPD-PCR using the M13 set of primers (Huey and Hall, 1989). The clustering was performed using the profiles obtained with the three sets of primers. The sizes of the DNA fragments were estimated by comparison against a DNA ladder (100 bp Roche Diagnostics GmBh, Germany).

Chemical Analysis of Final Wines

pH values were determined by a pH meter MicropH2000 (Crison Instruments, Barcelona, Spain). Sugars (glucose and fructose), acetic acid, citric acid, malic acid, tartaric acid, and glycerol were quantified using the Miura one enzymatic autoanalyzer (BioGamma I.S.E. S.r.L., Rome, Italy) with corresponding enzymatic kits (BioSystems S.A., Barcelona, Spain).

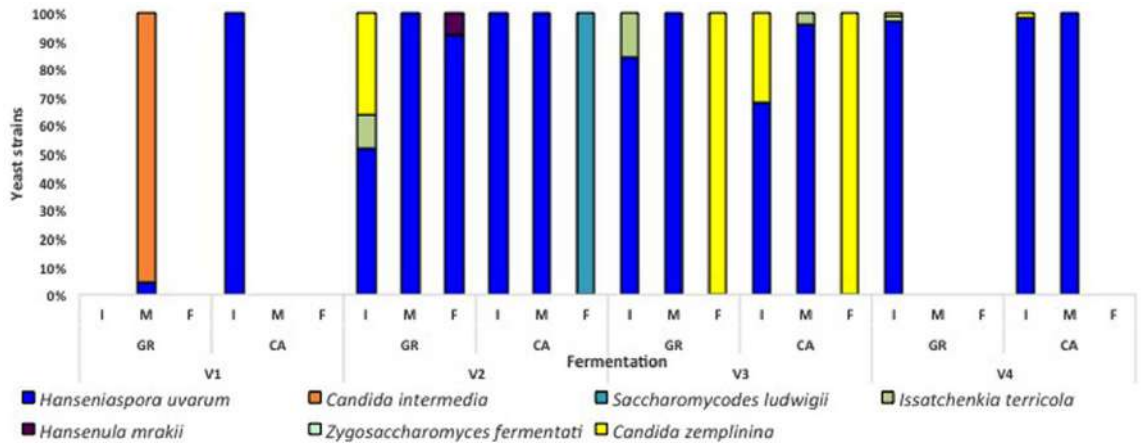

FIGURE 3 | Percentages of yeast isolates in lysine media along the different spontaneous fermentations. *I* (initial), *M* (mid), and *F* (final) refer to the analyzed fermentation stages. Absence of bars means no isolates could be recovered from plates.

TABLE 1 | Biodiversity indexes in the studied vineyards.

	V1-GR	V1-CA	V2-GR	V2-CA	V3-GR	V3-CA	V4-GR	V4-CA
S	6	6	5	4	4	5	5	3
H'	1.41	1.45	1.03	0.93	0.94	1.14	0.84	0.54
D	0.74	0.70	0.57	0.52	0.50	0.59	0.47	0.30

S, Species richness; H', Shannon–Weiner index; and D, Simpson index.

RESULTS

Fermentation Kinetics and Yeast Populations

Fermentation processes measured by must density are represented in **Figure 1**. In all cases, the initial must density was between 1,098 and 1,114 g/L. The fermentation kinetics determined by density monitoring indicated that the eight spontaneous alcoholic fermentations observed progressed differently, as three experiments were complete after 10–15 days (V2-GR, V3-GR, and V3-CA), two after 20 days (V4-GR and V4-CA) and three fermentations were incomplete after 20 days (V1-GR, V1-CA, and V2-CA). Except in V2, a similar trend was observed in the fermentation kinetics of experiments performed with grapes from the same vineyard but of a different variety.

Yeast counts were registered at different sampling points when possible due to the growth of filamentous fungi, which hampered proper yeast visualization and isolation. The initial yeast counts ranged from 10^4 to 10^6 CFU/mL in both growth media. In all cases, typical growth kinetics were observed with high total yeast viability until the end of fermentation, with values of approximately 10^7 CFU/mL. On the other hand, the growth of non-*Saccharomyces* species at this point was only observed in three fermentations, with values ranging from 10^5 to 10^7 CFU/mL. These species were present at the mid-fermentation point in all experiments, with counts between 10^6 and 10^8 CFU/mL.

Yeast Identification and Population Dynamics

A total of 1,401 yeasts were isolated and identified from samples taken during spontaneous alcoholic fermentation. Eleven non-*Saccharomyces* species, as well as *S. cerevisiae*, were found. The most abundant yeast species was *H. uvarum*, followed by *S. cerevisiae*, *C. zemplinina* and *A. pullulans*. Smaller quantities of other species such as *C. intermedia*, *S. ludwigii* and *I. terricola* were isolated.

Figure 2 shows the population dynamics during spontaneous vinifications of yeasts isolated in YPD medium. Obvious differences in species succession exist across the different experiments, influenced by the initial yeast load as well as by the endogenous vineyard microbiota. In the case of V4, only three species were identified in GR and CA, while the V1-GR and V1-CA fermentations were characterized by four common species and two species that were dependent on the grape variety. Between three and five different yeast species were involved in V2 and V3 fermentations.

Globally, the first stages of fermentation (Days 0, 1, and 2) were characterized by the presence of several non-*Saccharomyces* species, particularly *H. uvarum*. In the case of V1 fermentations, *A. pullulans* represented more than 50% of the isolates found at the beginning of the process. *S. cerevisiae* was present during this initial phase in fermentations V1-GR, V1-CA, and V2-GR, while in other experiments this species appeared at the mid (V4-GR and V4-CA) or final points of fermentation (V2-CA, V3-GR, and V3-CA). The clear dominance of *S. cerevisiae*

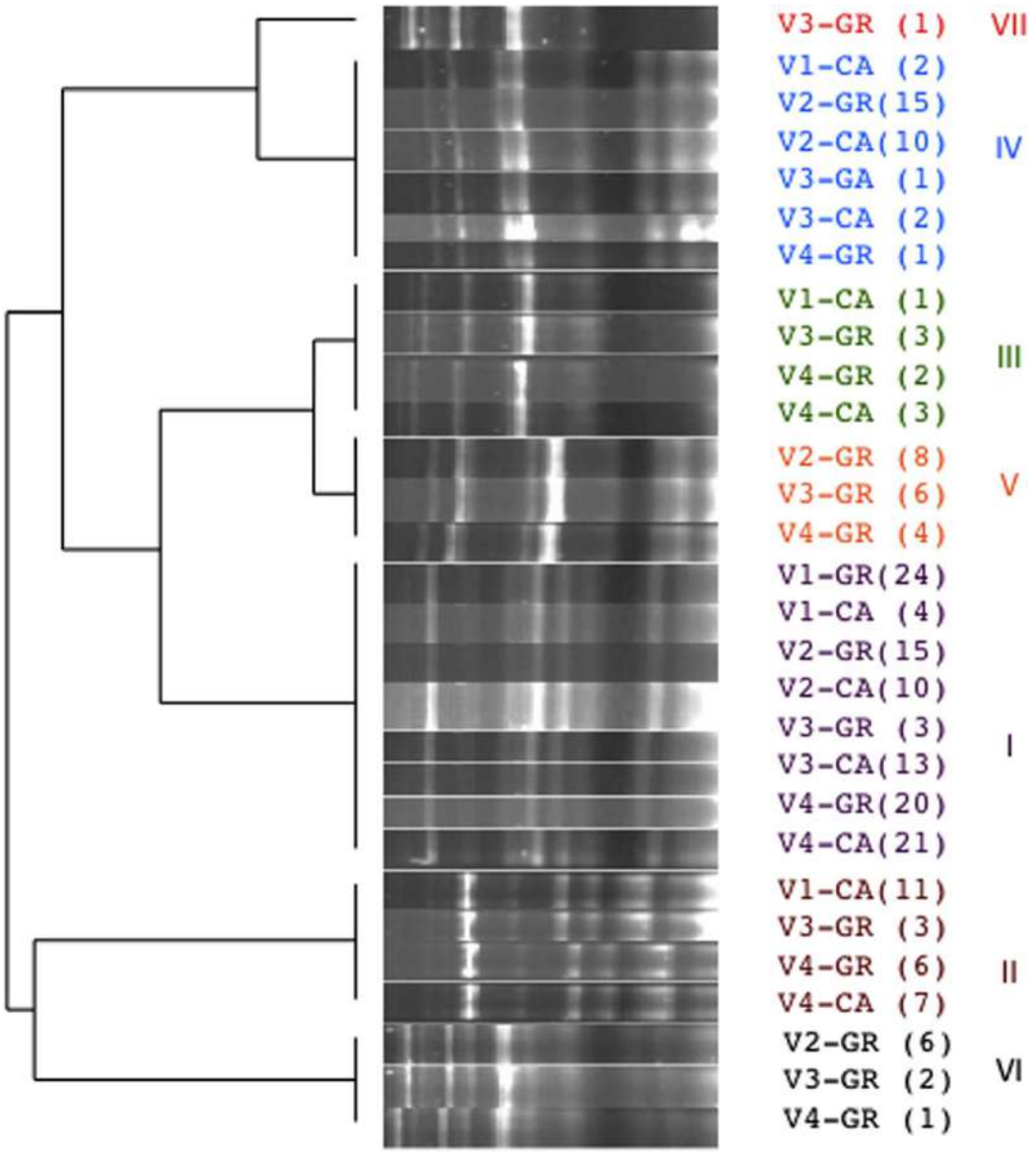

FIGURE 4 | Cluster analysis of the electrophoretic patterns of inter-delta PCR amplification obtained from representative isolates of *Saccharomyces cerevisiae* form each fermentation. In brackets number of isolates with the same profile.

(60–100%) at later sampling points was observed in all fermentations. However, the coexistence of non-*Saccharomyces* species, particularly *H. uvarum*, *C. zemplinina*, and *S. ludwigii,* and the appearance of *S. cerevisiae* at the end of the fermentation process is noticeable in different experiments (V1-GR, V2-CA, V3-GR, and V3-CA). When plated in lysine medium (**Figure 3**), the species *Hansenula mrakii* was also found.

To estimate yeast biodiversity we calculated species biodiversity by species richness and the indexes of Shannon–Weiner and Simpson (**Table 1**). It can be seen that the first vineyard (the only one certified organic) had the highest biodiversity indexes, whereas the last one, the only fully conventional one has the lowest biodiversity.

Yeast Typing

A total of 315 isolates were typified from different species: *S. cerevisiae* (205), *H. uvarum* (98), and *C. zemplinina* (9). Seven electrophoretic patterns were observed in *S. cerevisiae* (**Figure 4**). **Table 2** and **Figure 5** show the distribution of the inter-delta profiles of *S. cerevisiae* isolates from the eight spontaneous fermentations studied. Some fermentations contained only one or two strains (V1-GR, V2-CA, and V3-CA), while others included all strains (V3-GR). In all vinifications, inter-delta profile I was present and was the predominant profile in most vinifications, while III, VI, and VII were isolated in smaller numbers. Profiles I, II, and IV were present in all the fermentative processes studied, while V and VI were not found during the

TABLE 2 | Distribution of the different *Saccharomyces cerevisiae* strains among the fermenting grape musts and fermentation stages.

Profile	V1-GR			V1-CA			V2-GR			V2-CA			V3-GR			V3-CA			V4-GR			V4-CA			Σ
	I	*M*	*F*	*I*	*M*	*F*	*I*	*M*	*F*	*I*	*M*	*F*	*I*	*M*	*F*	*I*	*M*	*F*	*I*	*M*	*F*	*I*	*M*	*F*	
I	5	2	17	1	–	3	–	–	15	–	–	10	–	–	3	–	–	13	–	8	12	–	4	17	110
II	–	–	–	1	2	8	–	–	–	–	–	–	–	–	3	–	–	–	–	2	4	–	–	7	27
III	–	–	–	–	–	1	–	–	–	–	–	–	–	–	3	–	–	–	–	–	2	–	–	3	9
IV	–	–	–	–	–	2	6	3	3	–	–	10	–	–	1	–	–	2	–	–	1	–	–	–	31
V	–	–	–	–	–	–	–	5	3	–	–	–	–	–	6	–	–	–	–	–	4	–	–	–	18
VI	–	–	–	–	–	–	–	6	–	–	–	–	–	–	2	–	–	–	–	–	1	–	–	–	9
VII	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1	–	–	–	–	–	–	–	–	–	1
Σ		24			18			44			20			19			15			34			31		205

I, grape must; M, mid fermentation; F, End of fermentation.

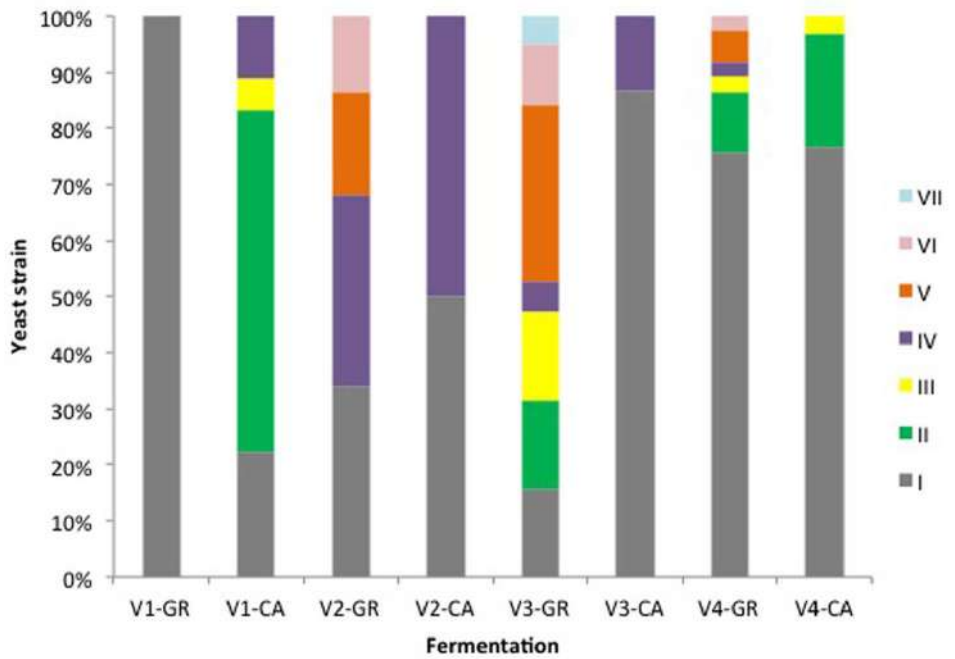

FIGURE 5 | Percentages of *S. cerevisiae* strains in different fermentations.

initial stages and III and VII were only isolated at the final fermentation sampling points.

Hanseniaspora uvarum isolates in V2 fermentations were typified by combining the results obtained from primer sets A and B and M13 RAPD-PCR. As a result of the genetic characterization of *H. uvarum* combining the results of the tipification tests 18 different strains were differentiated (**Figure 6**). Each strain pattern was composed of between one and 46 isolates, and only one strain included isolates from two different sampling points (profile E).

Candida zemplinina isolates from fermentation V2-GR were studied, and six different combinations of profiles were obtained: four isolates corresponded to the same strain pattern, while the other five were classified individually as single strains.

Chemical Analysis of Final Wines

The primary oenological parameters of the eight wines obtained are shown in **Table 3**. All wines contained less than 2 g/L of residual sugars and are thus considered dry. The only exception was V4-GR, which contained 4.46 g/L of residual sugars, which in laboratory scale fermentations is also often considered dry. The final pH values measured were between 2.92 and 3.45, and CA fermentations presented higher values than GR wines. Glycerol values ranged from 8.06 in V2-GR to 12.65 in V3-GR. The acidic contents were measured, and values close to 0.2 g/L were obtained for citric acid, while malic acid ranged from 0.53 to 2.16 g/L, the tartaric acid concentration varied from 0.3 to 2.48 g/L and acetic acid values were determined to be between 0.10 and 1.21 g/L. The quantification of ethanol was not consistent due to different lengths of fermentation and ethanol evaporation due to the small

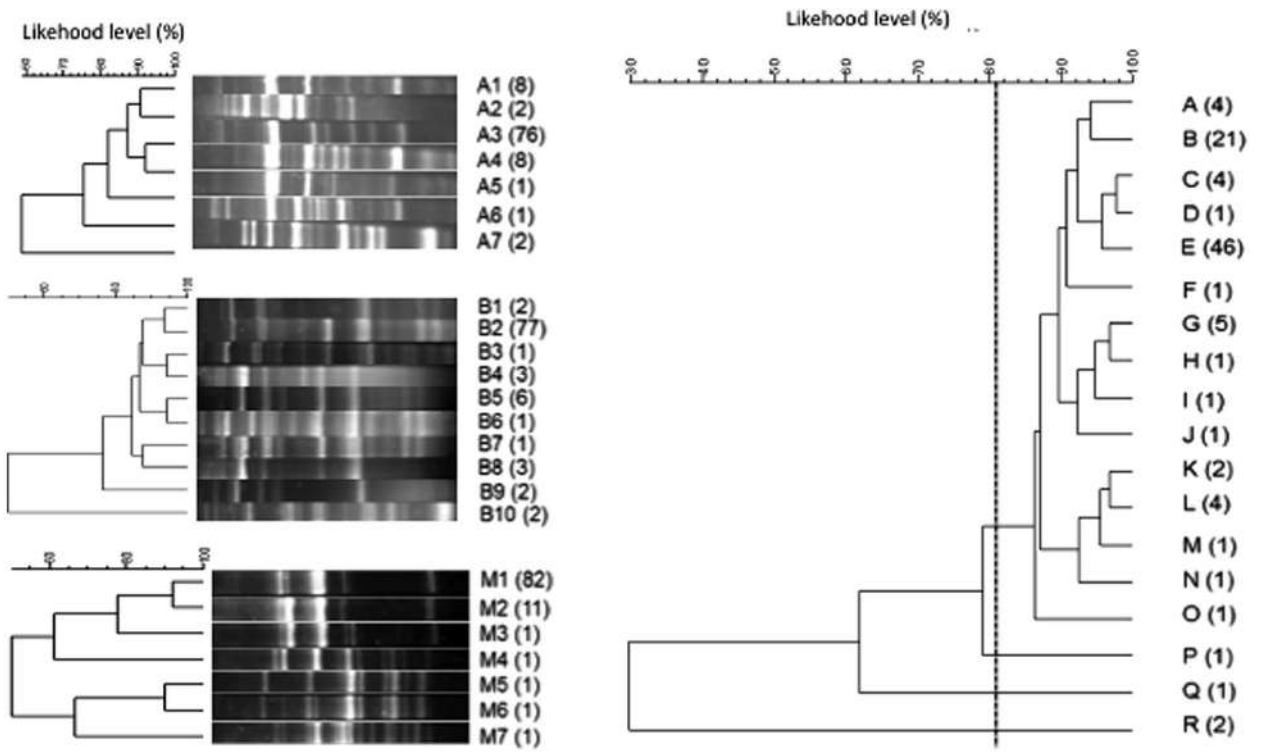

FIGURE 6 | Cluster analysis of the electrophoretic patterns obtained with three different sets of primers (RxA, RxB, and M13) of *Hanseniaspora uvarum* isolates. The cluster obtained with the combination of the three analyses is also represented (right).

TABLE 3 | Analytical parameters of final wines.

	Glucose + Fructose (g/L)	pH	Glycerol (g/L)	Malic acid (g/L)	Citric acid (g/L)	Tartaric acid (g/L)	Acetic acid (g/L)
V1-GR	0.56	2.92	10.09	1.20	0.26	2.48	1.20
V1-CA	0.02	3.40	10.91	2.16	0.34	0.88	0.80
V2-GR	1.12	2.99	8.06	0.67	0.20	1.34	0.77
V2-CA	0.10	2.99	10.99	0.53	0.13	2.41	1.21
V3-GR	0.06	3.22	12.65	0.67	0.12	0.78	0.97
V3-CA	0.07	3.31	11.09	0.87	0.22	0.01	0.71
V4-GR	4.46	2.98	9.90	0.80	0.17	1.50	0.30
V4-CA	0.29	3.45	12.49	0.91	0.13	0.30	0.10

volumes involved. However, considering the low levels of residual sugars, no fermentations were stuck.

DISCUSSION

In this work, the yeast population dynamics of eight different spontaneous vinifications of DOQ Priorat grapes were explored. Regarding fermentation kinetics, three different patterns were observed, as the fermentation lengths required for sugar consumption varied, from approximately 10–20 days and longer for sluggish fermentations.

Yeast isolates were identified by molecular techniques, and 11 non-*Saccharomyces* species as well as *S. cerevisiae* were found in the alcoholic fermentations, indicating that vineyards are an excellent source of yeast biodiversity. Although there are no enough number of vineyards analyzed, our results seems to indicate that organic handling could increase the biodiversity indexes, as observed by other authors (Setati et al., 2015). All yeast species isolated in this study have been previously described in grapes or in wine related environments (Renouf et al., 2005; Barata et al., 2012; Ortiz et al., 2013; Alessandria et al., 2014; Jolly et al., 2014). The main non-*Saccharomyces* yeast species isolated belong to the genera *Hanseniaspora* and *Candida*, which have been commonly associated with grape juice and are gradually replaced by *S. cerevisiae* during alcoholic fermentation (Fleet, 2003; Ocón et al., 2010). In this sense, yeast population dynamics along the eight DOQ Priorat spontaneous fermentations was examined. A typical species succession trend was observed in

all fermentations, although differences among the main non-*Saccharomyces* species were noticeable, such as the presence of *A. pullulans* in some of the experiments. This black yeast-like fungus is a common inhabitant on the surface of healthy grapes, which would explain its presence at the beginning of the fermentation process (Fleet, 2003; Sun et al., 2014). The only previous study performed in Priorat (Torija et al., 2001) studied the yeast population dynamics of GA fermentation over a period of three years, and determined that the main non-*Saccharomyces* species isolated was *C. stellata* (later known as *C. zemplinina* or *S. bacillaris*). An ecological analysis of yeast compositions conducted during six different years on different grape musts from nearby vineyards (although outside the DOQ Priorat) revealed that *H. uvarum* or *C. stellata* dominated the first stages of fermentation, depending on the experiment (Beltran et al., 2002). In the present study, *H. uvarum* was, excepting the cases where *A. pullulans* predominated, the dominant non-*Saccharomyces* species, as has been reported by other authors (Querol et al., 1990; Constanti et al., 1997; Bezerra-Bussoli et al., 2013).

In addition to being abundantly present during the beginning of spontaneous alcoholic fermentations, *H. uvarum* and *C. zemplinina* are considered interesting yeast species both for inclusion in starter cultures that aim to emulate natural fermentation, as well as from an aromatic point of view, as both yeast species are likely to affect the sensory properties of the final wine (Fleet, 2008; Jolly et al., 2014) . However, the production of volatile compounds and other molecules related to oenological parameters has proven to be strain dependent (Romano, 2003; Comitini et al., 2011; Loira et al., 2014), which highlights the relevance of conducting a strain characterization and selection process to obtain a desired outcome. Ecological studies generate large microbial collections that need to be genetically characterized to differentiate strains, simplify phenotypical characterization and provide a better conception of the winemaking process.

In the present study, *S. cerevisiae* isolates were typified by delta-elements PCR resulting seven different electrophoretic profiles from eight spontaneous fermentations. More than one strain was found in each experiment, indicating the coexistence of several strains during the vinification process, as has been specifically indicated in the same area (Torija et al., 2001) or widely reported (Fleet, 1993; Tofalo et al., 2013). These data support the idea of designing starter cultures that include more than one native strain of *S. cerevisiae* to mimic spontaneous fermentations. In fact, the practical application of this study has been the development of mixed inoculum containing the three main strains of *S. cerevisiae* observed in the present study (strains I, II, and IV). Additionally, some strains were exclusively found in one grape variety, even when harvested in different vineyards, which highlights the relationship between microbial diversity and varietal character. The absence of *S. cerevisiae* at the beginning of the grape must fermentation is well-known in culture-dependent studies, due to its near absence in grapes (Fleet, 1993); although in some cases it has been found when the sanitary status of the grapes was unusual (Beltran et al., 2002). However, its capacity to lead the fermentation process and interact with other non-*Saccharomyces* species leads to the recovery of only *S. cerevisiae* at the end of fermentation (Fleet, 1993).

The two main non-*Saccharomyces* species found, *H. uvarum* and *C. zemplinina* isolates from V2 spontaneous fermentations, were also typified. Both species included abundant strain patterns, although one main profile was found, and all *H. uvarum* strains were grape variety dependent. The biodiversity found among non-*Saccharomyces* isolates was much greater when compared with *S. cerevisiae*, as only four *S. cerevisiae* strains were found in V2 fermentations. The combination of different typing methods can result in very different results. In fact, using only one of the methods the profile diversity could be much lower than that from the combination of several methods. Some authors that applied combined analysis in *Saccharomyces*, already observed this fact (Fernández-Espinar et al., 2001; Schuller et al., 2004). However, the methods for Non-*Saccharomyces* analysis are still far from being standardized and thus, comparative studies have been recently reported (Masneuf-Pomarede et al., 2015; Albertin et al., 2016). In our hands, the combination of the three analyses has provided much higher polymorphism increasing from 7 or 10 different profiles to 18 profiles after the combination of different methods. Thus, we consider that so far the use of a single method for typing non-*Saccharomyces* is not conclusive enough.

In addition to the different molecular typing methods used, this difference may be due to the high populations of non-*Saccharomyces* species found at the beginning of alcoholic fermentation. Most ecological studies based on the microbial description of spontaneous fermentation are focused on the analysis of *S. cerevisiae* populations; therefore, the genetic typing of non-*Saccharomyces* isolates is often unexplored. One exception is a study published by Capece et al. (2011), based on the characterization, in wines, of non-*Saccharomyces* SO_2 tolerant yeasts by RAPD fingerprinting, with the aim of constructing a collection of wild strains capable of maintaining the specific sensory characteristics of Inzolia wine.

CONCLUSION

This study provides a testimony for the remarkable yeast species and strain heterogeneity associated with alcoholic fermentations carried out by the wild yeasts naturally present in four different DOQ Priorat vineyards and in two different red grape varieties: GA and CA. This yeast community is likely to leave a footprint in the final wines, which will be part of the distinctive characteristic of the wines of a given region. The defense of a given area typicality often leads to the use of spontaneous fermentations which may produce uncontrolled fermentations with unwanted and deleterious effects. A multi-strain and multi-species starter with selected yeast of the available and more characteristic

strains and species from a given region can provide the typicality of that region without the inconvenience of the uncontrolled fermentation. Thus, the description of this microbial diversity can be the first step of the selection of a consortium of native yeast microbiota emulating spontaneous fermentation that could be used for the production of wines exhibiting the Priorat footprint.

AUTHOR CONTRIBUTIONS

BP: Design experiments, perform experiments, analyze results, result discussion, and writing the manuscript. DG-F, BG, and IP: Perform experiments and analyze results. BE-Z and GB: design experiments, analyze results, and result discussion. AM: design experiments, analyze results, result discussion, writing the manuscript, and rise funding.

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REFERENCES

Albertin, W., Setati, M. E., Miot-Sertier, C., Mostert, T. T., Colonna-Ceccaldi, B., Coulon, J., et al. (2016). *Hanseniaspora uvarum* from winemaking environments show spatial and temporal genetic clustering. *Front. Microbiol.* 6:1569. doi: 10.3389/fmicb.2015.01569

Alessandria, V., Marengo, F., Englezos, V., Gerbi, V., Rantsiou, K., and Cocolin, L. (2014). Mycobiota of barbera grapes from the piedmont region from a single vintage year. *Am. J. Enol. Viticult.* 66, 244–250. doi: 10.5344/ajev.2014.14071

Baffi, M. A., dos Santos, C., Arévalo-Villena, M., Briones-Pérez, A. I., Gomes, E., and Da Silva, R. (2010). Isolation and molecular identification of wine yeasts from a brazilian vineyard. *Ann. Microbiol.* 61, 75–78. doi: 10.1007/s13213-010-0099-z

Barata, A., Malfeito-Ferreira, M., and Loureiro, V. (2012). The microbial ecology of wine grape berries. *Int. J. Food Microbiol.* 153, 243–259. doi: 10.1016/j.ijfoodmicro.2011.11.025

Barquet, M., Martín, V., Medina, K., Pérez, G., Carrau, F., and Gaggero, C. (2012). Tandem repeat-tRNA (TRtRNA) PCR method for the molecular typing of non-*Saccharomyces* subspecies. *Appl. Microbiol. Biotechnol.* 93, 807–814. doi: 10.1007/s00253-011-3714-4

Beltran, G., Torija, M. J., Novo, M., Ferrer, N., Poblet, M., Guillamón, J. M., et al. (2002). Analysis of yeast populations during alcoholic fermentation: a six year follow-up study. *Syst. Appl. Microbiol.* 293, 287–293. doi: 10.1078/0723-2020-00097

Bezerra-Bussoli, C., Alves Baffi, M., Gomes, E., and Da-Silva, R. (2013). Yeast diversity isolated from grape musts during spontaneous fermentation from a Brazilian winery. *Curr. Microbiol.* 67, 356–361. doi: 10.1007/s00284-013-0375-9

Bokulich, N. A., Thorngate, J. H., Richardson, P. M., and Mills, D. A. (2014). Microbial biogeography of wine grapes is conditioned by cultivar, vintage, and climate. *Proc. Natl. Acad. Sci. U.S.A.* 111, 139–148. doi: 10.1073/pnas.1317377110

Capece, A., Pietrafesa, A., and Romano, P. (2011). Experimental approach for target selection of wild wine yeasts from spontaneous fermentation of 'inzolia' grapes. *World J. Microb. Biot.* 27, 2775–2783. doi: 10.1007/s11274-011-0753-z

Carrascosa, A. V., Bartolome, B., Robredo, S., Leon, A., Cebollero, E., Juega, M., et al. (2012). Influence of locally-selected yeast on the chemical and sensorial properties of Albariño white wines. *LWT Food Sci. Technol.* 46, 319–325. doi: 10.1016/j.lwt.2011.09.011

Combina, M., Elía, A., Mercado, L., Catania, C., Ganga, A., and Martinez, C. (2005). Dynamics of indigenous yeast populations during spontaneous fermentation of wines from Mendoza, Argentina. *Int. J. Food Microbiol.* 99, 237–243. doi: 10.1016/j.ijfoodmicro.2004.08.017

Comitini, F., Gobbi, M., Domizio, P., Romani, C., Lencioni, L., Mannazzu, I., et al. (2011). Selected Non-*Saccharomyces* wine yeasts in controlled multistarter fermentations with *Saccharomyces cerevisiae*. *Food Microbiol.* 28, 873–882. doi: 10.1016/j.fm.2010.12.001

Constanti, M., Poblet, M., Arola, L., Mas, J., and Guillamón, J. M. (1997). Analysis of yeast populations during alcoholic fermentation in a newly established winery. *Am. J. Enol. Viticult.* 48, 339–344.

Díaz, C., Molina, A. M., Nähring, J., and Fischer, R. (2013). Characterization and dynamic behavior of wild yeast during spontaneous wine fermentation in steel tanks and amphorae. *Biomed Res. Int.* 2013, 540465. doi: 10.1155/2013/540465

Esteve-Zarzoso, B., Belloch, C., Uruburu, F., and Querol, A. (1999). Identification of yeasts by RFLP analysis of the 5.8S rRNA gene and the two ribosomal internal transcribed spacers. *Int. J. Syst. Bacteriol.* 49, 329–337. doi: 10.1099/00207713-49-1-329

Fernández-Espinar, M. T., Lopez, V., Ramon, D., Bartra, E., and Querol, A. (2001). Study of the authenticity of commercial wine yeast strains by molecular techniques. *Int. J. Food Microbiol.* 70, 1–10. doi: 10.1016/S0168-1605(01)00502-5

Fleet, G. H. (1993). "The microorganisms of winemaking-isolation enumeration and identification," in *Wine Microbiology and Biotechnology*, ed. G. H. Fleet (Chur: Harwood Academic Publishers), 1–27.

Fleet, G. H. (2003). Yeast interactions and wine flavour. *Int. J. Food Microbiol.* 86, 11–22. doi: 10.1016/S0168-1605(03)00245-9

Fleet, G. H. (2008). Wine yeasts for the future. *FEMS Yeast Res.* 8, 979–995. doi: 10.1111/j.1567-1364.2008.00427.x

Gonzalez, R., Quirós, M., and Morales, P. (2013). Yeast respiration of sugars by non-*Saccharomyces* yeast species: a promising and barely explored approach to lowering alcohol content of wines. *Trends Food Sci. Technol.* 29, 55–61. doi: 10.1016/j.tifs.2012.06.015

Harvey, M., White, L., and Frost, W. (2014). *Wine and Identity: Branding, Heritage, Terroir*. Routledge: University of Canterbury. doi: 10.4324/9780203067604

Huey, B., and Hall, J. (1989). Hypervariable DNA fingerprinting in *Escherichia coli*. Minisatellite probe from bacteriophage M13. *J. Bacteriol.* 171, 2528–2532.

Jolly, N. P., Varela, C., and Pretorius, I. S. (2014). Not your ordinary yeast: non-*Saccharomyces* yeasts in wine production uncovered. *FEMS Yeast Res.* 14, 215–237. doi: 10.1111/1567-1364.12111

Kurtzman, C. P., and Robnett, C. J. (1998). Identification and phylogeny of ascomycetous yeasts from analysis of nuclear large subunit (26S) ribosomal DNA partial sequences. *Antonie Van Leeuwenhoek* 73, 331–371. doi: 10.1023/A:1001761008817

Legras, J. L., and Karst, F. (2003). Optimisation of interdelta analysis for *Saccharomyces cerevisiae* strain characterisation. *FEMS Microbiol. Lett.* 221, 249–255. doi: 10.1016/S0378-1097(03)00205-2

Loira, I., Vejarano, R., Bañuelos, M. A., Morata, A., Tesfaye, W., Uthurry, C., et al. (2014). Influence of sequential fermentation with *Torulaspora delbrueckii* and *Saccharomyces cerevisiae* on wine quality. *LWT Food Sci. Technol.* 59, 915–922. doi: 10.1016/j.lwt.2014.06.019

Masneuf-Pomarede, I., Juquin, E., Miot-Sertier, C., Renault, P., Laizet, Y., Salin, F., et al. (2015). The yeast *Candida zemplinina* (Starmerella bacillaris) shows high

genetic diversity in winemaking environments. *FEMS Yeast Res.* 15:fov045. doi: 10.1093/femsyr/fov045

McDonald, D., and Dimmick, J. (2003). Diversity: conceptualization and measurement. *Commun. Res.* 30, 60–79. doi: 10.1177/0093650202239026

Mortimer, R., and Polsinelli, M. (1999). On the origins of wine yeast. *Res. Microbiol.* 150, 199–204. doi: 10.1016/S0923-2508(99)80036-9

Ocón, E., Gutiérrez, A. R., Garijo, P., Tenorio, C., López, I., López, R., et al. (2010). Quantitative and qualitative analysis of non-*Saccharomyces* yeasts in spontaneous alcoholic fermentations. *Eur. Food Res. Technol.* 230, 885–891. doi: 10.1007/s00217-010-1233-7

Ortiz, M. J., Barrajón, N., Baffi, M. A., Arévalo-Villena, M., and Briones, A. (2013). Spontaneous must fermentation: identification and biotechnological properties of wine yeasts. *LWT Food Sci. Technol.* 50, 371–377. doi: 10.1016/j.lwt.2012.09.019

Querol, A., Jiménez, M., and Huerta, T. (1990). Microbiological and enological parameters during fermentation of musts from poor and normal grape-harvests in the region of Alicante. *J. Food Sci.* 55, 1603–1606. doi: 10.1111/j.1365-2621.1990.tb03580.x

Renouf, V., Claisse, O., and Lonvaud-Funel, A. (2005). Understanding the Microbial Ecosystem on the Grape Berry Surface through Numeration and Identification. *Aust. J. Grape Wine Res.* 11, 316–327. doi: 10.1111/j.1755-0238.2005.tb00031.x

Romano, P. (2003). Function of yeast species and strains in wine flavour. *Int. J. Food Microbiol.* 86, 169–180. doi: 10.1016/S0168-1605(03)00290-3

Scacco, A., Oliva, D., Di Maio, S., Polizzotto, G., Genna, G., Tripodi, G., et al. (2012). Indigenous *Saccharomyces cerevisiae* strains and their influence on the quality of Cataratto, Inzolia and Grillo white wines. *Food Res. Int.* 46, 1–9. doi: 10.1016/j.foodres.2011.10.038

Schuller, D., Valero, E., Dequin, S., and Casal, M. (2004). Survey of molecular methods for the typing of wine yeast strains. *FEMS Microbol. Lett.* 231, 19–26. doi: 10.1016/S0378-1097(03)00928-5

Setati, M. E., Jacobson, D., and Bauer, F. F. (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

Sipiczki, M. (2004). Species identification and comparative molecular and physiological analysis of *Candida zemplinina* and *Candida stellata*. *J. Basic Microbiol.* 44, 471–479. doi: 10.1002/jobm.200410449

Sun, Y., Guo, J., Liu, F., and Liu, Y. (2014). Identification of indigenous yeast flora isolated from the five winegrape varieties harvested in Xiangning, China. *Antonie Van Leeuwenhoek* 105, 533–540. doi: 10.1007/s10482-013-0105-0

Tofalo, R., Perpetuini, G., Schirone, M., Fasoli, G., Aguzzi, I., Corsetti, A., et al. (2013). Biogeographical characterization of *Saccharomyces cerevisiae* wine yeast by molecular methods. *Front. Microbiol.* 4:166. doi: 10.3389/fmicb.2013.00166

Torija, M. J., Rozés, N., Poblet, M., Guillamón, J. M., and Mas, A. (2001). Yeast population dynamics in spontaneous fermentations: comparison between two different wine-producing areas over a period of three years. *Antonie Van Leeuvenhock* 79, 345–352. doi: 10.1023/A:1012027718701

Van Leeuwen, C., and Seguin, G. (2006). The concept of terroir in viticulture. *J. Wine Res.* 17, 1–10. doi: 10.1080/09571260600633135

White, T. J., Bruns, S., Lee, S., and Taylor, J. (1990). "Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics," in *PCR Protocols: A Guide to Methods and Applications*, eds M. Innis, D. Gelfand, J. Sninsky, and T. White (Orlando, FL: Academic Press), 315–322.

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Potential Role of Yeast Strains Isolated from Grapes in the Production of Taurasi DOCG

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Twelve samples of Aglianico grapes, collected in different locations of the Taurasi DOCG (Appellation of Controlled and Guaranteed Origin) production area were naturally fermented in sterile containers at room temperature. A total of 70 yeast cultures were isolated from countable WL agar plates: 52 in the middle of the fermentation and 18 at the end. On the basis of ITS-RFLP analysis and ITS sequencing, all cultures collected at the end of fermentations were identified as *Saccharomyces (S.) cerevisiae*; while, the 52 isolates, collected after 1 week, could be referred to the following species: *Metschnikowia* (*M.*) *pulcherrima*; *Starmerella* (*Star.*) *bacillaris*; *Pichia* (*P.*) *kudriavzevii*; *Lachancea* (*L.*) *thermotolerans*; *Hanseniaspora (H.) uvarum*; *Pseudozyma (Pseud.) aphidis*; *S. cerevisiae*. By means of Interdelta analysis, 18 different biotypes of *S. cerevisiae* were retrieved. All strains were characterized for ethanol production, SO_2 resistance, H_2S development, β-glucosidasic, esterasic and antagonistic activities. Fermentation abilities of selected strains were evaluated in micro-fermentations on Aglianico must. Within non-*Saccharomyces* species, some cultures showed features of technological interest. Antagonistic activity was expressed by some strains of *M. pulcherrima*, *L. thermotolerans, P. kudriavzevii*, and *S. cerevisiae.* Strains of *M. pulcherrima* showed the highest β-glucosidase activity and proved to be able to produce high concentrations of succinic acid. *L. thermotolerans* produced both succinic and lactic acids. The lowest amount of acetic acid was produced by *M. pulcherrima* and *L. thermotolerans;* while the highest content was recorded for *H. uvarum.* The strain of *Star. bacillaris* produced the highest amount of glycerol and was able to metabolize all fructose and malic acid. Strains of *M. pulcherrima* and *H. uvarum* showed a low fermentation power (about 4%), while, *L. thermotolerans, Star. Bacillaris*, and *P. kudriavzevii* of about 10%. Significant differences were even detected for *S. cerevisiae* biotypes with respect to H_2S production, antagonistic activity and β-glucosidase activity as well as for the production of acetic acid, glycerol and ethanol in micro-vinification experiments.

Keywords: grapes, yeast microflora, Aglianico, identification, biotyping, wine fermentation

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INTRODUCTION

Wine composition and quality are affected by several intrinsic and extrinsic variables, many of which are microbiologically mediated. Spontaneous alcoholic fermentation of grape must is a complex process owing to metabolic activities of different groups of microorganisms including filamentous fungi (i.e., *Botrytis* spp.), yeasts, and bacteria (lactic and acetic acid bacteria) originating from grapes, soil, and cellar equipment (Mills et al., 2008). The physiological properties of these complex microbial consortia lead to the formation of metabolites and to the transformation of grape molecules, thus influencing the sensorial properties (color, aroma, flavor, structure, and body) of the final product (Pretorius, 2000; Fleet, 2003). Due to the sequential action of different yeast species/strains, naturally present on the berries grapes or in the winery, the outcome of spontaneous alcoholic fermentation is difficult to predict and therefore, results are often unreproducible (Pretorius, 2000). To address this issue, many winemakers use pure yeast cultures (starters) of *S. cerevisiae* or *S. bayanus* species, which are inoculated into the must after pressing. The use of starter cultures allows a more rapid and complete grape must fermentation and a higher degree of reproducibility in the atmosphere of specific wines can be achieved (Pretorius, 2000; Fleet, 2008; Suarez-Lepe and Morata, 2012). However, there is some controversy about the use of commercial wine yeasts due to the lack of some desirable traits provided by natural or spontaneous fermentation (Pretorius, 2000). Moreover, the continuous use of a limited number of strains as commercial starter cultures by wine industry is causing the erosion of the microbial diversity. The study and the preservation of the wine yeasts biodiversity have recently become matter of growing interest (Di Maio et al., 2012). The maintenance of the biological patrimony is essential to obtain starter strains able to fully develop the typical sensory profile of wines originating from different grapevine cultivars, as well as to preserve a gene pool of paramount importance for any yeast-mediated process (Pretorius, 2000; Marinangeli et al., 2004). Such criticism is providing new challenges to enhance the appeal and value of wine produced by this fermentation technology. As reviewed by Fleet (2008), this may be achieved by selecting novel yeast starter cultures from natural wine environment and by leading the fermentations with mixtures of yeast species (including *Saccharomyces* and non-*Saccharomyces*) and strains, for flavor modulation, volatile acidity decreasing, malic and lactic acids production or degradation.

The present survey was focused on Taurasi DOCG (Appellation of Controlled and Guaranteed Origin), a wine produced within a small area of the Campania Region (Irpinia district) by a starter-led fermentation technology. Taurasi DOCG, as reported in the production specifications (Ministerial Decree 11 March 1993; G.U. n. 72 of 27 March 1993), is a red wine manufactured by *Vitis vinifera* cv. *Aglianico* (at least 85%) exclusively cultured in 17 municipalities (Taurasi, Bonito, Castelfranci, Castelvetere sul Calore, Fontanarosa, Lapio, Luogosano, Mirabella Eclano, Montefalcione, Montemarano, Montemileto, Paternopoli, Pietradefusi, Sant'Angelo all'Esca, San Mango sul Calore, Torre le Nocelle e Venticano) of the Avellino province. To explore the natural yeast diversity, grapes from 12 different vineyards were analyzed. Molecular methods were applied for isolates identification as well as for strains biotypization within the *S. cerevisiae* species. The potential winemaking role of isolated yeast strains was assessed by evaluation of oenological traits and of the behavior in micro-fermentation trials in Aglianico must.

MATERIALS AND METHODS

Sampling and Yeast Isolation

Samples of Aglianico grapes were collected (end of October 2012) in 12 vineyards located in the municipalities where this variety is cultivated (**Table 1**). The origin and °Babo of grape samples are reported in **Table 1**. Samples (about 20 bunches) were collected by using sterile gloves along the two diagonals of the vineyard, placed in sterile plastic bags and transferred in the laboratory within few hours. Grapes were manually pressed in the collection bag, and, after the addition of potassium metabisulfite (100 mg/kg), were incubated at room temperature (18–22°C). During incubation the sugar content (°Babo) was monitored and, after 9 days of fermentation, must samples were analyzed. After sampling, partially fermented musts were combined into one sterile container and left to ferment until complete sugars consumption (mix-wine).

Must samples and mix-wine were serially diluted in quarter strength Ringer's solution (Oxoid, Basingstoke, UK) and spread-plated on WL-nutrient agar (Oxoid). After incubation at 28°C for 5 days, countable plates (15–150 colonies/plate) were used for viable counts and yeasts isolation. Colonies showing different morphology and/or color were all selected, independently by their number. In mix-wine sample, all colonies (n°18) grown in one of the two countable plates were considered. Cultures were purified by repetitive streaking on WL-nutrient agar.

Yeast cultures were preserved on WL-nutrient agar slants, stored at 4°C and sub-cultured every 3 months. Before each test, strains were cultured twice in YPD (yeast extract 10 g/l, peptone 20 g/l, dextrose 20 g/l).

Yeast Strains Molecular Identification and Typing

DNA was isolated as previously reported by Aponte and Blaiotta (2016). Preliminary molecular identification of yeast strains was achieved by ITS (ITS1-5.8S-ITS2)-rDNA RFLP (Esteve-Zarzoso et al., 1999; Csoma and Sipiczki, 2008) analysis using restriction endonucleases *Hae* III, *Hinf* I, and *Cfo* I. In addition, enzymes *Dde* I and *Mbo* I were used for the characterization of *Hanseniaspora* and *Candida* spp, respectively. The identification of non-*Saccharomyces* cultures was obtained by ITS-rDNA sequencing. Genetic diversity within *Saccharomyces* isolates was assessed by Interdelta analysis (Legras and Karst, 2003).

Yeast Strains Technological Characterization

Ethanol tolerance was evaluated in YPD broth containing ethanol concentrations ranging from 4 to 15% (v/v).

TABLE 1 | Origin of grape samples and basic physico-chemical characteristics of the musts.

Grape Sample	Origin (Municipality)		Must characteristics		
			°Babo	pH	Total acidity[a]
1		Mirabella Eclano	20.9	3.18	9.57
2		Pietradefusi	20.8	3.41	9.26
3		Castelfranci	19.4	3.26	9.65
4		Montemarano	20.7	3.18	9.58
5		Lapio	20.3	3.22	9.05
6		Montemileto	21.6	3.21	8.78
7		Castelvetere sul Calore	20.6	3.35	8.56
8		Paternopoli	21.6	3,32	9.36
9		San Mango sul Calore	19.8	3.08	11.21
10		Luogosano	19.0	3.11	10.20
11		Taurasi	20.8	3.34	8.79
12		Fontanarosa	19.8	3.21	10.26

Location of Taurasi production area and of vineyards where grape sampling was carried out is reported on the map (collection sites are indicated by numbers).
[a]*g/l of tartaric acid (25 ml of wine sample and 0.25 N NaOH).*

After incubation at 20°C for 72 h, growth was assessed by spectrophotometry at white light (600 nm). Sulfur dioxide (SO_2) tolerance was evaluated in YPD broth adjusted at pH 3.30 with tartaric and malic acids (1:1) and containing potassium metabisulfite concentrations ranging from 50 to 200 mg/l. Growth was evaluated, after incubation at 20°C for 72 h, by spectrophotometry at white light (600 nm). Hydrogen sulfide (H_2S) production was estimated on Biggy agar (Oxoid) after incubation at 28°C for 48 h. For browning description, the following codes were used: low production, snow-white color; medium production, hazelnut-brown color; high production, rust-coffee color (Aponte and Blaiotta, 2016). Type of growth was estimated in tyndallized (100°C × 5 min × 3 times) must (21°Brix, pH 3.50) after 4 days at 25°C. Antagonistic activity was assessed as described by Sangorrin et al. (2001) using *S. cerevisiae* CECT 1890 as sensitive strain.

β-glucosidase activities were evaluated on media containing cellobiose (CELL), 4-methylumbelliferyl-b-D-glucopyranoside (MUG), arbutin (ARB), esculin (ESC), or p-nitrophenyl β-D-glucopyranoside (pNPG) (Fluka, Milan, Italy), according to the method proposed by Fia et al. (2005) and Hernandez et al. (2002). Esterase activity was evaluated on a medium containing Tween 80 as described by Slifkin (2000).

In order to estimate the percentage of similarity among isolates, data were subject to cluster analysis (Average Linkage Method). A correlation matrix was constructed using the formula described by Upholt (1977) and Nei and Li (1979): $F_{xy} = (2n_{xy})/(n_x+n_y)$ where F_{xy} is the proportion of common molecular markers of molecular biotypes compared (x and y), n_{xy} is the number characters shared by both isolates x and y and n_x and n_y are the total of number characters of observed in isolates x and y, respectively [in our case $(n_x + n_y) = (10 + 10) = 20$]. The resulting correlation matrix was analyzed by Systat 5.2.1 software.

Fermentation Performances of Selected Yeast Strains

Fermentation vigor (FV) and fermentation power (FP) were evaluated in micro-fermentation trials in Aglianico must (°Brix 24, pH 3.09; total acidity 9.98 g/l of tartaric acid). Strains, cultured twice in YPD medium, were used to inoculate (about 6 Log CFU/ml) 100 ml of tyndallized (100°C for 3 min for 3 times) must in 250 ml Erlenmeyer flasks closed with a Müller valve filled with sulfuric acid. During incubation (3 days at 23°C), flasks were handle stirred for 30 s every 12 h. Weight loss, due to CO_2 escaping from the system, was quantified to monitor the fermentation kinetics. Fermentation was considered concluded when no weight loss was any longer recorded within 24 h. FV was expressed as grams of CO_2 produced in 100 ml of must during the first 72 h of fermentation, while FP was expressed as grams of CO_2 produced until the end of fermentation. Each trial was performed in triplicate. At the end of micro-fermentations, concentrations of citric, tartaric, malic, lactic, and succinic acids and of glucose, fructose, glycerol, 2,3-butanediol and ethanol were determined by HPLC analyses as previously described by Aponte and Blaiotta (2016).

RESULTS

The aims of the present study were the yeast microbiota exploration of Aglianico grapes, grown in the Taurasi DOCG area and the evaluation of potential technological contribute of autochthonous yeast strains in winemaking. Grapes were sampled in 12 different vineyards located in area of production of this typical wine (**Table 1**); physico-chemical characteristics of relative musts are reported in **Table 1**. Musts showed a high sugar content (20.4 ± 0.8 °Babo as average value) and were characterized by low pH (3.2 ± 0.1) and high total acidity (9.5 ± 0.8 g/l). After 9 days of fermentation at room temperature, musts

showed highly different residual sugar contents (°Babo from 7 to 13) and alcoholic degrees (Malligand ebullioscope degree from 2 to 8% vol/vol) (**Table 2**). In fact, 9 musts out of 12 still contained more than a half of the initial sugar content. Viable yeast counts ranged from 6.4 to 8.2 Log CFU/ml (**Table 2**).

The mix-wine was obtained by joining partially fermented musts whose fermentation was allowed to proceed for further 30 days, namely until the sugar content did not change for 48 h. The mix-wine reached an alcoholic degree of 12.8% vol/vol (**Table 2**) and still contained a high concentration of residual sugars (10.7 g/l of glucose and 11.2 g/l of fructose) and acetic acid (3.2 g/L) as determined by HPLC analysis. Yeast loads were still high as well (around 5 Log CFU/ml). Fifty-two yeast cultures were isolated from partially fermented musts, on the basis of colony morphology and color on counting plates, and purified (**Table 2**). For mix-wine, all colonies (n°18) present in one countable plate seeded with the highest dilution (10^{-4}) were isolated (**Table 2**). According to ITS-RFLP analysis, yeast cultures could be clustered in seven groups (Supplementary Table S1). Forty-nine isolates were identified as *S. cerevisiae* on the basis of their ITS-RFLP patterns (Supplementary Table S1). Non-*Saccharomyces* entities were all subjected to ITS sequence analysis to confirm presumptive identification obtained according to ITS-RFLP (Supplementary Table S1). Yeast species isolated in each sample are summarized in **Table 2**. Since all types of colonies were selected, even those showing slight differences on WL agar, a medium supposed to be highly differential (Pallmann et al., 2001); and since colonies were all picked by plates seeded with the highest dilutions, species recorded could be confidently considered as components of the dominant cultivable microbiota in that environment. Specifically, in must samples characterized by an alcoholic degree higher than 5% (musts 1, 8, 10, 11, and 12), only *S. cerevisiae* or *S. cerevisiae* and *H. uvarum* (must 8) were isolated. In other samples, *S. cerevisiae* was co-isolated with at least further two yeast species (musts 2, 3, 4, and 7) or was not detected (musts 5, 6, and 9). In the latter case, yeast microbiota of musts appeared to be characterized by a mix yeast population (*P. kudriavzevii*, *L. thermotolerans,* and *H. uvarum* or *M. pulcherrima* and *L. thermotolerans*) or by a single species (*M. pulcherrima*) (**Table 2**).

As expected, in mix wine, with an alcoholic degree of 12.8%, only isolates referable to *S. cerevisiae* species were retrieved. The 49 *S. cerevisiae* isolates (38 from must samples and 18 from mix wine) were typed by Interdelta analysis to evaluate their genetic diversities and to determine their clonal relationships. Supplementary Figure S1 shows patterns displayed by *S. cerevisiae* isolates detected in mix-wine at the end of fermentation. In must samples (n°38), a total of 13 different biotypes were detected (**Table 3**, patterns "I"–"XIII"). In several musts (1, 2, 8, 10, 11, and 12) more than one *S. cerevisiae* biotype occurred. Nevertheless, in some cases, the same biotype was detected in different samples, i.e., "V" in musts 3 and 8; "VII" in musts 7 and 8; "XII" in musts 11 and 12 (**Table 3**). Moreover, it is noteworthy that must samples 3, 7, 8, and 11, 12 were produced from grapes collected in closely located vineyards (**Table 1**). In mix wine, a total of eight different biotypes, out of 18 isolates, were retrieved: three ("IV," "VII," and "XII") already detected in must samples and five new ("XIV"–"XVIII") (**Table 3**). The biotype "XIV" showed the highest occurrence: 10 isolates out 18 analyzed.

A total of 43 isolates (22 non-*Saccharomyces*, 13 *S. cerevisiae* isolates from musts samples and eight from mix-wine) were

TABLE 2 | Physico-chemical and microbiological characteristics of musts and mix wine after partial fermentation (9 and 30 days, respectively).

Must sample	°Babo	Ethanol (% vol/vol)[a]	Yeast loads (Log CFU/ml)[b]	No. of isolates[c]	Species[d]						
					M. pulcherrima	*Star. bacillaris*	*P. kudriavzevii*	*L. thermotolerans*	*H. uvarum*	*Pseud. aphidis*	*S. cerevisiae*
1	7.8	7.9	7.21 ± 0.21	4							4
2	13.8	4.2	8.16 ± 0.01	7				1		2	4
3	14.3	3.1	7.36 ± 0.05	4		1		1			2
4	17.3	2.1	7.33 ± 0.00	5	1				2		2
5	13.8	3.9	7.25 ± 0.15	5			3	1	1		
6	17.3	2.6	6.37 ± 0.02	2	2						
7	15.1	3.3	6.81 ± 0.51	5	1			1	1		2
8	10.8	6.4	6.80 ± 0.52	5					1		5
9	14.7	3.1	6.90 ± 0.44	3	1			2			
10	6.9	7.3	7.80 ± 0.06	4							4
11	11.7	5.4	7.77 ± 0.19	4							4
12	6.9	7.8	8.05 ± 0.04	4							4
Mix-wine	1.8	12.8	5.30 ± 0.06	18							18

[a] *Ebullioscopic (Malligand).*
[b] *Counts on WL Nutrient agar (28°C for 5 days).*
[c] *Selection on the basis of colony colur and morphology from countable plates (15-150 colonies/plate).*
[d] *Identifications obtained by ITS-RFLP and ITS sequencing analyses (see Supplementary Table S1).*

TABLE 3 | Distribution of *S. cerevisiae* biotypes, detected by Interdelta analysis, in analyzed samples.

Must	No. of isolates	*S. cerevisiae* biotypes																	
		I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV	XV	XVI	XVII	XVIII
1	4	2[a]	2																
2	4			2	2														
3	2					2													
4	2						2												
7	2							2											
8	5					3		2											
10	4								1	3									
11	4										1	1	2						
12	4												3	1					
Mix-wine	18				1			1					1		10	2	1	1	1

[a]*Number of isolates showing the same Interdelta pattern.*

characterized for biochemical features of oenological interest (**Table 4**). Strains belonging to the same species showed similar ethanol resistance: *M. pulcherrima* (4–5%)*; Pseud. aphidis* (a yeast like fungi, classified in the *Ustilaginales*) (6%)*; H. uvarum* (7%); *Star. bacillaris* (synonym *Candida zemplinina*), and *L. thermotolerans* (10%); *P. kudriavzevii* (10–12%)*; S. cerevisiae* (15–16%). All strains were able to grow in YPD (pH 3.30) containing 200 mg/l of potassium metabisulfite and most of them grew in tyndallized must as dispersed cells. One isolate (T28) of *P. kudriavzevii* grew on the surface; while the two *Pseud. aphidis* cultures were flocculent (**Table 4**). *H. uvarum* strains were low H_2S producers*, M. pulcherrima* fair producers, while the *Star. bacillaris* culture and those belonging to the species *Pseud. aphidis* were high producers (**Table 4**). Behavior within the species *P. kudriavzevii, L. thermotolerans*, and *S. cerevisiae* proved to be strain-dependent. Antagonistic activity was expressed by some isolates of *M. pulcherrima* (two out five)*, L. thermotolerans* (four out six), *P. kudriavzevii* (one out three), and *S. cerevisiae* (6 out 21). Cellobiose was hydrolyzed only by *M. pulcherrima* and *H. uvarum* isolates; while arbutin just by *M. pulcherrima.* For the other beta-glucosides used as precursors (4-methylumbelliferyl-b-D-glucopyranoside, esculin, and p-nitrophenyl β-D-glucopyranoside) different attitudes were recorded depending on the strain: the *Star. bacillaris* strain showed a low response, while *Pseud. aphidis* strains exhibited an high beta-glucosidase activity on these substrate (**Table 4**). Finally, only in *Pseud. aphidis* strains expressed esterase activity on a Tween 80-based medium.

The percentage of similarity among isolates, on the basis of technological traits, was evaluated by cluster analysis (Average Linkage Method) and the UPGMA dendrogram depicted in **Figure 1** was obtained. Isolates of the same species clustered at a similarity level higher than 75%, with the unique exception of *P. kudriavzevii* strains which were positioned in two different clusters: T24 and T25 in cluster 4 and T28 in cluster 6. Actually, T28 differed from the other two isolates for ethanol resistance (12%), type of growth (superficial), H_2S production (high), antagonistic activity (positive), and high beta-glucosidase activity (**Table 4**). In spite of the different origin and of the genetic diversity as emerged by Intedelta analysis, strains of *S. cerevisiae* grouped in a single cluster (cluster 5) with a quite high similarity level (80%) (**Figure 1**). No direct correlation between cluster position and origin of isolates was pointed out, even if, in some cases strains, strains with the same origin (T51 and T52, T8 and T5, T46 and T47; isolated from M11, M2, and M10, respectively) clustered very closely (>90 %) (**Figure 1**). Surprisingly, strains showing the same Interdelta pattern (T8 and MW3, pattern "IV"; T34 and MW16, pattern "VII"; T54 and MW5, pattern "XII") showed technological traits poorly different (**Figure 1**). Combining data of **Table 4** and **Figure 1**, 23 strains (10 non-*Saccharomyces* and 13 *S. cerevisiae*) were selected for the evaluation of the fermentation performances in Aglianico must containing about 240 g/l of reducing sugars and, therefore, an ethanolic potential of about 14% (vol/vol) (**Table 5**). Despite of their high beta-glucosidase and esterase activity, *Pseud. aphidis* strains were excluded because did not show fermentative activity. With exception of *P. kudriavzevii* isolates, strains of the same species showed similar FV values (*M. pulcherrima* 1.02–1.26 g CO_2/100 ml; *H. uvarum* 2.12–2.13; *Star. bacillaris* 2.84; *L. thermotolerans* 3.88-4.01; *S. cerevisiae* 5.11–5.89) (**Table 5**). *M. pulcherrima* and *H. uvarum* strains showed a FP value lower of 4 g CO_2/100 ml; all *L. thermotolerans* strains*,* the unique strain of *Star. bacillaris* and the strain T24 of *P. kudriavzevii* exhibited values ranging from 6.50 to 7.30; while *P. kudriavzevii* T28 a value of about 8.30. *S. cerevisae* strains, as expected, showed higher FP values, if compared to non-*Saccharomyces* (from 8.78 to 10.04 g CO_2/100 ml). By HPLC analysis of wines at the end of fermentation (no weight change of fermentation flasks, in 48 h), *M. pulcherrima* and of *H. uvarum* strains were able to produce <5% of ethanol (**Table 5**). However, both strains of *M. pulcherrima* produce undetectable (<0.15 g/l) amounts of acetic acid, a very high quantity of succinic acid (about 10.5 g/L), and a medium level of glycerol (about 5.5 g/l). By contrast, *H. uvarum* strains produced 1.0–1.2 g/l of acetic acid, 1.0–1.2 g/l of succinic acid and a lower amount of glycerol (4.1–4.7 g/l). *Star. bacillaris* strain T13 was able to produce a wine with about 10% of ethanol

TABLE 4 | Technological characteristics of yeasts collected during this study.

Species	No. of isolates	[a]Ethanol resistance	[b]$K_2O_5S_2$ resistance	[c]Type of growth	[d]H_2S production	[e]Antagonistic activity	Enzymatic activities					
							[f]CELL	[f]ARB	[f]ESC	[f]MUG	[f]pNPG	[g]EST
M. pulcherrima	5	4, 5	200	D	M	2	+	+	M	L, M, H	M	–
Star. bacillaris	1	10	200	D	H	–	–	–	L	L	L	–
P. kudriavzevii	3	10-12	200	D, S	M, H	1	–	–	L, H	M, H	L, M	–
L. thermotolerans	6	10	200	D	M, H	4	–	–	L	L, M, H	L	–
H. uvarum	5	6, 7	200	D	L	-	+	–	M, H	H	L, M	–
Pseud. aphidis	2	6	200	F	H	-	–	–	H	H	H	+
S. cerevisiae (musts)	13	15, 16	200	D	M, H	5	–	–	M, H	M, H	L	–
S. cerevisiae (mix wines)	8	15, 16	200	D	M, H	1	–	–	M	M, H	L, M	–

[a]*In YPD broth ethanol-added (4–16% vol).*
[b]*In YPD broth $K_2O_5S_2$–added (50–200 mg/l—50 mg/l increments).*
[c]*In tyndallized must (21°Brix, pH 3.50) after 4 days at 25°C: D, dispersed cells; S, surface growth; F, flocculent.*
[d]*On Biggy agar (Oxoid): L, low (Snow—White); M, medium (Hazelnut—Brown); H, high (Rust—Coffee).*
[e]*Number of isolates/strains showing antagonistic activity (Sangorrin et al., 2001).*
[f]*β-glucosidase activity evaluated on cellobiose (CELL) 4-methylumbelliferyl-b-D-glucopyranoside (MUG), arbutin (ARB), esculin (ESC) and p-Nitrophenyl β-D-glucopyranoside (pNPG) (Hernandez et al., 2002; Fia et al., 2005): +, positive; –, negative; L, low activity; M, medium activity; H, high activity.*
[g]*Esterase activity on Tween 80 (Slifkin, 2000).*

(9.91 ± 0.24), and to entirely metabolize fructose and malic acid. By contrast, the wine still contained unfermented glucose (about 60 g/l), glycerol (9.3 g/l), and acetic acid (0.8 g/l). In spite of the similar ethanol content (10.7–11.0%) *P. kudriavzevii* strains produced different fermentation by-products. In fact, the wine produced by strain T28 still contained about 45 g/l of glucose, 1.1 g/l of acetic and succinic acids and about 7.6 g/l of glycerol; while, that produced by strain T24 contained even more unfermented sugars (about 86 g/l), glycerol (6.6 g/l), lactic (0.8 g/l), and succinic acid (3.1 g/l) and did not contain detectable amount of acetic acid. *L. thermotolerans* produced slightly different wines depending on the strain: ethanol content ranged from 9.5 to 10.5%, residual sugars from 60 to 70 g/l, succinic acid from 2.3 to 3.0 g/l, lactic acid from 1.3 to 2.5 g/l, while glycerol was always around 6.5 g/l and acetic acid remained undetectable (<0.15 g/l). In wines produced by *S. cervisiae* strains some differences, depending on the strain used, emerged too. Two strains (MW16 and MW1), out 13, produced wines with a significant amount of unfermented fructose (15–20 g/l) and, as a consequence, with an alcoholic degree lower than 13% (11.92 and 12.75%, respectively). In the other, cases reducing sugars were detectable at low concentration (<4 g/l) (wines produced by strains T52 and MW6) or undetectable (**Table 5**). In fact, the mean alcoholic degree of wines, excluding those produced by strains MW16 and MW1, was 13.8 ± 0.28% (minimum 13.19 ± 0.25 %, maximum 14.17 ± 0.19%). The acetic acid production by *S. cerevisiae* strains ranged from 0.52 (T19) to 1.86 g/l (MW10), even if, more than 50% of the strains produced <0.6 g/l. Differences about glycerol production were also detected among wines produced by different strains of *S. cerevisiae*: from 5.35 of strain MW6 to 8.92 g/l of MW10, the high acetic acid producer. However, 60% of strains produced <6 g/l of glycerol. Different amount of succinic acid were produced by *S. cerevisiae* strains: from 0.92 (MW5) to 2.25 g/l (MW16). Finally, no significant differences in tartaric acid content were observed among wines produced by the different strains; by contrast, malic acid content of wines produced by strains MW17 and MW3 was significantly different: 3.63 ± 0.22 and 5.49 ± 0.49 g/l, respectively.

DISCUSSION

As recently reviewed by Barata et al. (2012), grapes are characterized by a complex microbial ecology including filamentous fungi, yeasts, and bacteria with different physiological characteristics that mightily affect wine quality. Some species (parasitic fungi and environmental bacteria) may be only found in grapes, while others microorganisms, such as yeast, lactic acid bacteria, and acetic acid bacteria, may survive and/or grow during winemaking process. The ratio occurring among groups of microorganisms depends on different ecology factors: climate conditions, viticulture practices, grape ripening stage, and health status of grapes that direct influences the availability of nutrients available for the epiphytic microflora. As matter of fact, health status is the main factor affecting the microbial ecology of grapes: damaged grapes possess higher microbial numbers and greater species diversity if compared to the healthy ones (Barata et al., 2012). This study focused on grape yeast microbiota able to survive and or to grow during both middle and final stages of wine fermentation, and, therefore, potentially able to impact on wine quality. Aglianico grape samples were collected in different vineyards full covering the production area of the Taurasi DOCG. Grapes may potentially host different genera of non-*Saccharomyces* yeasts mostly belonging to the following genera: *Metschnikowia, Dekkera, Pichia, Candida, Hanseniaspora, Kluyveromyces, Issatchenkia, Torulaspora, Debaryomyces, Saccharomycodes, Zygosaccharomyces,* and *Schizosaccharomyces* spp. (Mills et al., 2008). In must, strains of these genera are subjected to a selective pressure exerted by different factors including: high

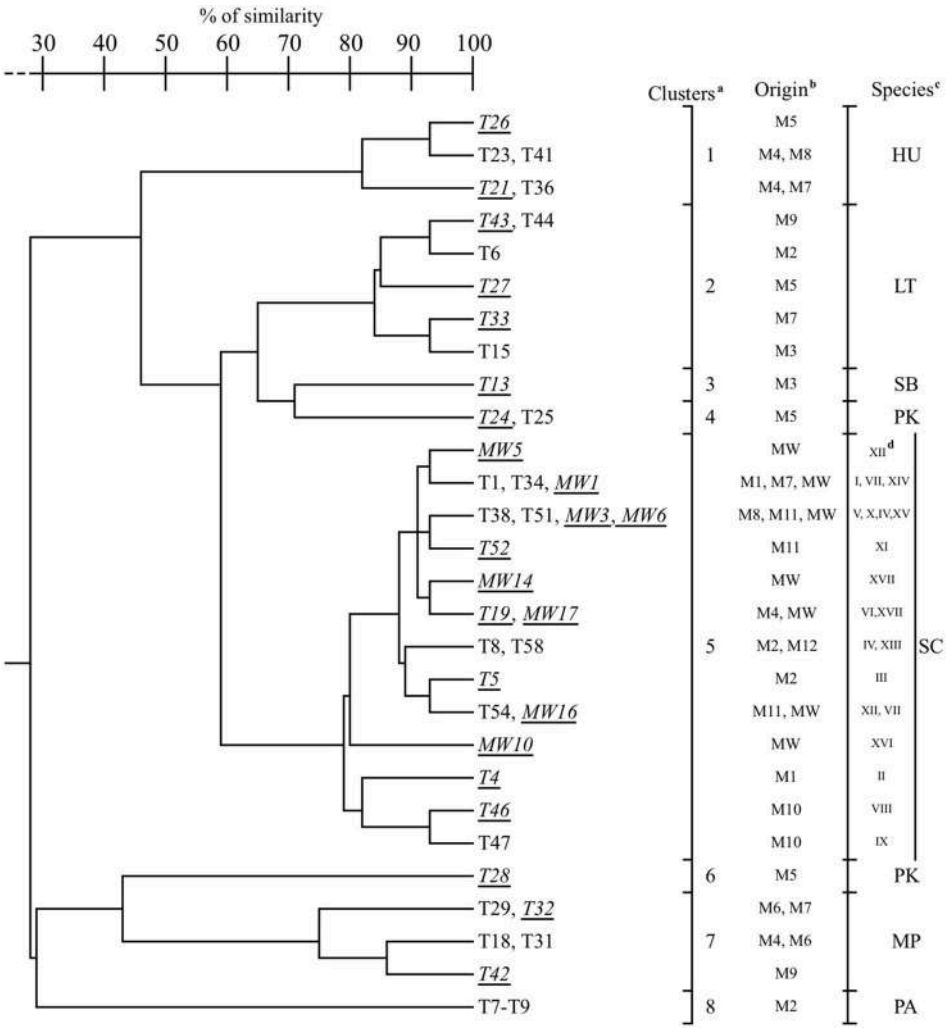

FIGURE 1 | UPGMA dendrogram obtained from the comparison of yeasts technological traits (see Table 4). [a]Clusters were defined at 75% of similarity. [b]Origin of isolates: M, must samples (see **Table 1**); MW, mix-wine. [c]MP, *M. pulcherrima*; SB, *Star. bacillaris*; PK, *P. kudriavzevii*; LT, *L. thermotolerans*; HU, *H. uvarum*; PA, *Pseud. aphidis;* SC, *S. cerevisiae*. [d]Interdelta biotypes of *S. cerevisiae* (I-XVIII). Isolates in underlined and *italicized* were used in the microfermentation trials (see **Table 5**).

sugar content, high acidity, nutrient availability, low oxygen tension, increasing ethanol concentrations, and presence of specific inhibitors (SO_2, botriticin, medium chain fatty acids) (Ribereau-Gayon et al., 2006; Mills et al., 2008). Therefore, after few days of fermentation, the occurrence of grape yeasts may vary depending on the must characteristics. Thereafter, in natural fermentation, is expected that *S. cerevisiae* (poorly occurring on grape) become dominant due to its high adaptation of must-wine environment. Despite of their progressive reduction during wine fermentation, non-*Saccharomyces* yeasts are considered important members of must-wine ecosystem and able to increase the "complexity" of the wines sensory profiles through the production of a massive range of sensory-active compounds, actually higher than that usually associated to *Saccharomyces* alone (Fleet, 2008). At technological maturity (°Babo higher then 19) Aglianico grapes still contain a high titratable acidity and low pH (Gambuti et al., 2009). Musts produced by grapes sampled during this study were characterized by different titratable acidity (8.6–11.2 g/l), pH (3.10–3.40), and sugar content (19.0–21.6). Moreover, due to their different origin of grapes samples, musts may likely contain different amounts of available nitrogen, phenolic compounds, pesticide residues, and fermentation inhibitors, also. Such diversity may be partly explained by the chemical and microbiological differences detected among the musts after 9 days of fermentation. The applied strategy allowed to detect both *Saccharomyces* and non-*Saccharomyces* entities. Species retrieved during this study, with the exception of *Pseud. aphids*, were frequently detected on grapes, cellar equipment and along wine fermentations (Mills et al., 2008). *Pseudozyma* spp., yeast-like fungi (*Ustilaginales*), mostly epiphytic or saprophytic, not pathogenic to plants (Buxdorf et al., 2013) and presumably disseminated by migratory birds (Francesca et al., 2012), have

already been detected on grape berries (Pantelides et al., 2015), and just once, by a culture-independent approach PCR-DGGE based, in commercial wines (Takahashi et al., 2014). In the present study, members of this genus were retrieved in one out of 12 musts at early stage of fermentation. Even if strains detected during this study showed high esterase and beta-glucosidase activities, they do not seem to play any oenological role. However, *Pseudozyma* species have been reported to exhibit biological activity against powdery mildews and *Botrytis cinerea* (Buxdorf et al., 2013) and, due to their enzymatic activities, may represent an important source of microbial lipases, surfactants (Dimitrijević et al., 2011; Dziegielewska and Adamczak, 2013) and glucosidases (this study).

Because of their several negative fermentation characteristics, such as low fermentation power and rate, low SO_2 resistance, and high production of acetic acid, ethyl acetate, acetaldehyde, and acetoin, non-*Saccharomyces* wine yeasts have been little considered as starter cultures in the past. However, as pointed out by this study, and previously by Comitini et al. (2011), some oenological traits of wine yeasts are species-specific (as ethanol resistance) and some are strain-specific (SO_2 resistance, type of growth, killer factor expression, H_2S production; enzymatic activities). Therefore, the strains selection among non-*Saccharomyces* may represent a profitable strategy to improve particular characteristics of wine (Suarez-Lepe and Morata, 2012). Despite of their low ethanol tolerance, as here reported, *M. pulcherrima* strains may exert antagonistic activity, high beta-glucosidase activity, low acetic acid production (Comitini et al., 2011), and high succinic acid accumulations (this study). *M. pulcherrima* strains may inhibit the growth of some spoilage yeasts (*Brettanomyces/Dekkera*, *Hanseniaspora,* and *Pichia*) (Oro et al., 2014) by pigment formation, which depletes the free iron in the medium thus generating an environment unsuitable for microorganisms requiring such element for the growth (Sipiczki, 2006).

Isolates of *H. uvarum* analyzed during this study proved to be high acetic acid producers, low H_2S producers and potentially expressing beta-glucosidase activities. In fact, a recent study of Albertin et al. (2016) reports several extracellular enzymatic activities of oenological relevance (pectinase, chitinase, protease, β-glucosidase) in *H. uvarum* strains.

The two isolates of *P. kudriavzevii* (synonymously known as *Issatchenkia orientalis*) showed very different traits. Strain T28, showing antagonistic activity and showing the ability to hydrolyze esculine, MUG and pNPG, was able to produce a wine with 11% of ethanol, high concentration of acetic acid (1.1 g/l) and medium-high of glycerol (7.6 g/l). By contrast, strain T24 (antagonistic activity positive and beta-glucosidase negative) produced a wine with a similar alcoholic degree, containing undetectable amounts of acetic acid, low quantity of lactic acid and relatively high concentration of succinic acid. Killer toxin expression, lactic and succinic production were recently highlighted in strains of *P. kudriavzevii* (Bajaj et al., 2013; Xiao et al., 2014).

Strains of *L. thermotolerans* (formerly known as *Kluyveromyces thermotolerans*) produced wines with about 10% of ethanol, low acetic acid, high lactic, and relative high succinic acids, thus confirming data already reported by Comitini et al. (2011). Moreover, four out six strains were able to express killer toxin, while no strain analyzed by Comitini et al. (2011) expressed this character. Killer toxin production by *Kluyveromyces thermotolerans* IFO 1778 was reported by Kono and Himeno (1997).

This study also confirmed the fructophilic nature, the high glycerol production and the relative low ethanol and acetic acid synthesis by *Star. bacillaris* (synonym *Candida zemplinina*) during wine fermentation (Tofalo et al., 2012; Englezos et al., 2015). In addition, Tofalo et al. (2012) proved that strains of this species can also metabolized about 40% of the malic acid of must. *Star. bacillaris* strain T13, isolated during this study, was able to entirely metabolize malic acid; being this metabolite undetectable (<0.25 g/l) by HPLC in the wine.

According to results, different biotypes of *S. cerevisiae* could be retrieved from the same grape sample; some biotypes could survive until the end of fermentation, while some other, not detectable in the grape or in must, become dominant in final product. In fact, as supposed by Sipiczki (2011), *S. cerevisiae* isolates of wine origin usually exhibit a significant biodiversity, due to the high propensity to genomic alteration of their genomes. In spite of the genetic diversity, *S. cerevisiae* strains exhibited an humble variability regarding their technological features and fermentation performances. Similar results were obtained by Capece et al. (2012): only three clusters out of 132 *S. cerevisiae* strains were obtained by statistical management of strains technological characterization. However, some strains isolated during this study showed undesirable characteristics as high H_2S and acetic acid production, and high residual fructose in wine.

As recently reviewed by Capozzi et al. (2015), the utilization of non-*Saccharomyces*/*Saccharomyces* multi-starter has been suggested by different researchers in order to mimic the spontaneous fermentation process and to avoid the risks of stuck or sluggish fermentations; in fact, the last years numerous investigations dealt with the biodiversity of non-*Saccharomyces* yeast isolated from grape juice and their use in multi-starter fermentations. Moreover, there is an increasing demand for autochthonous yeast, with the aim to select starter cultures better adapted to a definite grape must, thus exploiting the biodiversity of a specific "*terroir*" (see Capozzi et al., 2015). As consequence, specific selection projects are required in order to prevent negative impact autochthonous yeast on wine fermentation and to exploit their beneficial contributions to wine quality. In this study, the yeasts diversity occurring in grapes of a restrict area where high quality wines are produced was explored. By evaluating oenological traits, the potential of some isolated strains (non-*Saccharomyces* and *S. cerevisae*) in combination to modulate quality of Taurasi DOCG wine was highlighted.

In conclusion, apart from the local relevance of the present study, obtained outcomes clearly confirm that *S. cerevisiae* is a member of the vineyard microbiota. Moreover, the hypothesis formulated by Sipiczki (2011) according to which the genome of *S. cerevisiae* can change during fermentation (Fast Adaptive Evolution) seems to gain a further proof.

TABLE 5 | Fermentation performances of selected yeast strains in Aglianico must.

Species	Strain	Weight loss[a]		HPLC analysis[b]								
		FV	FP	TART	GLU	FRU	MAL	SUC	LACT	GLYC	ACET	ETHA
M. pulcherrima	T32	1.02	3.35	4.74 ± 0.27	45.8 ± 0.5	65.0 ± 0.5	NQ[c]	10.58 ± 0.36	<0.15	5.31 ± 0.05	<0.15	4.20 ± 0.07
M. pulcherrima	T42	1.26	3.70	4.79 ± 0.40	52.8 ± 0.5	89.0 ± 0.5	NQ	10.71 ± 0.28	<0.15	5.73 ± 0.15	<0.15	4.53 ± 0.03
Star. bacillaris	T13	2.84	6.97	4.00 ± 0.16	59.3 ± 0.5	<0.25	<0.25	0.83 ± 0.24	<0.15	9.34 ± 0.10	0.76 ± 0.01	9.91 ± 0.24
P. kudriavzevii	T28	2.17	8.25	4.40 ± 0.17	2.70 ± 0.23	43.9 ± 0.5	NQ	1.14 ± 0.15	<0.15	7.64 ± 0.21	1.15 ± 0.27	11.02 ± 0.35
P. kudriavzevii	T24	4.01	6.70	4.71 ± 0.21	30.6 ± 0.5	56.6 ± 0.5	NQ	3.14 ± 0.36	0.84 ± 0.30	6.66 ± 0.55	<0.15	10.74 ± 0.16
L. thermotolerans	T27	3.90	6.49	4.79 ± 0.33	28.1 ± 0.5	52.1 ± 0.5	NQ	2.28 ± 0.21	1.29 ± 0.43	6.45 ± 0.14	<0.15	10.42 ± 0.23
L. thermotolerans	T33	3.88	6.91	5.01 ± 0.21	23.1 ± 0.5	43.8 ± 0.5	NQ	3.03 ± 0.17	1.24 ± 0.52	6.33 ± 0.05	<0.15	9.46 ± 0.54
L. thermotolerans	T43	4.01	7.26	4.86 ± 0.55	19.2 ± 0.5	40.8 ± 0.5	NQ	2.55 ± 0.28	2.56 ± 0.65	6.71 ± 0.36	<0.15	10.24 ± 0.35
H. uvarum	T26	2.13	3.45	4.35 ± 0.22	70.0 ± 0.5	77.9 ± 0.5	NQ	1.01 ± 0.07	<0.15	4.77 ± 0.18	1.08 ± 0.03	4.41 ± 0.15
H. uvarum	T21	2.12	3.58	4.70 ± 0.47	69.2 ± 0.5	69.2 ± 0.5	NQ	1.28 ± 0.25	<0.15	4.12 ± 0.16	1.20 ± 0.16	4.36 ± 0.05
S. cerevisiae	T4	5.77	9.64	5.40 ± 0.59	<0.25	<0.25	4.03 ± 0.36	1.23 ± 0.03	<0.15	6.02 ± 0.21	0.55 ± 0.00	13.82 ± 0.14
S. cerevisiae	T5	5.83	9.53	5.60 ± 0.12	<0.25	<0.25	4.25 ± 0.42	1.67 ± 0.36	<0.15	5.61 ± 0.23	0.70 ± 0.06	13.89 ± 0.13
S. cerevisiae	T19	5.59	8.38	5.42 ± 0.13	<0.25	<0.25	4.15 ± 0.29	1.50 ± 0.03	<0.15	5.92 ± 0.15	0.52 ± 0.03	14.12 ± 0.26
S. cerevisiae	T46	5.49	9.50	5.79 ± 0.04	<0.25	<0.25	4.96 ± 0.31	2.08 ± 0.04	<0.15	6.19 ± 0.11	0.78 ± 0.01	13.78 ± 0.06
S. cerevisiae	T52	5.79	9.43	5.81 ± 0.00	1.44 ± 0.36	<0.25	4.20 ± 0.39	1.71 ± 0.21	<0.15	5.68 ± 0.19	0.77 ± 0.14	13.63 ± 0.21
S. cerevisiae	MW1	5.25	8.78	5.63 ± 0.48	1.21 ± 0.27	15.20 ± 0.42	4.75 ± 0.38	1.69 ± 0.26	<0.15	6.88 ± 0.09	0.95 ± 0.07	12.75 ± 0.12
S. cerevisiae	MW3	5.68	9.55	5.76 ± 0.46	<0.25	<0.25	5.49 ± 0.49	1.36 ± 0.35	<0.15	5.89 ± 0.01	0.53 ± 0.01	13.60 ± 0.38
S. cerevisiae	MW5	5.26	9.51	4.83 ± 0.14	<0.25	<0.25	4.38 ± 0.14	0.92 ± 0.62	<0.15	5.67 ± 0.12	0.61 ± 0.04	13.79 ± 0.08
S. cerevisiae	MW6	5.67	9.42	5.14 ± 0.51	<0.25	3.68 ± 0.01	4.60 ± 0.28	1.27 ± 0.02	<0.15	5.35 ± 0.17	0.68 ± 0.00	14.00 ± 0.29
S. cerevisiae	MW10	5.11	9.38	5.36 ± 0.32	<0.25	<0.25	4.59 ± 0.47	1.19 ± 0.29	<0.15	8.92 ± 0.16	1.86 ± 0.16	13.19 ± 0.25
S. cerevisiae	MW14	5.75	9.48	5.40 ± 0.26	<0.25	<0.25	4.05 ± 0.53	1.34 ± 0.13	<0.15	6.31 ± 0.00	0.61 ± 0.00	14.17 ± 0.19
S. cerevisiae	MW16	5.89	8.93	5.67 ± 0.00	<0.25	19.70 ± 0.66	4.62 ± 0.38	2.25 ± 0.03	<0.15	8.01 ± 0.34	0.57 ± 0.03	11.92 ± 0.25
S. cerevisiae	MW17	5.75	10.04	5.43 ± 0.06	<0.25	<0.25	3.63 ± 0.22	1.59 ± 0.34	<0.15	5.93 ± 0.25	0.56 ± 0.05	13.77 ± 0.14

Results are reported as mean (±SD).

[a] *FV, fermentation vigor (g CO_2/100 ml of must in 72 h). FP, Fermentation power (gr. CO_2/100 ml of must until the end of fermentation).*

[b] *TART, tartaric acid (g/l); GLU, glucose (g/l); FRU, fructose (g/l); MAL, malic acid (g/l); SUC, succinic acid (g/l); LACT, lactic acid (g/l); GLYC, glycerol (g/l); ACET, acetic acid (g/l); ETHA, ethanol (% vol/vol).*

[c] *NQ, unquantifiable, due to the high residual fructose concentration.*

Finally, results support the idea, already reported by several authors (Comitini et al., 2011; Rantsiou et al., 2012; Domizio et al., 2014; Zuehlke et al., 2015), that must fermentation with mixed cultures may improve the quality and complexity of the final product.

AUTHOR CONTRIBUTIONS

GB designed the project. MA and GB performed all the experiments, wrote and edited the manuscript.

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SUPPLEMENTARY MATERIAL

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REFERENCES

Albertin, W., Setati, M. E., Miot-Sertier, C., Mostert, T. T., Colonna-Ceccaldi, B., Coulon, J., et al. (2016). *Hanseniaspora uvarum* from winemaking environments show spatial and temporal genetic clustering. *Front. Microbiol.* 6:1569. doi: 10.3389/fmicb.2015.01569

Aponte, M., and Blaiotta, G. (2016). Selection of an autochthonous *Saccharomyces cerevisiae* strain for the vinification of "Moscato di Saracena," a southern Italy (Calabria Region) passito wine. *Food Microbiol.* 54, 30–39. doi: 10.1016/j.fm.2015.10.019

Bajaj, B. K., Raina, S., and Singh, S. (2013). Killer toxin from a novel killer yeast *Pichia kudriavzevii* RY55 with idiosyncratic antibacterial activity. *J. Basic Microbiol.* 53, 645–656. doi: 10.1002/jobm.201200187

Barata, A., Malfeito-Ferreira, M., and Loureiro, V. (2012). The microbial ecology of wine grape berries. *Inter. J. Food Microbiol.* 153, 243–259. doi: 10.1016/j.ijfoodmicro.2011.11.025

Buxdorf, K., Rahat, I., Gafni, A., and Levy, M. (2013). The epiphytic fungus *Pseudozyma aphidis* induces jasmonic acid- and salicylic acid/nonexpressor of PR1-independent local and systemic resistance. *Plant Physiol.* 161, 2014–2022. doi: 10.1104/pp.112.212969

Capece, A., Romaniello, R., Siesto, G., and Romano, P. (2012). Diversity of *Saccharomyces cerevisiae* yeasts associated to spontaneously fermenting grapes from an Italian "heroic vine-growing area." *Food Microbiol.* 31, 159–166. doi: 10.1016/j.fm.2012.03.010

Capozzi, V., Garofalo, C., Chiriatti, M. A., Grieco, F., and Spano, G. (2015). Microbial terroir and food innovation: the case of yeast biodiversity in wine. *Microbiol. Res.* 181, 75–83.

Comitini, F., Gobbi, M., Domizio, P., Romani, C., Lencioni, L., Mannazzu, I., et al. (2011). Selected non-*Saccharomyces* wine yeasts in controlled multistarter fermentations with *Saccharomyces cerevisiae*. *Food Microbiol.* 28, 873–882. doi: 10.1016/j.fm.2010.12.001

Csoma, H., and Sipiczki, M. (2008). Taxonomic reclassification of *Candida stellata* strains reveals Frequent occurrence of *Candida zemplinina* in wine fermentation. *FEMS Yeast Res.* 8, 328–336. doi: 10.1111/j.1567-1364.2007.00339.x

Di Maio, S., Polizzotto, G., Di Gangi, E., Foresta, G., Genna, G., Verzera, A., et al. (2012). Biodiversity of indigenous *Saccharomyces* populations from old wineries of south-eastern Sicily (Italy): preservation and economic potential. *PLoS ONE* 7:30428. doi: 10.1371/journal.pone.0030428

Dimitrijević, A., Velickovic, D., Bezbradica, D., Bezbradica, F., Jankov, R., and Milosavic, N. (2011). Production of lipase from *Pseudozyma aphidis* and determination of the activity and stability of the crude lipase preparation in polar organic solvents. *J. Serb. Chem. Soc.* 76, 1081–1092. doi: 10.2298/JSC110428096D

Domizio, P., Liu, Y., Bisson, L. F., and Barile, D. (2014). Use of non-*Saccharomyces* wine yeasts as novel sources of mannoproteins in wine. *Food Microbiol.* 43, 5–15. doi: 10.1016/j.fm.2014.04.005

Dziegielewska, E., and Adamczak, M. (2013). Evaluation of waste products in the synthesis of surfactants by yeasts. *Chem. Papers* 67, 1113–1122. doi: 10.2478/s11696-013-0349-1

Englezos, V., Rantsiou, K., Torchio, F., Rolle, L., Gerbi, V., and Cocolin, L. (2015). Exploitation of the non-*Saccharomyces* yeast *Starmerella bacillaris* (synonym *Candida zemplinina*) in wine fermentation: physiological and molecular characterizations. *Intern. J. Food Microbiol.* 199, 33–40. doi: 10.1016/j.ijfoodmicro.2015.01.009

Esteve-Zarzoso, B., Belloch, C., Uruburu, F., and Querol, A. (1999). Identification of yeast by RFLP of 5.8 rRNA gene and two ribosomal internal transcribed spacers. *Int. J. Syst. Bacteriol.* 49, 329–337. doi: 10.1099/00207713-49-1-329

Fia, G., Giovani, G., and Rosi, I. (2005). Study of β-glucosidase production by wine-related yeasts during alcoholic fermentation. A new rapid fluorimetric method to determine enzymatic activity. *J. Appl. Microbiol.* 99, 509–517. doi: 10.1111/j.1365-2672.2005.02657.x

Fleet, G. H. (2003). Yeast interactions and wine flavor. *Int. J. Food Microbiol.* 86, 11–22. doi: 10.1016/S0168-1605(03)00245-9

Fleet, G. H. (2008). Wine yeasts for the future. *FEMS Yeast Res.* 8, 979–995. doi: 10.1111/j.1567-1364.2008.00427.x

Francesca, N., Canale, D. E., Settanni, L., and Moschetti, G. (2012). Dissemination of wine-related yeasts by migratory bird. *Environ. Microbiol. Rep.* 4, 105–112. doi: 10.1111/j.1758-2229.2011.00310.x

Gambuti, A., Capuano, R., Lecce, L., Fragasso, M. G., and Moio, L. (2009). Extraction of phenolic compounds from 'Aglianico' and 'Uva di Troia' grape skins and seeds in model solutions: Influence of ethanol and maceration time. *Vitis* 48, 193–200.

Hernandez, L. F., Espinosa, J. C., Fernandez-Gonzales, M., and Briones, A. (2002). β-glucosidase activity in a *Saccharomyces cerevisiae* wine strain. *Int. J. Food Microbiol.* 80, 171–176. doi: 10.1016/S0168-1605(02)00149-6

Kono, I., and Himeno, K. (1997). A novel killer yeast effective on *Schizosaccharomyces pombe*. *Biosc. Biotech. Bioch.* 61, 563–564. doi: 10.1271/bbb.61.563

Legras, J. L., and Karst, F. (2003). Optimisation of interdelta analysis for *Saccharomyces cerevisiae* strain characterisation. *FEMS Microbiol. Lett.* 221, 249–255. doi: 10.1016/S0378-1097(03)00205-2

Marinangeli, P., Angelozzi, D., Ciani, M., Clementi, F., and Mannazzu, I. (2004). Minisatellites in *Saccharomyces cerevisiae* genes encoding cell wall proteins: a new way towards wine strain characterisation. *FEMS Yeast Res.* 4, 427–435. doi: 10.1016/S1567-1356(03)00172-7

Mills, D. A., Phister, T., Neeley, E., and Johannsen, E. (2008). "Wine Fermentation," in *Molecular Techniques in the Microbial Ecology of Fermented Foods*, eds L. Cocolin and D. Ercolini (New York, NY: Springer), 161–192.

Nei, M., and Li, W. (1979). Mathematical model for studying genetic variation in thers of restriction endonucleases. *Proc. Nat. Acad. Sci. U.S.A.* 76, 5269–5273. doi: 10.1073/pnas.76.10.5269

Oro, L., Ciani, M., and Comitini, F. (2014). Antimicrobial activity of *Metschnikowia pulcherrima* on wine yeasts. *J. Appl. Microbiol.* 116, 1209–1217. doi: 10.1111/jam.12446

Pallmann, C. L., Brown, J. A., Olineka, T. L., Cocolin, L., Mills, D. A., and Bisson, L. F. (2001). Use of WL medium to profile native flora fermentations. *Am. J. Enol. Vitic.* 52, 198–203.

Pantelides, I. S., Christou, O., Tsolakido, M.-D., Tsaltas, D., and Ioannou, N. (2015). Isolation, identification and *in vitro* screening of grapevine yeasts for the control of black aspergilli on grapes. *Biol. Contr.* 88, 46–53. doi: 10.1016/j.biocontrol.2015.04.021

Pretorius, I. S. (2000). Tailoring wine yeast for the new millennium: novel approaches to the ancient art of winemaking. *Yeast* 16, 675–729. doi: 10.1002/1097-0061(20000615)16:8<675::AID-YEA585>3.0.CO;2-B

Rantsiou, K., Dolci, P., Giacosa, S., Torchio, F., Tofalo, R., Torriani, S., et al. (2012). *Candida zemplinina* can reduce acetic acid produced by *Saccharomyces cerevisiae* in sweet wine fermentations. *Appl. Environ. Microbiol.* 78, 1987–1994. doi: 10.1128/AEM.06768-11

Ribereau-Gayon, P., Dubourdieu, D., Doneche, B., and Lonvaud, A. (eds.). (2006). *Handbook of Enology: The Microbiology of Wine and Vinifications, 2nd Edn.*, Vol. 1. Chichester: John Wiley & Sons Ltd.

Sangorrin, M. P., Zajonskovsky, I. E., Lopes, C. A., Rodriguez, M. E., Giraudo de Van Broock, M. R., and Caballero, A. C. (2001). Killer behaviour in wild wine yeasts associated with Merlot and Malbec type musts spontaneously fermented from Northwestern Patagonia (Argentina). *J. Bas. Microbiol.* 41, 105–113. doi: 10.1002/1521-4028(200105)41:2<105::AID-JOBM105>3.0.CO;2-W

Sipiczki, M. (2006). *Metschnikowia* strains isolated from botrytized grapes antagonize fungal and bacterial growth by iron depletion. *Appl. Environ. Microbiol.* 72, 6716–6724. doi: 10.1128/AEM.01275-06

Sipiczki, M. (2011). Diversity, variability and fast adaptive evolution of the wine yeast (*Saccharomyces cerevisiae*) genome e a review. *Ann. Microbiol.* 61, 85–93. doi: 10.1007/s13213-010-0086-4

Slifkin, M. (2000). Tween 80 opacity test responses of various *Candida* species. *J. Clin. Microbiol.* 38, 4626–4628.

Suarez-Lepe, J. A., and Morata, A. (2012). New trends in yeast selection for winemaking. *Trend. Food Sci. Technol.* 23, 39–50. doi: 10.1016/j.tifs.2011.08.005

Takahashi, M., Ohta, T., Masaki, K., Mizuno, A., and Goto-Yamamoto, N. (2014). Evaluation of microbial diversity in sulfite-added and sulfite-free wine by culture-dependent and -independent methods. *J. Biosci. Bioeng.* 117, 569–575. doi: 10.1016/j.jbiosc.2013.10.012

Tofalo, R., Schirone, M., Torriani, S., Rantsiou, K., Cocolin, L., Perpetuini, G., et al. (2012). Diversity of *Candida zemplinina* strains from grapes and Italian wines. *Food Microbiol.* 29, 18–26. doi: 10.1016/j.fm.2011.08.014

Upholt, W. B. (1977). Estimation of DNA sequence divergence from comparison of restriction endonuclease didigests. *Nuc. Acids Res.* 4, 1257–1265. doi: 10.1093/nar/4.5.1257

Xiao, H., Shao, Z., Jiang, Y., Dole, S., and Zhao, H. (2014). Exploiting *Issatchenkia orientalis* SD108 for succinic acid production. *Microb. Cell Fact.* 13, 121. doi: 10.1186/s12934-014-0121-4

Zuehlke, J. M., Childs, B. C., and Edwards, C. G. (2015). Evaluation of *Zygosaccharomyces bailii* to metabolize residual sugar present in partially-fermented red wines. *Fermentation* 1, 3–12. doi: 10.3390/fermentation1010003

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Microbial Terroir in Chilean Valleys: Diversity of Non-conventional Yeast

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In this study, the presence of non-conventional yeast associated with vineyards located between latitudes 30°S and 36°S was examined, including the valleys of Limarí, Casablanca, Maipo, Colchagua, Maule, and Itata. The microbial fingerprinting in each valley was examined based on the specific quantification of yeast of enological interest. Grape–berries were sampled to evaluate the presence and load of non-conventional yeast with enological potential, such as *Metschnikowia*, *Hanseniaspora*, *Torulaspora*, *Debaryomyces*, *Meyerozyma,* and *Rhodotorula*. These yeasts were present in all vineyards studied but with varying loads depending on the valley sampled. No identical fingerprints were observed; however, similarities and differences could be observed among the microbial profiles of each valley. A co-variation in the loads of *Metschnikowia* and *Hanseniaspora* with latitude was observed, showing high loads in the Casablanca and Itata valleys, which was coincident with the higher relative humidity or rainfall of those areas. Non-conventional yeasts were also isolated and identified after sequencing molecular markers. Potentially good aromatic properties were also screened among the isolates, resulting in the selection of mostly *Metschnikowia* and *Hanseniaspora* isolates. Finally, our results suggest that microbial terroir might be affected by climatic conditions such as relative humidity and rainfall, especially impacting the load of non-conventional yeast. In this study, the microbial fingerprint for yeast in Chilean vineyards is reported for the first time revealing an opportunity to study the contribution of this assembly of microorganisms to the final product.

Keywords: wine, non-*Saccharomyces*, *Hanseniaspora*, *Metschnikowia*, *Torulaspora*

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INTRODUCTION

Grape–berries are a great reservoir for microorganisms such as yeasts, lactic acid bacteria, and acetic acid bacteria. Yeasts play a fundamental role in the process of alcoholic fermentation because they are responsible for the transformation of sugars into ethanol, carbon dioxide, and other metabolites (Ribereau-Gayon et al., 2006). Due to their role in alcoholic fermentation, in enology, yeasts are usually divided into two categories: *Saccharomyces* and non-*Saccharomyces*. The latter category includes a wide array of different genera, also termed non-conventional yeasts. *Saccharomyces* has a high fermentative capacity and predominates during alcoholic fermentation (Ribereau-Gayon et al., 2006), whilst non-conventional yeasts proliferate during the first stage of spontaneous fermentation as they can tolerate low concentrations of ethanol, approximately 4% v/v (Fleet, 1993).

Non-conventional yeasts are relevant for their ability to influence the varietal flavors of wines by transforming non-volatile compounds into volatile aromas through enzymatic action

(Carrau et al., 2005; Varela et al., 2009; Ciani et al., 2010). Additionally, non-conventional yeasts might influence the fermentative flavor by generating metabolites derived from the fermentation processes (Fleet, 2003; Cordente et al., 2012). Moreira et al. (2008) examined the role of *Hanseniaspora guilliermondii* and *H. uvarum* in pure and mixed starter cultures with *Saccharomyces cerevisiae*. Their results showed that the growth of those yeasts during the early days of fermentation enhanced the production of desirable compounds, such as esters, and had no negative influence through the production of undesirable compounds. Viana et al. (2008) investigated 38 yeast strains, including the *Candida, Hanseniaspora, Pichia, Torulaspora,* and *Zygosaccharomyces* genera, for acetate ester formation. They identified *H. osmophila* as a good candidate for mixed cultures because this yeast has a glycophilic nature, the ability to produce acetaldehyde within a range compatible for wine and acetate ester production. Medina et al. (2013) evaluated the use of a native *H vineae* in Chardonnay must. They found that the aroma sensory analysis indicated a significant increase in fruit intensity, described as banana, pear, apple, citric fruits, and guava, when *H. vineae* was used.

On the grape surface, Fleet (2003) reported very low loads of *Saccharomyces cerevisiae,* at 10^2 CFU/g of grape–berry, while Beltran et al. (2002) reported that only in very healthy grapes could they recover *S. cerevisiae* in the must. Instead, high loads of non-conventional yeasts ranging from 10^6–10^8 CFU/g in grape–berries have been reported several times (Constantí et al., 1997; Torija et al., 2001; Barata et al., 2012). Using Next Generation Sequencing, Setati et al. (2012) and Bokulich et al. (2014) added that non-conventional yeasts on grape–berry, that is, the majority component of the microbiome, will have a potential influence on the organoleptic quality of wine and can even be considered the "microbial terroir" (Gilbert et al., 2014).

In Chile, most vineyards are located between latitudes 30°S and 36°S, along a longitudinal stretch of ca. 1300 km that offers a wide variety of climatic conditions and hence a variety of viticultural areas that may influence the microbial terroir, thus potentially contributing to the distinctive organoleptic properties of wine from each region. To the best of our knowledge, no studies on yeast diversity have been conducted in Chilean vineyards covering a wide range of climate conditions. Therefore, the aim of this work was to understand the diversity and geographic distribution of the microbial communities associated with grape–berries in Chilean valleys. Culture independent approach based on qPCR and DNA extracted directly from grapevines, was used to study the presence and load of yeast of enological interest. This was complemented with the isolation, identification, and characterization of non-conventional yeasts that were also performed to explore the enological potential of native isolates.

MATERIALS AND METHODS

Sampling and Culture Conditions

Grape–berry samples from healthy vines were obtained from several vineyards located in different Chilean valleys between March and May of 2015 (harvest period) with sugar content around 23° Brix. The vineyards were located between latitudes 30°S and 36°S, representing the Limarí, Casablanca, Maipo, Colchagua, Maule, and Itata valleys. Approximately 1 kg of grapes was obtained from at least five plants of each vineyard, as detailed in **Table 1**.

The samples were loaded into sterile Stomacher® bags and transported to the laboratory in a coolbox containing ice pads. Once in the laboratory, each sample was transferred into a fresh Stomacher® bag, in which the berries were pressed by hand for 3 min. Then, 5 mL of juice was separated for culture analysis, while 5 mL was centrifuged at 12.000 × *g* for 15 min, and the pellets obtained were frozen at −20°C until DNA extraction.

Yeast Isolation

Samples for the isolation and identification of yeasts were taken from the juice samples described above. Several decimal dilutions (10^{-2} to 10^{-4}) of each sample (0.1 mL) were plated on YEPD agar medium (1% yeast extract, 2% peptone, 2% glucose, and 2% agar by w/v, Merck) with 25 ppm of cycloheximide (Merck) and incubated at 28°C for 2 days. Where possible, 4–6 representatives of each colony-morphology were isolated from plates with ≤200 colonies and purified through two rounds of streak plating onto fresh agar plates. In addition, unique but infrequent colonies that were observed on plates with >200 colonies were also isolated. The isolates were maintained in a cryobank at −80°C.

TABLE 1 | Vineyard locations and grape cultivars sampled.

Approximate vineyard location	Valleys	Grape cultivar's
30°39′S–71°19′W	Limarí	Chardonnay
		Pinot Noir
		Muscat of Alexandria
		Carmenere
		Merlot
33°21′S–71°20′W	Casablanca	Pinot noir
		Sauvignon Blanc
		Chardonnay
		Merlot
33°34′S–70°38′W	Maipo	Malbec
		Sauvignon Blanc
		Cabernet Sauvignon
		Carmenere
		Cabernet Franc
		Carmenere
34°39′S–71°12′W	Colchagua	Merlot
		Cabernet Sauvignon
		Cabernet Franc
		Cabernet Sauvignon
35°50′S–71°75′W	Maule	Petit Verdot
		Alicante Bouschet
		Torontel
		Mencia
36°30′S–74°42′W	Itata	País
		Cinsault

DNA Extraction from Grape–Berry Samples and Yeast Isolates

The initial step for our culture independent approach was the extraction of DNA directly from grapevines. The pellets obtained after grape juice centrifugation were re-hydrated with 480 μL Phosphate-buffered saline (PBS), with vigorous agitation. A 20 μL aliquot of 20 mg/mL lyticase (Sigma) was added to the samples, which were subsequently incubated at 37°C for 20 min. Then, the samples were treated with 2.5 μL volume of 20 mg/mL Proteinase K (Merck) incubated at 37°C for 45 min. The Power Soil DNA Isolation Kit (Mo-Bio Laboratories, Inc.) was used for DNA extraction according to the manufacturer's instructions.

In the case of yeast isolates, each of the colonies selected was suspended in 200 μL PBS, with vigorous agitation, followed by centrifugation at 5.000 × *g* for 5 min. The pellets formed were washed with TE-NaCl (Tris 10mM pH7, EDTA 1 mM, NaCl 0.15 M) and centrifuged at 5.000 × *g* for 5 min. Subsequently, a 20 μL volume of 20 mg/mL lyticase (Sigma) was added to the samples, which were subsequently incubated at 37°C for 20 min. Finally, the samples were treated with 2.5 μL volume of 20 mg/mL Proteinase K (Merck) incubated at 37°C for 45 min. The Power Soil DNA Isolation Kit (Mo-Bio Laboratories, Inc.) was used for DNA extraction according to the manufacturer's instructions. All the DNA obtained was froze at −20°C until processed.

Identification of Yeast Isolates

The identification of yeast isolates (non-conventional yeast and *Saccharomyces*) were done by ITS 5.8S rRNA and D1/D2 sequence. The ITS 5.8S-rRNA were amplified using primers ITF1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITF4 (5′-TCCTCCG CTTATTGATATGC-3′; Esteve-Zarzoso et al., 1999). The partial 26S-rRNA gene sequences (D1/D2 domains) were amplified using primers NL-1 (5′-GCATATCAATAAGCGGAGGAAAAG-3′) and NL-4 (5′-GGTCCGTGTTTCAA GACGG-3′; Kurtzman and Robnett, 1998). DNA sequencing was performed by Macrogen (USA). Also, a BLAST (Basic Alignment Search Tool) analysis[1] was performed for the sequences obtained. The identification of each isolate was performed based on the closest relative sequence found in the database (GenBank). Isolates were identified and respective sequences were deposited in GenBank (KU350312–KU350496).

Primer Design

Primers were designed by aligning of the variable D1/D2 domains of the 26S rRNA gene sequences from different yeast species. Sequences were obtained from the GenBank database and alignment was performed with the Clustal W multiple sequence alignment. The final selection of the primers was performed using the Primer-Blast program[2]. A BLAST search was used to check the specificity of each primer as described in **Table 2**.

Quantitative PCR (qPCR) and Standard Curves

Specific qPCR reactions were carried out to examine the presence and load of yeast of enological interest. The qPCR reactions were performed using an AriaMx real-time PCR System (Agilent Technologies) using primers and programs described in **Table 2**, for the following yeast: *Saccharomyces, Hanseniaspora, Torulaspora, Metschnikowia, Rhodotorula, Debaryomyces, and Meyerozyma*. Standard curves were built for each yeast species in triplicate using 10-fold serial dilutions of fresh cultures.

PCR amplification was performed in 10 μL of mix containing 1 μL of DNA 0.5 pmol/μL of each respective primer, 8 μL of

[1]http://www.ncbi.nlm.nih.gov/blast/Blast.cgi

[2]http://www.ncbi.nlm.nih.gov/tools/primer-blast/

TABLE 2 | Primers and programs for quantitative PCR.

	Programs	Primer	Sequences 5′–3′	Reference
Total yeast	95°C 10 s; 60°C 10 s; 72°C 15 s. Cycle: 50	NL1	GCATATCAATAAGCGGAGGAAAAC	Mills et al., 2002
		NL4	GGTCCGTGTTTCAAGACGG	
Saccharomyces	95°C 10 s; 60°C 10 s; 72°C 10 s. Cycle: 35	Sac F	GAAAACTCCACAGTGTGTTG	
		Sac R	GCTTAAGTGCGCGGTCTTG	
Aureobasidium	95°C 10 s; 60°C 10 s; 72°C 10 s. Cycle: 40	Aur F	CGCATCGATGAAGAACGCAG	This study
		Aur R	CAACTAAGGACGGCACCCAA	
Rhodotorula	95°C 10 s; 60°C 10 s; 72°C 10 s. Cycle: 50	Rho F	ACCTTGCGCTCCTTGGTATT	This study
		Rho R	TCCTTTAACCCAACTCGGCT	
Meyerozyma	95°C 10 s; 60°C 10 s; 72°C 10 s. Cycle: 40	Mey F	AGATAGGTTGGGCCAGAGGT	This study
		Mey R	GCATTTCGCTGCGTTCTTCA	
Torulaspora	95°C 10 s; 60°C 10 s; 72°C 10 s. Cycle: 40	Tor F	CAAAGTCATCCAAGCCAGC	This study
		Tor R	TTCTCAAACAATCATGTTTGGTAG	
Metschnikowia	95°C 10 s; 60°C 10 s; 72°C 10 s. Cycle: 40	Met F	CAACGCCCTCATCCCAGA	This study
		MetR	AGTGTCTGCTTGCAAGCC	
Debaryomyces	95°C 10 s; 57°C 10 s; 72°C 10 s. Cycle: 50	Deb F	TGAAGAACGCAGCGAAATGC	This study
		Deb R	GCCGAGCCTAGAATACCGAG	
Hanseniaspora	95°C 10 s; 57°C 10 s; 72°C 10 s. Cycle: 50	HanF	CCCTTTGCCTAAGGTACG	This study
		HanR	CGCTGTTCTCGCTGTGATG	

LightCycler 480 SYBR Green I Master (Roche) and 1 μL of Milli-Q sterile H_2O. All of the amplifications were carried out in optical-grade, 96-well plates, AriaMx real-time PCR System (Agilent Technologies). All samples were analyzed in triplicate. Yeast load were compared using grouped analysis performed with GraphPad Prism version 6.00 for Mac (GraphPad Software, La Jolla CA, USA[3]).

Phylogenetic Analysis

The nucleotide sequences of the 5.8S ITS rDNA region and D1/D2 domain part gen 26S rRNA were compared with those available in the GenBank database using the BLAST method in order to investigate their approximate phylogenetic affiliation, and their sequence similarities were determined at the National Center for Biotechnology Information, USA (Altschul et al., 1997)[4]. Phylogenetic and molecular evolutionary analyses were performed using MEGA software, version 6.0 Beta. The phylogenetic tree for 5.8S ITS and D1/D2 domain part 26S rRNA gen were constructed by UPGMA (unweighted pair-group method with arithmetic mean) method. The evolutionary distances were computed using the Maximum Composite Likelihood method, using the Mega 6 (version 6.0) software package obtained from the website[5].

Laboratory-Scale Fermentations and Yeast Selection

With the yeast isolates obtained from each of the grape–berry samples, a series of micro-fermentations were conducted for an initial assessment of the fermentation capacity and aromatic attributes of the isolates. Fifty milliliters of synthetic must (**Table 3**) supplemented with 20 mg/L of SO_2 was inoculated to a final concentration of 10^8 cells/mL. Micro-fermentations were conducted at 18°C with stirring in an orbital shaker at a rate of 150 rpm. The evolution of the alcoholic fermentation was evaluated by monitoring weight loss every two days. At the end of the fermentation, the concentrations of glucose/fructose were measured using enzymatic kits (Boehringer Mannheim), according to the manufacturer's instructions.

Sensory Analyses

The resulting micro-fermentations were evaluated at controlled room temperature (20°C), in individual booths. The sensory analyses were carried out by olfactory evaluation in the Sensory evaluation laboratory and all panelists were winemakers belonging to the Enology Department, Universidad de Chile. Terpenes, thiols, and higher alcohols were represented as aromatic descriptors such as "fruit" and/or "flower" and were order as aromatic groups: fermentative, tropical fruit, citrus fruit, stone fruits, berries, flower, dried fruit, cooked fruits, and sweet aromas. The sensory panel first smelled several fresh aromatic references to choose those samples which best fitted their aroma. The strategy for data analysis was a descriptive

[3]www.graphpad.com

[4]http://ncbi.nlm.nih.gov/BLAST

[5]www.megasoftware.net

TABLE 3 | Synthetic must composition.

Components	
Glucose	100 g
Fructose	100 g
Citric acid	0.5 g
Malic acid	5 g
Tartaric acid	3 g
KH_2PO_4	0.750 g
K_2SO_4	0.500 g
$MgSO_2$ 7 H_2O	0.250 g
$CaCl_2$ 2 H_2O	0.155 g
NaCl	0.200 g
Nitrogen	408 mg/L
NH_4Cl (120 mgN/l)	0.460 g
Amino acid stock ***	13.09 mL
Oligo-elements stock *	1 mL
Vitamins stock **	10 mL
Distilled H_2O	1 L
Vitamins Stocks (for 1 liter)**	
Myo-inositol	2 g
Pantothenate calcium	0.15 g
Thiamine, hydrochloride	0.025 g
Nicotinic acid	0.2 g
Pyridoxine	0.025 g
Biotine	3 mL
Distilled H_2O	csp 1 L
Oligo-elements stock (1 liter)*	
$MnSO_4$, H_2O	4 g
$ZnSO_4$, 7 H_2O	4 g
$CuSO_4$, 5 H_2O	1 g
KI	1 g
$CoCl_2$, 6 H_2O	0.4 g
H_3BO_3	1 g
$(NH_4)_6MO_7O_{24}$	1 g
Distilled H_2O	
Stock anaerobiosis factors (100 mL)	
Ergosterol	1.5 g
Oleic Acid	0.5 mL
Tween 80	50 mL
Ethanol	cps 100 mL
Amino acids stocks (for 1 liter solution Na_2CO_3 2%) ***	
Tyrosine (Tyr)	1.5 g
Tryptophan (Trp)	13.4 g
Isoleucine (Ile)	2.5 g
Aspartic Acid (Asp)	3.4 g
Glutamic Acid (Glu)	9.2 g
Arginine (Arg)	28.3 g
Leucine (Leu)	3.7 g
Threonine (Thr)	5.8 g
Glycine (Gly)	1.4 g
Glutamine (Gln)	38.4 g
Alanine (Ala)	11.2 g
Valine (Val)	3.4 g
Methionine (Met)	2.4 g
Phenylalanine (Phe)	2.9 g

(Continued)

TABLE 3 | Continued

Amino acids stocks (for 1 liter solution Na_2CO_3 2%) ***	
Serine (Ser)	6.0 g
Histidine (His)	2.6 g
Lysine (Lys)	1.3 g
Cysteine (Cys)	1.5 g
Proline (Pro)	46.1 g
Distilled H_2O	cps 1 L
Total	138 g

method (attribute score versus frequencies of citation). The number of times each attribute was cited as negative frequency and positive frequency for each sample were counted up. Once all data were collected, the list of yeast was ranked according to their citation frequency to identify the most relevant attribute of each fermented product.

Climate Data

Monthly weather data were extracted from the Agromet INIA[6] and Red Agroclimática Nacional[7] databases. Data were collected from seven different weather stations representing each vineyard. Monthly measurements were extracted for average temperature, maximum temperature, minimum temperature, rainfall, and average relative humidity during 2015 (**Figure 2**).

[6]http://agromet.inia.cl

[7]www.agromet.cl

RESULTS

Non-conventional Yeast in Different Chilean Valleys and Climatic Conditions

A total of twenty-five samples of grape–berries from six Chilean viticultural areas were analyzed (**Table 1**), screening for *Saccharomyces* and six non-conventional yeast genera *Torulaspora, Hanseniaspora*, *Metschnikowia, Rhodotorula, Debaryomyces,* and *Meyerozyma*. All of these yeast were present at different loads depending on the examined samples. The yeast population (log_{10} scale) was represented for each valley and it revealed the microbial fingerprint for each area, ordered from north to south (**Figure 1**). No identical fingerprints were observed, however, similarities and differences could be observed among the microbial profiles of each valley. For example, *Hanseniaspora* and *Metschnikowia* were present in Limarí, Casablanca, and Itata

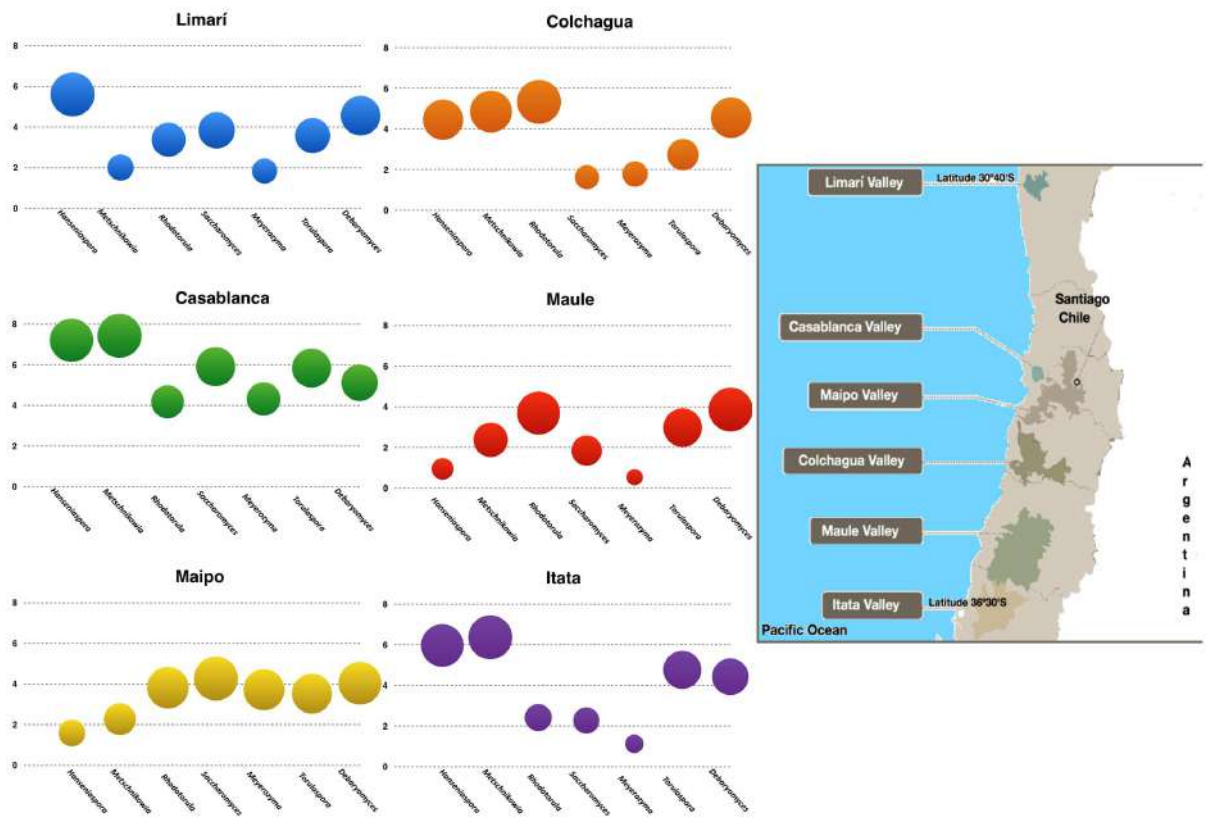

FIGURE 1 | Microbial terroir in different Chilean Valleys. The graphics represent the load of the yeast of enological interest as were quantified by qPCR in the different valleys examined (log_{10} scale). The insert in the right corresponds to a map showing the location of the valleys.

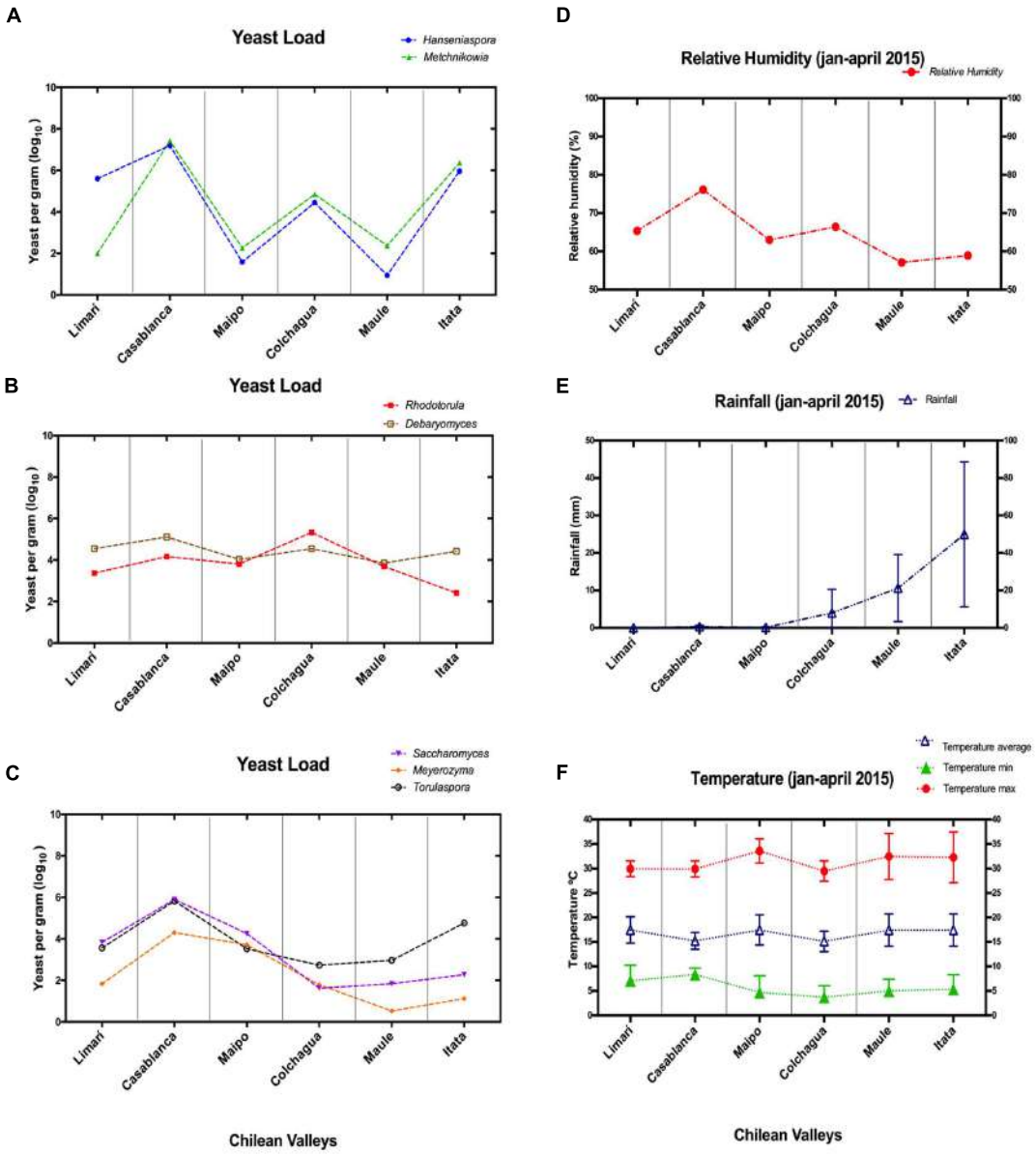

FIGURE 2 | Non-conventional yeast loads and climatic factors in Chilean valleys. (A–C) Correspond to qPCR data expressed as yeast per grams (scale $\log_{10}$). **(A)** *Hanseniaspora/Metschnikowia*; **(B)** *Torulaspora/Saccharomyces/Meyerozyma*; **(C)** *Rhodotorula/Debaryomyces*. **(D–F)** Correspond to climatic factors. **(D)** Relative humidity (%); **(E)** Rainfall (mm); **(F)** Temperature (Celsius degree).

valleys at comparatively high loads with respect to Maipo and Maule valleys. Interestingly, the following three sets of yeast showed load patterns that were similar across valleys: *Hanseniaspora* and *Metschnikowia; Torulaspora, Saccharomyces, and Meyerozyma*; *and Rhodotorula and Debaryomyces* (**Figure 2**). The vineyard samples with the highest yeast load were the ones collected in Casablanca Valley, with a dominant presence of *Hanseniaspora* and *Metschnikowia*, with 10^7 yeasts per gram of fruit. *Torulaspora/Saccharomyces/Meyerozyma* showed a similar population trend along latitude, with high loads in Casablanca (10^5 yeasts per gram), lower toward the South (about $<10^3$ yeasts per gram), with a slight increase for the Itata vineyards. In contrast, the population load of *Rhodotorula* and *Debaryomyces* presented a similar behavior along latitude, maintaining loads between 10^3 and 10^5 yeasts per gram of fruit.

To try to explain the differences observed in population patterns depending on vineyard location, the relative humidity, rainfall, and temperatures of these areas were explored (**Figure 2**). The high population loads of *Hanseniaspora/Metschnikowia* observed in the Casablanca and Itata valleys were coincident with the highest relative humidity and rainfall observed for those valleys, respectively. Similarly, the load pattern of

Torulaspora/Saccharomyces/Meyerozyma could also be linked with the types of climate variations. They showed the highest load in valleys with low rainfall (Limarí, Casablanca, and Maipo), but their load was reduced in valleys with high rainfall (Colchagua, Maule, and Itata valleys). Finally, *Rhodotorula/Debaryomyces* seemed to be independent of relative humidity and rainfall, and their load might be linked to the temperatures observed in the valleys.

Identification of Yeast Isolates

More than 200 yeasts were isolated, and the results are summarized in **Figure 3**. Among them, 164 different sequences were identified as non-conventional yeasts matching 15 different yeast genera (97.3%) and *Saccharomyces* (2.7%). These results showed an important presence of non-conventional yeast isolates in Chilean valleys. The dimorphic *Aureobasidium* (24%) black yeast-like fungus was widely distributed in the Maipo and Maule valleys. The predominant non-conventional genera were *Metschnikowia* (21%), *Hanseniaspora* (18%), and *Rhodotorula* (13%). Minority genera included *Cryptococcus* (6%), *Hyphopichia* (2%), and *Candida* (2%). Other isolates corresponded to *Lachancea, Zygosaccharomyces, Sporidiobolus, Pichia, Meyerozyma, Debaryomyces, and Torulaspora*, which represented less than 1% as a group.

Among the total samples isolated, 61 yeast sequences were included in a phylogenetic analysis. **Supplementary Figure S1** shows the tree generated by the D1/D2 domain of the partial 26S rRNA sequences from *Metschnikowia* isolates, in which 16 different phylotypes can be observed, indicating the high genetic diversity of *Metschnikowia*. On the other hand, the analysis of *Rhodotorula* and *Hanseniaspora* based on D1/D2 sequences (**Supplementary Figure S2**) showed limited genetic diversity, with four phylotypes for *Rhodotorula* and six for *Hanseniaspora*. The observed diversity of phylotypes seems to be randomly distributed among vineyards rather than corresponding to the predominance of specific genotypes depending on the geographical areas.

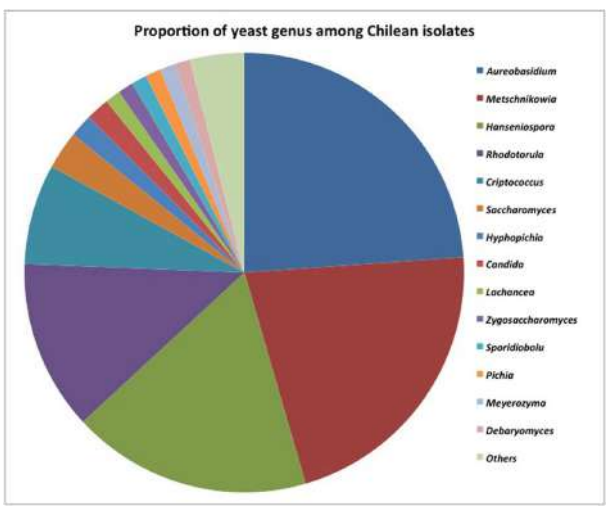

FIGURE 3 | Distribution of isolates retrieved from different Chilean valleys among yeast genera.

Selection of Isolates Using Micro-fermentations

To select non-conventional yeast with enological potential, all of the strains were tested for their resistance to SO_2. Micro-fermentations were performed in synthetic must, which was supplemented with metabisulfite to address this criterion. Only 106 isolates were able to tolerate metabisulfite. Then, the enological potential of these isolates was evaluated based on their ability to produce desirable aromas during micro-fermentation.

The attributes most frequently observed in the micro-fermentations were those belonging to the fruity, flowery, and fermentative aromas. Nineteen non-conventional yeasts were selected based on descriptive aromatic profiles (**Table 4**). These isolates corresponded to *Metschnikowia* (Casablanca and Itata valleys), *Hanseniaspora* (Maipo Valley), *Rhodotorula* (Limarí and Maipo valleys), *Hyphopichia* (Maipo and Itata valleys), *Candida* (Maule valley), *Lachancea* (Limarí valley), *Pichia* (Maipo valley), *Debaryomyces* (Maipo Valley), and *Citeromyces* (Limarí valley). The main aromatic attribute in *Debaryomyces* was dried fruits; in *Hanseniaspora* isolates were stone, tropical fruits, and sweet aromas; in *Pichia* isolates were tropical fruits and berries. Interestingly, several *Metschnikowia* isolates offered different attributes such as fermentative and sweet aromas, tropical, and stone fruits.

The fermentation abilities of the isolates were also tested. **Table 4** shows the residual sugar concentration at the end of the micro-fermentation. Some isolates such *Hanseniaspora* consumed almost all the sugar, while others consumed an intermediate range (*Metschnikowia*, *Lachancea*, and *Citeromyces*). Other isolates can be considered as poor fermenters, with less than 50% sugar consumption.

DISCUSSION

This study evaluated the load and diversity of non-conventional yeasts in Chilean vineyards and was the starting point of a study whose final goal is take advantage of the native microbiome by selection of local strains with interesting enological properties. Similarly to the animal microbiome, the plant microbiome may have important roles for their host, such as improving the availability of organic matter and preventing the growth pathogens through competition for space and nutrients (Gilbert et al., 2014). Studying the microbial ecology in the context of viticulture and wine, offers the opportunity to discover the denominated "microbial terroir" and the contribution of this assembly of microorganisms to the whole process and the final product. The microbial fingerprint for yeast in the Chilean vineyards has not been previously reported.

This study has covered a wide viticultural region (from 30°S to 36°S latitude approximately) that included the most important Chilean valleys and described the microbial fingerprinting based on the yeast of enological interest by using a culture independent approach. In contrast, Bokulich et al. (2014) used

TABLE 4 | Sensory description evalutaion and residual sugar from select isolates.

Isolates	Identification	Aromatic groups	Frequency of citation	Residual sugar g/L
PS PN2	*Candida*	Flowers/sweet aromas	100%	143.4
CHLI 14	*Citeromyces*	Fermentative aromas	66,6%	76.1
CARHP5	*Debaryomyces*	Dried fruits	100%	133.3
SB1 MP3	*Hanseniaspora*	Stone fruits/Tropical fruits	100%	74.0
CarLi 3C 1	*Hanseniaspora*	Tropical fruits	100%	21.5
SB2 MP1	*Hanseniaspora*	Stone fruits	100%	9.7
MALI66N 1	*Hanseniaspora*	Sweet aromas	83,3%	21.1
PS MG7	*Hyphopichia*	Cooked fruits	83,3%	84.2
CS AN2	*Hyphopichia*	Sweet aromas	100%	130.3
PNLI 29	*Lachancea*	Fermentative aromas	66,6%	77.8
ML CB1	*Metschinikowia*	Sweet aromas	83,3%	75.4
PN CB1	*Metschinikowia*	Sweet aromas	100%	85.8
PS MG4	*Metschinikowia*	Tropical fruits	100%	102.4
PS MG2	*Metschinikowia*	Stone fruits	100%	75.1
SB3 MP5	*Metschinikowia*	Sweet aromas	83,3%	142.2
SB2 MP5	*Pichia*	Tropical fruits/Berry fruits	100%	125.6
SB2 MP6	*Pichia*	Tropical fruits	100%	136.1
MB AN8	*Rhodotorula*	Flowers	100%	146.7

a high-throughput, short-amplicon sequencing approach to test the regional distribution of fungal and bacterial communities associated to vineyards in Napa and Sonoma valleys, which are located in the same latitude, covering from N 38°6.8′ to N 38°50.40′ approximately. The next generation sequencing approach allowed a deep examination of microbial communities (Bokulich et al., 2014) and detection of the differences between these close locations (Gilbert et al., 2014). Using a simpler approach to cover more distant locations (Hierro et al., 2006), we were able to establish that grape-surface microbial communities were different among Chilean regions. These differences in the microbial profiles may be related to climatic factors, as northern and southern regions of Chile present important discrepancies in the wheatear conditions.

Additionally, our results allowed us to identify co-variations in the loads of *Metschnikowia*/*Hanseniaspora, Torulaspora*/*Saccharomyces*/*Meyerozyma,* and *Rhodotorula*/*Debaryomyces* that were observed along latitude and associated with relative humidity, rainfall and temperature, respectively. These observations are consistent with studies by Gayevskiy and Goddard (2012), reporting that the proportions of these non-conventional yeasts varied in each sampling zone. Several authors attribute these changes to the geographical location and climatic conditions of the vineyard (Parish and Carroll, 1985; Longo et al., 1991), and it has also been suggested that rainfall could be among the most important factors affecting the load of non-conventional yeasts (Rousseau and Doneche, 2001). Combina et al. (2005), reported that rain near harvest can induce changes in yeast populations, affecting *Metschnikowia* and *Hanseniaspora*. Similarly, Itata valley showed the highest rainfall during the harvest period, which coincided with a high load of *Metschnikowia*/*Hanseniaspora*. Recent studies by Brilli et al. (2014) showed the influence of relative humidity on non-conventional yeast populations, indicating that an increase in relative humidity might induce higher loads of non-conventional yeast during grape ripening. Similar studies by Van der Westhuizen et al. (2000a,b), confirmed that yeast loads observed in coastal areas were higher than in the inland area. Therefore, those studies support the idea that higher load of *Metschnikowia*/*Hanseniaspora* in Casablanca valley, located near to the coast, may be related to relative humidity, as this parameter is highest due to the pronounced maritime influence. Those reports showed that rainfall and relative humidity favored a prevalence of *Hanseniaspora* and *Metschnikowia* yeast on the grape–berries, which could explain the co-variation observed in the Chilean valleys. Contrasting results were obtained for *Torulaspora*/*Saccharomyces*/*Meyerozyma,* which seems to vary inversely with rainfall. However, these results have not been reported previously and demand more research efforts to define the negative influence of the rainfall on the load of those yeasts.

Based on the culture dependent approach, our results showed that *Hanseniaspora*, *Metschnikowia,* and *Aureobasidium* were the main genera present on grape–berries in all of the vineyards studied in Chile. These observations are consistent with the findings reviewed by Bisson and Joseph (2009) and Barata et al. (2012) establishing that those genera, along with *Candida*, were the main ones present on grape–berries examined in Spain, Canada, and Argentina. Molecular biology techniques provide a simple and rapid method to differentiate yeasts based on their genetic background (Granchi et al., 1998; Torija et al., 2001). The modern taxonomy of yeasts has been improved by molecular biology techniques providing reliable methods to differentiate yeasts based on their genetic background, mainly the phylogenetic analysis of conserved DNA and protein sequences. Repeats of the chromosomal rDNA sequences have been widely used for the identification and barcoding of yeast genera and

species. The D1/D2 rDNA sequences are frequently used in the phylogenetic analysis of yeast, where yeast isolates differing by more than 1% substitutions in the D1/D2 domain represent separate species (Kurtzman and Robnett, 1998). The comparison of D1/D2 domains in our *Metschnikowia* isolates from different Chilean valleys showed that the high genetic diversity was related to 26 polymorphic sites that generate 16 phylotypes. These results agreed with the findings of Sipiczki et al. (2013), which reported 18 and 25 substitutions in the D1/D2 domain in *Metschnikowia* species.

Non-conventional yeasts influenced the wine aroma: terpenes, thiols, esters, and higher alcohols are the most typical aromatic compounds that contribute to the enhancement of sweet-fruity aromas in wines (Mason and Dufour, 2000; Clemente-Jimenez et al., 2004; Sumby et al., 2010; Viana et al., 2011; Gobbi et al., 2013; Jolly et al., 2014). Most of the evidence of the effect of non-conventional yeasts has been obtained from co-inoculation studies combining non-conventional yeast and *Saccharomyces* in different wines. Medina et al. (2012) indicated that the inoculation of *Hanseniaspora* isolates in Chardonnay produced a uniquely fruity character, such as banana, pear apple, citric fruits, grape fruit, and guava. *Metschnikowia* has been used in base wine for sparkling wine production, improving the aromatic profile by increasing smoky and flowery notes (González-Royo et al., 2015). In another example, it was reported that *Debaryomyces* increased the concentrations of citronellol, nerol, and geraniol, which resulted in floral and citrus-type aromas in wine (Garcia et al., 2002); however, the *Debaryomyces* isolate obtained in this study showed a different property, improving dried fruit aromas. These studies have demonstrated that non-conventional yeast can be selected based on their ability to produce aromatic secondary metabolites that contribute to improving the quality of wine, which is presently a very important area of applied interest in oenology.

In our study, we focused on the selection of different non-conventional yeasts as a basis for the selection of interesting strains to be used by the wine industry. In our hands, the most promising yeast strains corresponded to the *Hanseniaspora, Metschnikowia,* and *Debaryomyces* genera. Therefore, our isolates might influence the wine quality through the release of several aromatic compounds, such as esters, higher alcohols, acids, and monoterpenes. Thus, further research to analyze the potential of these strains is underway to select new starter presentations for the wine industry. Furthermore, these non-conventional yeasts reveal the potential of the yeast microbiome to contribute to the complexity and typicality of the wine and conferring the aromatic profiles of specific regions. This possibility is another aspect that is currently being developed in our laboratories.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: CJ, VL, AM, and JR. Performed the experiments: CJ and JR. Generated and analyzed the data: CJ, VL, AM, and JR. Wrote the paper: CJ, VL, AM, and JR.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2016.00663

FIGURE S1 | Phylogenetic analysis of Chilean of *Metschnikowia* using D1/D2 domain. Two sequences from GenBank were included as reference (KM249369 and KM658981).

FIGURE S2 | Phylogenetic analysis of Chilean isolates of using D1/D2 domain for *Hanseniaspora* and *Rhodotorula*.

REFERENCES

Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W., et al. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* 25, 3389–3402. doi: 10.1093/nar/25.17.3389

Barata, A., Malfeito-Ferreira, M., and Loureiro, V. (2012). The microbial ecology of wine grape berries. *Int. J. Food Microbiol.* 153, 243–259. doi: 10.1016/j.ijfoodmicro.2011.11.025

Beltran, G., Torija, M. J., Novo, M., Ferrer, N., Poblet, M., Guillamón, J. M., et al. (2002). Analysis of yeast populations during alcoholic fermentation: a six year follow-up study. *Syst. Appl. Microbiol.* 293, 287–293. doi: 10.1078/0723-2020-00097

Bisson, L. F., and Joseph, C. M. L. (2009). "Yeasts," in *Biology of Microorganisms on Grapes, in Must and in Wine*, eds H. König, G. Unden, and J. Fröhlich (Berlin: Springer), 47–60.

Bokulich, N. A., Thorngate, J. H., Richardson, P. M., and Mills, D. A. (2014). microbial biogeography of wine grapes is conditioned by cultivar, vintage, and climate. *Proc. Natl. Acad. Sci. U.S.A.* 111, 139–148. doi: 10.1073/pnas.1317377110

Brilli, L., Buscioni, G., Moriondo, M., Bindi, M., and Vincenzini, M. (2014). Influence of interannual meteorological variability on yeast content and composition in Sangiovese grapes. *Am. J. Enol. Vitic.* 65, 375–380. doi: 10.5344/ajev.2014.13116

Carrau, F. M., Medina, K., Boido, E., Farina, L., Gaggero, C., Dellacassa, E., et al. (2005). De novo synthesis of monoterpenes by *Saccharomyces cerevisiae* wine yeasts. *FEMS Microbiol. Lett.* 243, 107–115. doi: 10.1016/j.femsle.2004.11.050

Ciani, M., Comitini, F., Mannazzu, I., and Domizio, P. (2010). Controlled mixed culture fermentation: a new perspective on the use of non-*Saccharomyces* yeasts in winemaking. *FEMS Yeast Res.* 10, 123–133. doi: 10.1111/j.1567-1364.2009.00579.x

Clemente-Jimenez, J. F., Mingorance-Cazorla, L., Martínez-Rodríguez, S., Las Heras-Vázquez, F. J., and Rodríguez-Vico, F. (2004). Molecular characterization and oenological properties of wine yeasts isolated during spontaneous fermentation of six varieties of grape must. *Food Microbiol.* 21, 149–155.

Combina, M., Elía, A., Mercado, L., Catania, C., Ganga, A., and Martinez, C. (2005). Dynamics of indigenous yeast populations during spontaneous fermentation of wines from Mendoza, Argentina. *Int. J. Food Microbiol.* 99, 237–243. doi: 10.1016/j.ijfoodmicro.2004.08.017

Constantí, M., Poblet, M., Arola, L., Mas, A., and Guillamón, J. M. (1997). Analysis of yeast populations during alcoholic fermentation in a newly established winery. *Am. J. Enol. Viticult.* 48, 339–344.

Cordente, A. G., Curtin, C. D., Varela, C., and Pretorius, I. S. (2012). Flavour-active wine yeasts. *Appl. Microbiol. Biotechnol.* 96, 601–618. doi: 10.1007/s00253-012-4370-z

Esteve-Zarzoso, B., Belloch, C., Uruburu, F., and Querol, A. (1999). Identification of yeasts by RFLP analysis of the 5.8S rRNA gene and the two ribosomal internal transcribed spacers. *Int. J. Syst. Bacteriol.* 49, 329–337. doi: 10.1099/00207713-49-1-329

Fleet, G. (2003). Yeast interactions and wine flavour. *Int. J. Food Microbiol.* 86, 11–22. doi: 10.1016/S0168-1605(03) 00245-9

Fleet, G. H. (1993). "The microorganisms of winemaking-isolation enumeration and identification," in *Wine Microbiology and Biotechnology*, ed. G. H. Fleet (Chur: Harwood Academic Publishers), 1–27.

Garcia, A., Carcel, C., Dulau, L., Samson, A., Aguera, E., Agosin, E., et al. (2002). Influence of a mixed culture with *Debaryomyces vanriji* and *Saccharomyces cerevisiae* on the volatiles of a Muscat wine. *J. Food Sci.* 67, 1138–1143. doi: 10.1111/j.1365-2621.2002.tb09466.x

Gayevskiy, V., and Goddard, M. R. (2012). Geographic delineations of yeast communities and populations associated with vines and wines in New Zealand. *ISME J.* 6, 1281–1290. doi: 10.1038/ismej.2011.195

Gilbert, J. A., van der Lelie, D., and Zarraonaindia, I. (2014). Microbial terroir for wine grapes. *Proc. Natl. Acad. Sci. U.S.A.* 111, 5–6. doi: 10.1073/pnas.1320471110

Gobbi, M., Comitini, F., Domizio, P., Romani, C., Lencioni, L., Mannazzu, I., et al. (2013). *Lachancea thermotolerans* and *Saccharomyces cerevisiae* in simultaneous and sequential co-fermentation: a strategy to enhance acidity and improve the overall quality of wine. *Food Microbiol.* 33, 271–281. doi: 10.1016/j.fm.2012.10.004

González-Royo, E., Pascual, O., Kountoudakis, N., Esteruelas, M., Esteve-Zarzoso, B., Mas, A., et al. (2015). Oenological consequences of sequential inoculation with non-*Saccharomyces* yeasts (*Torulaspora delbrueckii* or *Metschnikowia pulcherrima*) and *Saccharomyces cerevisiae* in base wine for sparkling wine production. *Eur. Food Res. Technol.* 240, 999–1012. doi: 10.1007/s00217-014-2404-8

Granchi, L., Ganucci, D., Messini, A., Rosellini, D., and Vicenzini, M. (1998). Dynamics of yeast populations during the early stages of natural fermentations for the production of Brunello de Montalcino wines. *Food Technol. Biotechnol.* 36, 313–318.

Hierro, N., Esteve-Zarzoso, B., González, A., Mas, A., and Guillamón, J. M. (2006). Real-time quantitative PCR (QPCR) and reverse transcription-QPCR (RT- QPCR) for the detection and enumeration of total yeasts in wine. *Appl. Environ. Microbiol.* 72, 7148–7155. doi: 10.1128/AEM.00388-06

Jolly, N. P., Varela, C., and Pretorius, I. S. (2014). Not your ordinary yeast: non-*Saccharomyces* yeasts in wine production uncovered. *FEMS Yeast Res.* 14, 215–237. doi: 10.1111/1567-1364.12111

Kurtzman, C. P., and Robnett, C. J. (1998). Identification and phylogeny of Ascomycetous yeasts from analysis of nuclear large subunit (26S) ribosomal DNA partial sequences. *Antonie van Leeuwenhoek* 73, 331–371. doi: 10.1023/A:1001761008817

Longo, E., Cansado, J., Agrelo, D., and Villa, T. G. (1991). Effect of climatic conditions on yeast diversity in grape musts from Northwest Spain. *Am. J. Enol. Vitic.* 42, 141–144.

Mason, A. B., and Dufour, J. P. (2000). Alcohol acetyltransferases and the significance of ester synthesis in yeast. *Yeast* 16, 1287–1298. doi: 10.1002/1097-0061(200010)16:14<1287::AID-YEA613>3.0.CO;2-I

Medina, K., Boido, E., Dellacassa, E., and Carrau, F. (2012). Growth of non-*Saccharomyces* yeasts affects nutrient availability for *Saccharomyces cerevisiae* during wine fermentation. *Int. J. Food Microbiol.* 157, 245–250. doi: 10.1016/j.ijfoodmicro.2012.05.012

Medina, K., Boido, E., Fariña, L., Gioia, O., Gomez, M. E., Barquet, M., et al. (2013). Increased flavour diversity of Chardonnay wines by spontaneous fermentation and co-fermentation with *Hanseniaspora vineae*. *Food Chem.* 141, 2513–2521. doi: 10.1016/j.foodchem.2013.04.056

Mills, D. A., Johannsen, E. A., and Cocolin, L. (2002). Yeast diversity and persistence in botrytis-affected wine fermentations. *Appl. Environ. Microbiol.* 68, 4884–4893. doi: 10.1128/AEM.68.10.4884-4893.2002

Moreira, N., Mendes, F., de Pinho, R. G., Hogg, T., and Vasconcelos, I. (2008). Heavy sulphur compounds, higher alcohols and esters production profile of *Hanseniaspora uvarum* and *Hanseniaspora guilliermondii* grown as pure and mixed cultures in grape must. *Int. J. Food Microbiol.* 124, 231–238. doi: 10.1016/j.ijfoodmicro.2008.03.025

Parish, M. E., and Carroll, D. E. (1985). Indigenous yeasts associated with Muscadine (*Vitis rotundifolia*) grapes and musts. *Am. J. Enol. Vitic.* 36, 165–169.

Ribereau-Gayon, P., Dubourdieu, D., Doneche, B., and Lonvaud, A. (2006). *The Microbiology of Wine and Vinifications: Handbook of Enology*. Chichester: John Wiley & Sons Ltd.

Rousseau, S., and Doneche, B. (2001). Effects of water activity (aw) on the growth of some epiphytic micro-organisms isolated from the grape berry. *Vitis* 40, 75–78.

Setati, M. E., Jacobson, D., Andong, U.-C., and Bauer, F. (2012). The vineyard yeast microbiome, a mixed model microbial map. *PLoS ONE* 7:e52609. doi: 10.1371/journal.pone.0052609

Sipiczki, M., Pfliegler, W. P., and Holb, I. J. (2013). *Metschnikowia* species share a pool of diverse rRNA genes differing in regions that determine hairpin-loop structures and evolve by reticulation. *PLoS ONE* 8:e67384. doi: 10.1371/journal.pone.0067384

Sumby, K. M., Paul, R., Grbin, P. R., and Jiranek, W. (2010). Microbial modulation of aromatic esters in wine: current knowledge and future prospects. *Food Chem.* 121, 1–16. doi: 10.1016/j.foodchem.2009.12.004

Torija, M. J., Rozés, N., Poblet, M., Guillamón, J. M., and Mas, A. (2001). Yeast population dynamics in spontaneous fermentations: comparison between two different wine-producing areas over a period of three years. *Antonie Van Leeuwenhoek* 79, 345–352. doi: 10.1023/A:1012027718701

Van der Westhuizen, T. J., Augustyn, O. P. H., Khan, W., and Pretorius, I. S. (2000a). Seasonal variation of indigenous *Saccharomyces cerevisiae* strains isolated from vineyards of the Western Cape in South Africa. *S. Afr. J. Enol. Vitic.* 21, 10–16.

Van der Westhuizen, T. J., Augustyn, O. P. H., and Pretorius, I. S. (2000b). Geographical distribution of indigenous *Saccharomyces cerevisiae* strains isolated from South African vineyards in the cooler, coastal regions of the Western Cape in South Africa. *S. Afr. J. Enol. Vitic.* 21, 3–9.

Varela, C., Siebert, T., Cozzolino, D., Rose, L., McLean, H., and Henschke, P. A. (2009). Discovering a chemical basis for differentiating wines made by fermentation with 'wild' indigenous and inoculated yeasts: role of yeast volatile compounds. *Aust. J. Grape Wine Res.* 15, 238–248. doi: 10.1111/j.1755-0238.2009.00054.x

Viana, F., Belloch, C., Vallés, S., and Manzanares, P. (2011). Monitoring a mixed starter of *Hanseniaspora vineae–Saccharomyces cerevisiae* in natural must: impact on 2-phenylethyl acetate production. *Int. J. Food Microbiol.* 151, 235–240. doi: 10.1016/j.ijfoodmicro.2011.09.005

Viana, F., Gil, J. V., Genovés, S., Vallés, S., and Manzanares, P. (2008). Rational selection of non-*Saccharomyces* wine yeasts for mixed starters based on ester formation and enological traits. *Food Microbiol.* 25, 778–785. doi: 10.1016/j.fm.2008.04.015

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Production of Volatile and Sulfur Compounds by 10 *Saccharomyces cerevisiae* Strains Inoculated in Trebbiano Must

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In wines, the presence of sulfur compounds is the resulting of several contributions among which yeast metabolism. The characterization of the starter *Saccharomyces cerevisiae* needs to be performed also taking into account this ability even if evaluated together with the overall metabolic profile. In this perspective, principal aim of this experimental research was the evaluation of the volatile profiles, throughout GC/MS technique coupled with solid phase micro extraction, of wines obtained throughout the fermentation of 10 strains of *S. cerevisiae*. In addition, the production of sulfur compounds was further evaluated by using a gas-chromatograph coupled with a Flame Photometric Detector. Specifically, the 10 strains were inoculated in Trebbiano musts and the fermentations were monitored for 19 days. In the produced wines, volatile and sulfur compounds as well as amino acid concentrations were investigated. Also the physico-chemical characteristics of the wines and their electronic nose profiles were evaluated.

Keywords: ***Saccharomyces cerevisiae*, sulfur compounds, volatile compounds, Trebbiano wine, electronic nose**

INTRODUCTION

The wine flavor and aroma are the result of several interactions between a huge amount of chemical compounds and sensory receptors. The wine flavor can be the sum of varietal (deriving from the grapes), pre-fermentative (deriving from grape crushing and must conditioning), fermentative (generated during fermentations by yeasts and/or bacteria), and post-fermentative flavors (generated by the wood release or the chemical transformation during conservation; Swiegers et al., 2005).

However, volatiles from fermentation largely dominate wine flavor, since yeasts metabolize grape sugars and other components into ethanol, carbon dioxide, and hundreds of secondary end-products, contributing to the wine character (Fleet, 2003). The aroma compounds from yeast metabolisms are constituted by higher alcohols, esters, organic acids and aldehydes (Lambrechts and Pretorius, 2000; Vernocchi et al., 2011, 2015). The amount of these compounds constitutes the overall expression of the fermentative flavor and, if in excess, some of them (i.e., acetic acid, acetaldehyde) may also be regarded as undesirable (Liu and Pilone, 2000; Styger et al., 2011).

Also sulfur compounds, which can be considered a "double-edged sword," can contribute positively or negatively to wine aroma (Vichi and Cortes-Francisco, 2015). Positive examples are furfurylthiol (roast coffee' aroma) (Tominaga et al., 2000) and the "fruity" polyfunctional thiols 3-mercaptohexan-1-ol (3MH), 4-mercapto-4-methyl-pentan-2-one (4MMP), and 3-mercaptohexyl acetate (3MHA), that impart passion fruit, grapefruit, gooseberry, guava, and "box hedge" aromas (Swiegers et al., 2005; Swiegers and Pretorius, 2007). In particular, these thiols affect the distinctive sensory characteristics of wines made from the grape variety Sauvignon Blanc (Harsch and Gardner, 2013). On the contrary, the highly volatile sulfur compounds (HVSC) have a negative impact in wine because, with their low odor threshold (in the order of ppb), they imparts a powerful odor described as soup-like, meaty, boiled potato, rotten egg-like off-flavor, and cooked cabbage aroma (Vermeulen and Gus, 2005; Davis and Qian, 2011; Franco-Luesma and Ferreira, 2014). Commonly found HVSCs include methanethiol, dimethyl sulphide (DMS), dimethyl disulphide (DMDS), dimethyl trisulphide (DMTS), 3-methylthio-1-propanal (methional), 3-methylthio-1-propanol (methionol), and *S*-methylthioesters of short-chain fatty acids (acetate, propanoate, and butanoate). Also the production of H_2S represents in winemaking a global problem, resulting in a loss of wine quality and a rejection from the consumers. During alcoholic fermentation, *Saccharomyces cerevisiae* can be responsible for the production of several sulfur compounds *via* the sulfate reduction pathway (Swiegers and Pretorius, 2007), but the majority of H_2S produced during winemaking occurs as a result of the biosynthesis of the sulfur containing amino acids, methionine, and cysteine, which occur in low concentrations in grape juice, through the sulfate reduction sequence (SRS). However, for sulfides as for all the other classes of volatile compounds, the yeast strain used for fermentation is the main factor influencing their production (Rainieri and Pretorius, 2000; Fleet, 2008). However, their perception in wine is related to the other volatile compounds, and it is also the result of the interaction with non-volatile molecules. Because sulfur compounds are present in wine at very low concentrations, they are usually determined by gas-chromatographic techniques and their detection represents a methodological challenge. Headspace techniques are generally preferred due to the high volatility of these compounds and their relatively low solubility in organic solvents. Consequently, simple static headspace or headspace solid phase microextraction are generally used for their extraction and the sulfur chemiluminiscence (SCD) and the flame photometric detectors (FPD) for their detection (Franco-Luesma and Ferreira, 2014).

Thus, the main goal of this research was characterize 10 strains of *S. cerevisiae*, endowed for good oenological properties, producers of H_2S in strain dependent way, also for the production of HVSC, by using a suitable and reliable technique, since they are fundamental for the sensory wine features but deeply investigated. Because, as previously underlined, the wine volatile profiles are the outcome of the ratio and interaction of several molecules (volatile and not), also the production of volatile compounds and electronic nose profiles were investigated to evaluate the strain volatile fingerprinting and their effects on wine features. In order to verify their potential use for the production of Trebbiano wine, the strains were inoculated in Trebbiano must determining also the fermentation kinetics and the aminoacidic compositions.

MATERIALS AND METHODS

Strains

Ten *S. cerevisiae* strains (L234, L288, L674, L951, M630, M692, U5298, 6944, 7541, 6644), able to produce in strain dependent way H_2S belonging to ASTRA srl, Faenza, Italy, were employed in the research (**Table 1**).

Before using, the frozen strains were sub-cultured three times in Sabouraud broth medium (Oxoid, Basingstoke, UK) at 28°C for 48 h.

Micro-Vinifications

Grape must of Trebbiano variety (vintage 2011) was used to test the effects of the different strains of *S. cerevisiae* on wine characteristics. Until the use, the must was kept frozen. The Trebbiano must features are reported in **Table 2**. Before inoculation, the must was flash pasteurized (70°C for 20 s). The fermentations were carried out in 500-ml flasks filled with 400 ml of Trebbiano must. For each strain considered, three different micro-vinifications were performed. Each strain was inoculated at level of about 6 Log cfu ml^{-1}. The inoculations were performed using 48-h pre-cultures in the same must. The temperature was kept at 18°C during alcoholic fermentation. The weight lost was used to follow the fermentation process. After the completion of alcoholic fermentation, the different wine samples were separated by filtration.

Chemical Analyses

Residual sugars, SO_2, ethanol, pH, and total acidity were performed according to the Official EU Methods (OJEU, 2010).

TABLE 1 | Main features of the *Saccharomyces cerevisiae* strains used in the research.

Identifier	Strain	H2S Production
A	L234	++++*
B	L288	+**
C	L674	+++***
D	L951	+
E	M630	−****
F	M692	+++
G	U5298	+++
H	6944	+
I	7541	++
L	6644	−

*Very high producer; **Low producer; ***High producer; ****no production.

TABLE 2 | Enological features of Trebbiano wines in relation to the strain used for fermentation.

Strain	Sugars (g l^{-1})	SO_2 (mg l^{-1})	Total acidity (expressed as g l^{-1} of tartaric acid)	Succinic acid (g l^{-1})	Malic acid (g l^{-1})	Lactic acid (g l^{-1})	ABV (%vol) ABV (%vol)	pH pH
Must	227 ± 11^{A}	–*	9.66 ± 0.25^{A}	0.05 ± 0.01^{A}	7.49 ± 1.13^{A}	0.06 ± 0.02^{A}	–*	3.21 ± 0.01^{A}
A	0.75 ± 0.15^{B}	5.0 ± 1.0^{A}	6.98 ± 0.15^{B}	1.74 ± 0.02^{B}	1.90 ± 0.45^{B}	0.80 ± 0.10^{B}	13.56 ± 0.55^{A}	3.18 ± 0.01^{B}
B	0.86 ± 0.13^{B}	5.0 ± 0.8^{A}	5.48 ± 0.21^{C}	0.59 ± 0.05^{C}	0.56 ± 0.10^{C}	0.02 ± 0.0^{C}	$13.48 \pm 0.60^{A,B}$	3.22 ± 0.02^{A}
C	1.03 ± 0.20^{BC}	5.0 ± 1.1^{A}	5.93 ± 0.18^{D}	$0.37 \pm 0.09^{D,E}$	$0.40 \pm 0.08^{C,D}$	0.07 ± 0.0^{A}	$13.40 \pm 0.45^{A,B}$	3.27 ± 0.01^{C}
D	8.58 ± 0.45^{D}	8.0 ± 0.5^{B}	10.65 ± 0.45^{E}	0.19 ± 0.03^{F}	0.05 ± 0.01^{E}	0.43 ± 0.04^{D}	12.70 ± 0.15^{C}	3.14 ± 0.01^{D}
E	1.57 ± 0.10^{E}	8.0 ± 0.7^{B}	$10.20 \pm 1.1^{E,A}$	1.02 ± 0.09^{G}	0.22 ± 0.01^{F}	0.61 ± 0.06^{E}	13.21 ± 0.25^{B}	3.20 ± 0.0^{A}
F	2.54 ± 0.25^{F}	8.0 ± 1.1^{B}	7.20 ± 0.13^{B}	0.28 ± 0.03^{D}	0.17 ± 0.05^{F}	0.35 ± 0.01^{F}	$13.11 \pm 1.12^{A,B,C}$	$3.13 \pm 0.02^{D,E}$
G	3.12 ± 0.18^{G}	8.0 ± 1.2^{B}	12.00 ± 0.15^{F}	0.38 ± 0.04^{E}	0.03 ± 0.01^{E}	0.07 ± 0.01^{A}	$12.98 \pm 0.95^{A,B,C}$	3.11 ± 0.01^{E}
H	1.62 ± 0.10^{E}	10.0 ± 0.9^{C}	7.76 ± 0.85^{B}	0.94 ± 0.05^{G}	1.32 ± 0.03^{G}	0.03 ± 0.0^{G}	$13.18 \pm 0.39^{A,B,C}$	3.05 ± 0.02^{F}
I	1.26 ± 0.08^{C}	13.0 ± 1.1^{D}	$5.10 \pm 1.10^{C,D}$	0.72 ± 0.02^{H}	0.31 ± 0.04^{D}	0.09 ± 0.0^{H}	$13.38 \pm 0.25^{A,B}$	3.27 ± 0.01^{C}
L	3.49 ± 0.10^{H}	5.00 ± 0.2^{A}	7.91 ± 0.94^{B}	0.29 ± 0.01^{D}	0.88 ± 0.02^{H}	0.02 ± 0.0^{C}	$13.20 \pm 1.01^{A,B,C}$	3.06 ± 0.02^{F}

**not performed; For each column considered, values with the same superscript letter are not statistically different ($P > 0.05$).*

Determination of Volatile Compound Profiles

The volatile molecule profiles of Trebbiano wines were analyzed by solid-phase microextraction coupled with gas chromatography-mass spectrometry (SPME-GC-MS) according to the method of Vernocchi et al. (2015). A polyacrylate-coated fiber (85 μm; Supelco, Bellefonte, PA) and a manual SPME holder (Supelco) were used after preconditioning, according to the manufacturer's guidelines. Before each head-space sampling, the fiber was exposed to the gas chromatograph inlet for 5 min for thermal desorption at 250°C in a blank sample. Five milliliter of wine samples were placed in 10 ml glass vials, with 1 g NaCl and 10 μL 4-methyl-2-pentanol (initial concentration of 10000 mg l^{-1})(Sigma, Milan, Italy) as internal standard. The samples were then heated for 10 min at 45°C. The SPME fiber was exposed to each sample for 40 min. Both the equilibration and absorption phases were carried out under stirring. The fiber was then inserted into the injection port of the gas chromatograph for a 5-min sample desorption. GC–MS analyses were performed on an Agilent 7890A (Agilent Technologies, Palo Alto, CA) coupled to an Agilent 5975C mass selective detector operating in electron ionization mode (ionization voltage 70 eV) and using a Chrompack CP-Wax 52 CB capillary column (50 m, 0.32 mm i.d.; Chrompack, Middelburg, Netherlands). Volatile compounds were separated using helium as carrier gas (1 ml min^{-1}). The temperature program was 50°C for 2 min, then programmed at 1.5°C min^{-1} to 65°C, and finally at 4.5°C min^{-1} to 220°C, which was maintained for 20 min. Injector, interface, and ion source temperatures were 250, 250, and 230°C, respectively. Identification of the compounds detected in the wine samples was performed comparing mass spectra of compounds with those contained in an available database (NIST version 2005) and those of pure standards.

Determination of Sulfur Compounds

The extraction of sulfur compounds from wine was performed by using the method proposed by Moreira et al. (2002). Briefly, 50 ml of wine were extracted twice with 5 ml of dichloromethane after the addition of 4 grams of sodium sulfate and of 500 μl of i.s. [ethyl (methylthio)acetate] at 500 μg l^{-1} to have a final concentration of 50 μg l^{-1}. The two organic phases were mixed and the solution was concentrated to 1/10 under a nitrogen flow. Finally, 2 μL of the extract was injected into the chromatograph.

For the analyses, a gas-chromatograph equipped with a Flame-Photometric-Detector (Clarus 500, Perkinelmer) fitted with a 30 m Elite-5 (Supelco, Bellefonte, PA, USA) (i.d. 0.53 mm) column was used. The identification was based on the comparison of the peak retention times with those of pure standards while the quantification was performed by using calibration curves, obtained with reagents Pure standards (>95%) of methanethiol and ethanethiol from Fluka (Steinheim, Germany), dimethylsufide from Merck (Darmstadt, Germany), sodium sulfide, ethylmethylsulfide, 1-propanethiol, thiophene, diethyldisulfide, dimethyldisulfide, diethylsulfide from Sigma-Aldrich (Steinheim, Germany).

Determination of the Amino Acids Release in Wine

The analysis of amino acids in wine was performed according to the method proposed by Ndagijimana et al. (2010, unpublished data).

One ml of of NaOH 1% was added to 1 ml of standard solution or to 1 ml of freeze dried samples supernatants in a silanized micro reaction vessel and vortexed for 10 s. Two-hundred microliter of the mixture were collected in a new micro reaction vessel, added with methanol and pyridine and vortexed for 10 s in presence of 10 μL of decanoic acid (10.000 ppm—solution in ethanol 70%). The following ratios of aqueous phase/methanol/pyridine was used 6:2.1.

An increasing volume of ECF (18 ul) was then added to the mixture to evaluate the efficiency of the derivatizing agent and the mixture was vortexed for 20 s. The same procedure was repeated twice. In order to extract the derivatized analytes, 400 ul of chloroform were added and the mixture vortexed

for 20 s. The control of the pH of the reaction medium was performed by means of addition of 400 ul of sodium bicarbonate 50 mM. In order to remove traces of water, anhydrous sodium sulfate was added then the organic phase was carefully collected in a glass silanized conical tube and subjected to GC/MS analysis. The derivatized extracts (both form culture and from standards) were analyzed with a Agilent 7890 gas chromatograph coupled with a 5973C mass spectrometer (Agilent Technologies, USA). One microliter of the extracts was injected into a SPB5 capillary column coated with 5% diphenyl cross-linked 95% dimethylpolysiloxane (60 m × 250 μm i.d., 0.25-μm film thickness; Supelco, Palo Alto, USA) in the split mode (30:1). Preliminary experiments, described in the results, permitted to choose the subsequent conditions. The injection and interface temperatures were set to 250°C and the ion source temperature was adjusted to 200°C. Initial GC oven temperature was 80°C; 2 min after injection, the GC oven temperature was raised to 140°C with 10°C min^{-1}, to 240°C at a rate of 4°C min^{-1}, to 280°C with 10°C min^{-1} again, and finally held at 280°C for 3 min. Helium was used as the carrier gas with a flow rate of 1 mL min^{-1}. The analyses were performed with electron impact ionization (70 eV) in the full scan mode (m/z 30–550).

The identification of analytes was performed by comparison of their retention times and their mass spectra data with those of pure standards analyzed under the same conditions. Moreover, the retention index of the analytes of interest was calculated by means of results related to a mixture of n-alcanes (C10-C24) analyzed under the GC-MS conditions above described. The following equation was used for the calculation of retention index:

$$RI\,(x) = 100 \times z + 100 \times \frac{\mathrm{RT(x)} - \mathrm{RT(z)}}{\mathrm{RT}\,(z+1) - \mathrm{RT}(z)}$$

where *RI(x)* is the retention index of the unknown analyte, z is the number of carbon atoms of the n-alkane eluting before the analyte unknown and (z + 1) is the number of carbon atoms of the n-alkane eluting after the peak of interest, RT(x) is the retention time of analyte unknown, RT(z) is the retention time of the n-alkane eluting before the analyte unknown and RT(z+1) is the retention time of of the n-alkane eluting after the peak of interest. All the GC–MS raw files were converted to netCDF format via Chemstation (Agilent Technologies, USA) and subsequently processed by the XCMS toolbox (http://metlin.scripps.edu/download/). XCMS software allows an automatic and simultaneous retention time alignment, matched filtration, peak detection, and peak matching. The resulting table containing information such as peak index (retention time-m/z pair) and normalized peak area was exported into R (www.r-project.org) for subsequent statistical analysis.

Determination of Electronic Nose Profiles

The electronic nose profiles of the different Trebbiano wines were recorded using a Pen2 Electronic Nose (Airsense Analytics GmbH, Schwerin, Germany) composed of an array of 10 temperature-moderated metal-oxide sensors (MOS), a sampling system, a data acquisition system, and a data processing system. Each sensor is sensible to different kind of volatile molecules For the analysis, 5 ml of wine sample was placed in 40 ml glass vials hermetically sealed and warmed at 28°C for 1 h. After warming, injections were performed at 180°C. For each sample, three repetitions were performed.

Ten different sensors were used: s1 (WMA-CCTO1), s2 (WMA-US5), s3 (WMA-CCTO2), s4 (WMA-US6), s5 (WMA-CCTO3), s6 (WMA-US1), s7 (WMA-CW1), s8 (WMA-US2), s9 (WMA-CW3), and s10 (WMA-U3). Each sensor is sensible to different kind of volatile molecules i.e., s1 for aromatic, s2 for generic compounds, s3 for aromatic, s4 for hydrogenated, s5 for aromatic-aliphatic, s6 for hydrocarbons, s7 for sulfur, s8 for alcohols, s9 for sulfur chlorides, s10 for hydrocarbons-aliphatic. During the analysis the response of the sensors were monitored at 1 s intervals for an overall time of 95 s at a flow rate of 400 mL/min. The sensor data were expressed as the ratio between signal sensor and minimum signal sensor recorded (data not showed). The signal evaluation was done following the method reported by Sado Kamden et al. (2007), in order to find out which are the most indicative signals for the evaluation of the differences among the samples.

Statistical Analysis

Microvinification were performed in triple. The data obtained are the mean of three independent repetitions. The electronic nose analyses, for each independent experiment, were repeated five times.

The oenological were analyzed by 1-way Anova using the statistical package Statistica for Window (Statsoft Inc. Tulsa, OK). The ability of each parameter to discriminate among the samples was investigated according to the *post-hoc* comparison of the Anova.

For volatile compounds and amino acids the variability coefficient was reported.

The raw data obtained for electronic nose were subjected to principal component analysis (PCA) by using Statistica (Package for Window).

RESULTS

Fermentation Kinetics and Wine Analytical Profile

In order to evaluate the effects of yeast strain on the physicochemical wine characteristics, Trebbiano musts were inoculated with the 10 strains at level of about 6 Log cfu ml^{-1}.

The fermentation kinetics were evaluated measuring the weight loss of musts during fermentation at 18°C, as shown by **Figure 1**. Data obtained indicated that strains L674 (C), L951(D), M692(F), U5298 (G), 7541(I), and 6644 (L) had similar kinetics, characterized by a reduced amount of fermented sugars, in particular for strain D. On the other hand, strains L234 (A), L288 (B), M630 (E), and H (6944) fermented faster and with a deep sugars consumption. This behavior is confirmed by data of **Table 2**, where, for yeast A, B, E, and H, the highest alcohol contents are shown. As expected, the yeast strains have produced, in strain dependent way, succinic acid, which ranged from 0.19 (sample fermented with strain D) to 1.74 g l^{-1} (sample fermented with strain A). Total acidity, ranged between 5.10

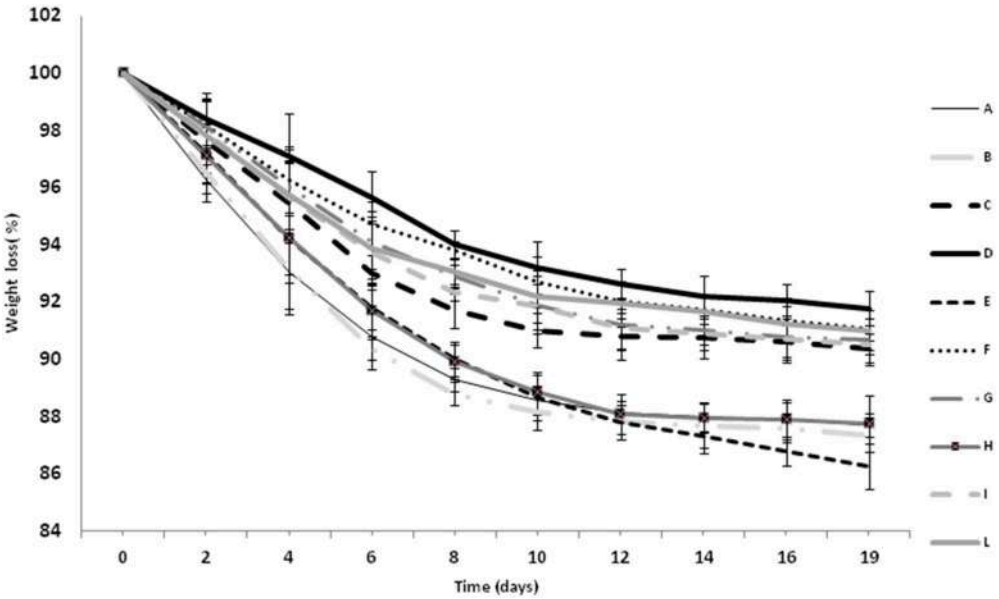

FIGURE 1 | Sample weight loss during fermentation in relation to the strain used.

and 12.0 g l^{-1}. Several differences, in strain dependent way, were reported also for the malic and lactic acid (**Table 2**). A significant decrease of malic acid was observed, comparing to the must, for all the inoculated samples. The lowest decrease in mailc acid concentration were observed in wines produced with the strains H (6944) and A (L234). The decrease of malic acid was not always accompanied by the increase of lactic acid. The pH values ranged between 3.05 and 3.27 according to the strain used.

Analysis of Volatile Compounds

The gas-chromatographic analyses permitted the identification of molecules belonging to different chemical classes such as aldehydes, lactones, higher alcohols, esters, short chain fatty acids, and terpenes (**Table 3**).

Regarding aldehydes, the strain F produced the highest amounts of acetaldehyde, while the strains A and H produced nonanal, having a great sensorial impact. For what concern ketons, quantitative and qualitative differences were observed among the samples, in relation to the strain used. For example, the wine produced with the strains C, E, and H were characterized by great amount of butyrolactone. The strain C, E, and G have produced in wines high amount of acetoin, absent in wines produced by strains B, I, and L. Only the strains B, C, and E produced low amount of 2,3-butanedione.

Great differences were detected among wines in produced alcohols. The strains C, G, and I produced in wines levels higher than 100 mg l^{-1}, associated to production of phenylethyl alcohol higher than 50 mg l^{-1}. Low amounts of isoamylic alcohols distinguished the sample fermented by strains A, B, D, F, and L. The wine samples H and I did not presented ethylphenol, a molecule of great impact at low concentration.

Regarding esters, high amounts were detected in all the samples, independently on the strain employed. The most presents were ethylacetate and ethylester of medium chain fatty acids such as hexanoic, octanoic, and decanoic acids. Ethylacetate was highly produced by strain D, F, and G. On the contrary, low productions were detected for strains A, B, C, E, H, I, and L. In general, the highest production of esters (excluding ethylacetate) were detected in wines obtained by strain A, H, and I.

Terpenic alcohols, molecules of great sensorial impact, were detected in wines obtained by fermentation of strain A, C, E, and H. These wines showed an accuulation of linalool, α-terpineol, and citronellol.

The tested strains resulted different also in the organic acid release. In particular, acetic, isobutyric, decanoic, 3-methylbutyric, octanoic, and 2,2, dimethyloctanoic acid productions were different in relation to the strain employed. The strongest producers of acetic acid were the strains C, D, E, and H.

Sulfur Compounds

The use of a photometric flame detector permitted to detect and quantify in wine samples methantiol (MT), dimethylsulfur (DMS), dimethyldisulfur (DMDS), dimethyltrisulfur (DMTS), 3-methyl-tio-propanol (MO), ethyl 3-methylpropanoate (EMTP), and 4-isopropyltiophenol (IPTF). The detected sulfur compounds, deriving from yeast metabolism, were found in all the samples. All the strains were able to produce high amounts of IPTF (from 45 to 233 μg l^{-1}; **Figure 2**). However, the strains C, G and L produced more than 200 ppb. The strains A, E, I, and G produced high levels of methionol while EMPT was produced at level of 11.82 μg l^{-1} and 10.61 μg l^{-1} in wines produced by strain A and I, respectively. The highest amounts of MT, DMDS,

TABLE 3 | Volatile molecules (expressed as mg l^{-1}) identified by GC-MS/SPME in Trebbiano wines in relation to the strain used.

	A	B	C	D	E	F	G	H	I	L
ALDEHYDES										
Acetaldehyde	0.7	1.6	0.4	2.1	1.4	3.0	2.3	1.0	2.3	1.9
Nonanal	0.2	–*	–	–	–	–	–	0.1	–	–
KETONS										
2,3-butanedione	–	0.1	0.1	–	–	–	–	–	–	–
Methylisobuthyl ketone	–	0.2	0.2	0.4	0.1	0.4	0.3	0.1	0.2	0.2
Acetoin	0.1	–	0.4	0.1	0.4	–	0.4	0.2	–	–
Butyrolactone	0.1	–	0.5	0.1	0.8	–	0.1	1.8	0.1	0.1
ALCOHOLS										
1-propanol	–	0.3	0.3	0.3	0.1	0.3	0.3	0.7	0.4	1.3
Isobutanol	2.8	4.3	4.2	3.9	4.5	5.2	7.3	3.5	6.0	2.8
1-butanol	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.2	0.1	0.1
Isoamylic alcohol	83.2	80.3	100.5	68.4	85.7	78.1	111.0	93.7	108.3	84.0
3-methyl pentanol	–	0.1	–	–	0.3	0.1	0.2	0.3	0.2	0.3
1-hexanol	7.6	6.4	8.5	4.8	8.0	5.9	5.6	6.7	7.8	7.2
(Z)-3-hexenol	0.2	0.2	0.1	0.0	0.2	0.2	0.2	0.2	0.2	0.2
(E)-3-hexenol	0.3	0.3	0.4	0.2	0.4	0.3	0.3	0.3	0.3	0.3
1-heptanol	0.3	0.4	–	48.8	–	14.9	12.9	–	0.2	–
1-octanol	0.2	0.1	–	0.2	0.2	0.1	0.5	–	0.7	0.2
Nonanol	0.3	0.2	0.1	0.2	0.3	0.1	0.2	0.3	0.2	0.2
Phenylethanol	72.3	43.2	54.6	72.3	52.5	38.6	61.1	81.6	71.6	72.1
Ethylphenol	0.3	0.1	0.5	0.3	0.1	0.2	0.1	–	–	0.7
ESTERS										
Ethyl acetate	12.8	7.8	6.3	103.0	12.5	73.4	29.9	11.6	14.7	10.4
Isoamyl acetate	0.9	1.5	0.8	2.1	1.3	1.8	1.5	1.2	1.8	0.9
Ethyil hexanoate	1.7	2.5	2.8	1.0	2.4	1.7	2.4	2.7	3.1	2.2
Hexyl acetate	0.1	0.3	0.2	–	0.1	–	–	0.2	–	0.1
Ethyl octanoate	7.5	4.1	4.6	2.5	6.7	2.4	6.2	5.5	9.0	4.0
Ethyl hydroxy caproate	–	0.2	–	–	–	–	–	–	–	–
Ethyl decanoate	2.4	1.3	1.1	1.3	1.8	0.6	2.1	2.6	3.5	2.1
Diethyl succinate	1.1	1.0	1.9	2.7	1.1	2.4	1.8	2.4	1.9	2.8
Ethyl 9 decenoate	5.3	1.5	1.5	1.3	3.2	0.7	2.4	3.0	3.4	1.9
Ethyl phenyl acetate	0.2	0.2	2.4	0.7	0.1	0.9	0.6	0.2	0.1	0.1
Phenyl acetate	3.1	2.8	2.8	6.4	2.7	2.3	2.2	6.5	4.0	4.9
Ethyl 9 octadecenoate	0.8	–	0.2	0.1	0.3	–	–	0.3	–	–
TERPENIC ALCOHOLS										
Linalool	3.2	0.2	2.8	1.7	1.7	0.3	0.3	1.7	0.2	0.6
α-terpineol	1.1	0.1	0.9	0.7	0.6	0.1	0.1	1.2	0.1	0.1
Citronellol	2.7	0.3	2.3	1.1	1.4	0.1	0.2	2.6	0.3	0.3
ACIDS										
Acetic acid	13.9	2.7	14.3	20.2	17.7	11.7	12.2	18.1	8.1	8.3
Isobutyric acid	0.2	0.3	0.1	0.7	0.4	0.4	0.8	0.4	0.3	0.2
Decanoic acid	3.9	2.8	4.3	5.2	3.6	1.6	4.4	6.0	3.2	6.5
3-methyl butyric acid	2.2	1.7	1.5	4.6	2.1	2.7	5.5	2.0	1.9	1.8
2,2-dimethyl octanoic acid	–	0.9	1.4	2.5	0.9	0.4	2.8	3.0	1.2	2.4
Octanoic acid	3.4	10.6	24.8	18.4	27.9	19.2	29.0	33.6	17.2	22.2

**Under the detection limit; The coefficient of variability, defined as the ratio of the standard deviation to the mean, ranged between 5 and 7%.*

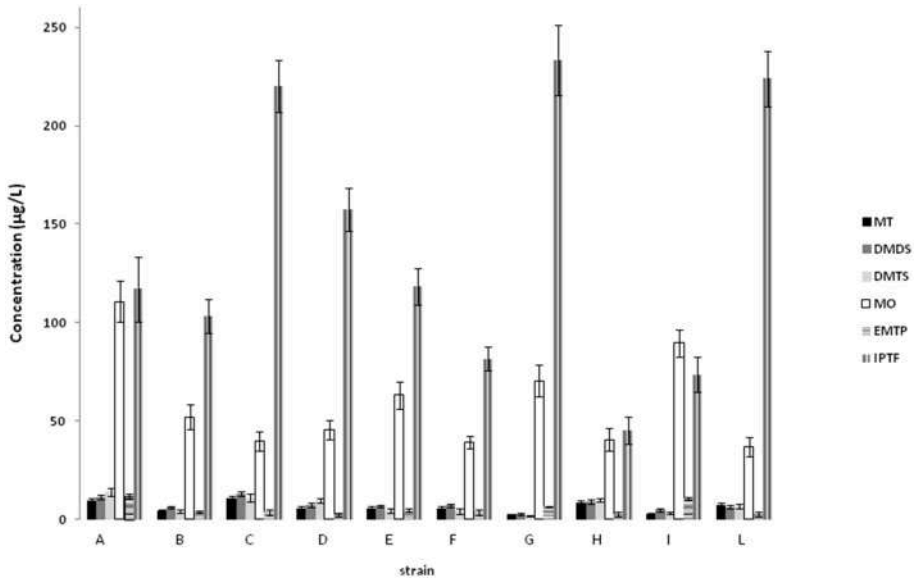

FIGURE 2 | Sulfur compounds (expressed as $\mu g\ l^{-1}$) detected in wine samples in relation to the strain used. Methantiol (MT), dimethylsulfur (DMS), dimethyldisulfur (DMDS), dimethyltrisulfur (DMTS), 3-methyl-tio-propanol (MO), ethyl 3-methylpropanoato (EMTP), and 4-isopropyltiophenol (IPTF).

and DMTS were detected in wines deriving from fermentation of the yeasts A and C.

Amino Acid Release

Data obtained showed a low level of amino acid content in produced wines, while the must was characterized by high level of all the investigated amino acids (**Table 4**). Alanine, cystein, methionine (present at low level also in must), proline, leucine, isoleucine, valine, threonine were completely metabolized independent on the considered strain. On the contrary, in wine produced by strain A, with respect to the must, there was an increase of arginine and tryptophan. Arginine was found at low level in wine produced by strain D, E, G, and L.

Electronic Nose

Because the sensorial profile of a wine is the resulting of volatile and not volatile molecule interaction, the wines were subjected to the electronic nose analysis. The data from electronic nose were obtained by using 10 different probes able to detect different classes of compounds, as reported in Materials and Methods, and give a wine sensory evaluation. The raw data obtained were analyzed by PCA able to discriminate the samples in three different macro-groups in relation to the affinity with the probes used (**Figure 3**). The first group included wine obtained by strains B, E, G, and H; the second group contained wines from strain D, I, and L, while the wines from strain A, F, and C were grouped together. In particular, the group 2 distinguished for the probes 2 and 7, detecting NO and sulfur compound, respectively. The cluster 3 was formed on the basis of probes 3 and 5, detecting NH_3 and aromatic compounds and low-polarity aromatic compounds, respectively.

DISCUSSION

The results of the present work showed that all the *S. cerevisiae* strains were able to drive the fermentation although with different kinetics. As expected, the strains have produced wines characterized by different amounts of succinic acid. On the other hand, the production of this acid, normally absent in the must, is related to yeast metabolism during alcoholic fermentation (Rainieri et al., 2003). Also the difference in total acidity, ranging from 5.1 to 12.65 g l^{-1}, can be associated to the strain ability to produce different amount of several acids (succinic, acetic, lactic, and malic acids) but also to the release of different amount of mannoproteins, during the fermentation. The significant decrease of malic acid detected in wine samples, compared to the initial must, can be attributed in the major part of sample to the ability of *S. cerevisiae* strains to degrade malic acid more than malo-lactic fermentation, (Styger et al., 2011). In fact it is well know that *S. cerevisiae* strains are able to degrade or produce malic acid in a strain dependent way. A wide literature have shown that these polymers, produced in strain-dependent way during the yeast growth, fermentation and autolysis, can affect also the tartaric acid concentration and its stability (Caridi, 2006; Palomero et al., 2007).

Until a few decades ago, wine yeasts were selected basically on their ability to quickly transform grape sugars into ethanol, on their resistance to sulfur dioxide and on the low acetic acid production. Actually, their role has been significantly expanded by the advent of modern oenological microbiology and their selection has therefore involved the development of techniques for detecting strains that might improve wines in terms of color, aroma, structure, technological, and also healthy properties. In the present work, in addition to test the yeast

TABLE 4 | Amino acid content (mg l^{-1}) detected in Trebbiano wines in relation to the strain used.

	Must	A	B	C	D	E	F	G	H	I	L
Alanine	234.34	1.31	3.48	5.36	–	0.41	0.05	1.21	0.15	1.03	–
Proline	622.81	0.23	0.22	0.4	0.09	0.03	0.05	0.04	0.04	0.12	–
Methionine	1.07	–*	–	–	–	–	–	–	–	–	–
Cysteine	1.51	–	–	–	–	–	–	–	–	–	–
Leucine	29.55	1.92	2.53	4.97	–	–	–	–	–	–	–
iso-Leucine	23.28	0.7	0.86	1.64	–	–	–	–	–	–	–
Valine	49.41	3.58	1.42	2.37	–	0.17	0.04	0.45	–	0.95	0.14
Threonine	70.71	–	–	–	–	–	–	–	–	–	–
Ornithine	–	–	–	0.06	–	–	–	–	0.06	–	–
Triptophan	319.3	111.64	20.25	18.1	19.22	42.63	27.37	21.83	22.61	24.33	15.3
Phenyl alanine	5.08	–	1.01	–	–	–	–	0.4	–	–	–
Tyrosine	3.76	0.66	3.25	3.75	–	–	–	0.03	–	–	–
Arginine	207.37	640.04	114.04	216	102.53	165.36	129.41	40.29	192.6	353.13	107.03
Glutamic acid	95.52	5.34	0.59	–	–	–	–	–	–	–	–
γ-Aminobutyric acid	128.43	–	–	–	–	–	–	–	–	–	–

**Under the detection limit; The coefficient of variability, defined as the ratio of the standard deviation to the mean, ranged between 5 and 7%.*

fermentation power, also the strain ability to produce wine with characterizing flavor was investigated. In particular, the volatile sulfur compounds production, and in general the volatile molecule profiles produced by starter cultures have a main role in the strain selection and in the product characterization. Some researchers have suggested that these profiles can be regarded as footprints or "aromagrams" and can in the future be used for identification and quality control purposes (Styger et al., 2011). These aromagrams are not only composed of various chemical classes of compounds (alcohols, esters, aldehydes, ketones, acids, and sulfur- and nitrogen-containing compounds), but these compounds have a very wide concentration range in the wine varying between the gram to the nanogram per liter (Bonino et al., 2003). Moreover, it is their ratio which plays an important role in the final wine flavor and taste.

The GC-MS volatile molecules profiles obtained in this work resulted strain dependent and the results are in accordance with Vernocchi et al. (2015) who demonstrated that Trebbiano wines fermented with wild *S. cerevisiae* strains were characterized by proper unique aromatic profiles. Also Mauriello et al. (2009) found that a great variability in volatile molecules produced among the tested wild wine yeasts, emphasizing the potential role of this parameter as trait for starter culture selection. Moreover, Romano et al. (2015) found that volatiles detected by mass spectra techniques represent a strain fingerprinting. Also Tufariello et al. (2014) found that yeast species and, within each species, different strains exhibit wide differences in volatile compound profiles in the production of Negroamaro wines. In this research, for example, the strain F produced the highest amounts of acetaldehyde, while the strains A and H produced nonanal, having a great sensorial impact. The wine produced with the strains C, E, and H were characterized by great amount of butyrolactone. In the obtained wines, also terpenic compounds and esters were found. In general, esters are formed by yeasts during the alcoholic fermentation and they are responsible for the fruity odor, while terpenic and nor-isoprenoid compounds are the most important constituent of the varietal aroma of grapes and confer a flowery odor to the wine (Vararu et al., 2016). In wines obtained by the strains A, C, E, and H linalool, α-terpineol, and citronellol, able to impart citrus and peach flavor notes, were found. In general, these are released in wine also by the yeast ß-glucosidase activities (Pedersen et al., 2003; Fia et al., 2005). By now, numerous works have shown that yeasts involved in vinification processes possess β-glucosidase activity, and this is greater in non-*Saccharomyces* yeast strains than in *S. cerevisiae* ones (Fia et al., 2005). Also volatile esters constitute one of the most important classes of aroma compounds and are largely responsible for the fruity aromas associated with wine and other fermented beverages (Vararu et al., 2016). Their formation differs widely between yeast strains and other external factors such as fermentation temperature, nutrient availability, pH, unsaturated fatty acid/sterol levels, and oxygen levels all playing an important part in determining the end levels of esters in a wine (Lilly et al., 2000). Our data suggested that the highest production of esters (excluding ethylacetate) was detected in wines obtained by strains L284 (A), 6944 (H), and 7541 (I). For example this last strain produced high amount of ethyl hexanoate (whose odor descriptor corresponds to fruit, pineapple), ethyl octanoate (apricot). Also higher alcohols play a fundamental role since they have usually a strong pungent smell. Differently, 2-phenylethanol is an aroma carrier and its presence may contribute to the floral nuance of wines, especially for white wines. The aroma characterized by this compound changes with its oxidation from a rose to a hyacinth bouquet (Duarte et al., 2010). The strains I (7541), A (L284), D (L951), H (6944), L (6644) were able to produces in Trebbiano wines the highest amounts, contributing to positively affect the final aroma. Also sulfur-containing compounds play an important role in wine aroma. Sulfur compounds contribute mainly to unpleasant aromas in wines, although some of them have been reported to have a positive contribution to wine (4-mercapto-4-methyl-2-pentanone, 3-mercaptohexyl acetate,

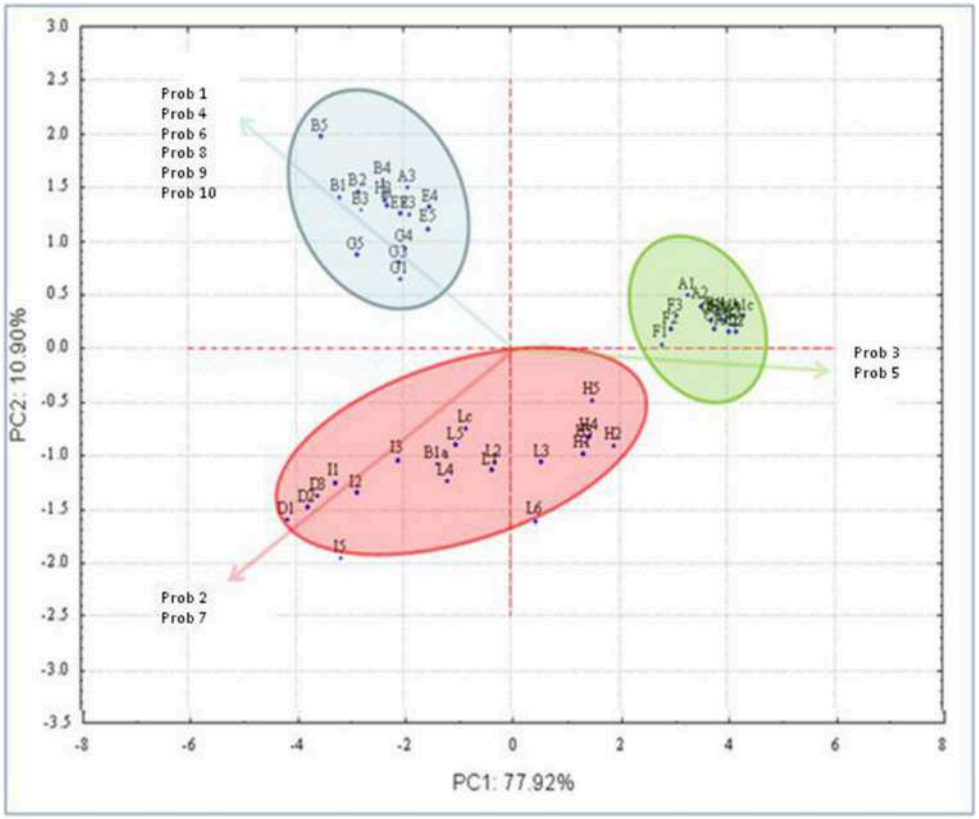

FIGURE 3 | Principal component analysis loading plot of electronic nose data in relation to the strain used in fermentation. A (strain L234), B (strain L288), C (strain L674), D (strain L951), E (strain M630), F (strain M692), G (strain U5298), H (strain 6944), I (strain 7541), L (strain 6644).

3-mercapto-l-hexanol,4-mercapto-4-methyl-2-pentanol, and 3-mercapto-3-methyl-l-butanol). In this research, methionol was the heavy sulfur compound present in wines in the highest concentrations with IPTF. Similar results were obtained by Moreira et al. (2010) for monovarietal white wines. According to Falqué et al. (2002), methionol concentration was one of the variables responsible for the differentiation of wines from Loureiro, Dona Branca, and Trajadura cultivars from the Galicia region (Spain). Methionol is produced by yeast from methionine, via deamination, followed by decarboxylation (Ehrlich reaction); the aldehyde thus formed, 3-(methylthio)-1-propanal (methional), is then reduced to the alcohol (methionol) or oxidized to the acid (3-(methylthio)propionic acid). The reaction of methionol with acetic acid yields 3-(methylthio)propyl acetate (Rauhut, 1993). The content of methionol increased considerably in wines with reduction defects (Mestres et al., 2002), contributing odors of potato, cauliflower, and cooked vegetables/cabbage. In our research, the highest amounts of methionol were produced by strain A (110 $\mu g\, l^{-1}$), I (89 $\mu g\, l^{-1}$), and G (70 $\mu g\ l^{-1}$). However, the data of the present research showed that the impact of the sulfur compounds detected is not so strong because in relation and *in equilibrium* with other volatile and not volatile compounds. In fact, the PCA analysis, performed on the data from electronic nose, divided the wine samples only in three homogeneous clusters. On the other hand, this kind of analysis can account the sensory profiles of a wine and reflect the interaction between volatile and not volatile molecules.

CONCLUSIONS

The present work showed that the omic technique adopted (GC/FPD and GC/MS-SPME) can be used as fingerprinting tools and, since they are successfully combinable with those produced by conventional analysis techniques, they can allow to discriminate among the tested strains, in order to select the best candidate in relation to the desiderated wine sensory features. In fact volatile compounds and HVSC, are fundamental for the characterization and definition of the wine sensory properties. The data obtained in this research outline the importance of strain aromagramma in the yeast strain selection for winemaking. In fact, the data contributed to the non-conventional characterization of the employed *S. cerevisiae* strains. In fact, although all the strains showed potential to ferment Trebbiano must, different profiles for volatile and sulfur compounds were identified and fundamental for strain discrimination. Although these preliminary data can useful for the selection of strains in Trebbiano winemaking, further studies regarding other technological features, such as the mannoprotein release and the production of molecule of

health importance, such as ethylcarbammate, can be performed. Moreover, additional investigations regarding the genes involved in the sulfur production from the selected yeasts need to be investigated.

AUTHOR CONTRIBUTIONS

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

REFERENCES

Bonino, M., Schellino, R., Rizzi, C., Aigotti, R., Delfini, C., and Baiocchi, C. (2003). Aroma compounds of an talian wine (Ruche) by HS–SPME analysis coupled with GC–ITMS. *Food Chem.* 80, 125–133. doi: 10.1016/S0308-8146(02)00340-0

Caridi, A. (2006). Enological functions of parietal yeast mannoproteins. *Antonie Van Leeuwenhoek.* 89, 417–422. doi: 10.1007/s10482-005-9050-x

Davis, P. M., and Qian, M. C. (2011). "Progress on volatile sulfur compound analysis in wine" in *Volatile Sulfur Compounds in Food*, Vol. 1068, eds M. C. Qian, X. Fan, and K. Mahattanatawee (Washington, DC: American Chemical Society), 93–115. doi: 10.1021/bk-2011-1068.ch005

Duarte, W. F., Dias, D. R., Oliveira, J. M., Vilanova, M., Teixeira, J. A., Almeida, J. B., et al. (2010). Raspberry (*Rubus idaeus* L.) wine: yeast selection, sensory evaluation and instrumental analysis of volatile and other compounds. *Food Res. Int.* 43, 2303–2314. doi: 10.1016/j.foodres.2010.08.003

Falqué, E., Fernández, E., and Dubourdieu, D. (2002). Volatile components of Loureira, Dona Branca, and Treixadura wines. *J. Agr. Food Chem.* 50, 538–543. doi: 10.1021/jf010631s

Fia, G., Giovani, G., and Rosi, I. (2005). Study of β-glucosidase production by wine-related yeasts during alcoholic fermentation. A new rapid fluorimetric method to determine enzymatic activity. *J. App. Microbiol.* 99, 509–517. doi: 10.1111/j.1365-2672.2005.02657.x

Fleet, G. H. (2003). Yeast interactions and wine flavour. *Int. J. Food Microbiol.* 86, 11–22. doi: 10.1016/S0168-1605(03)00245-9

Fleet, G. H. (2008). Wine yeasts for the future. *FEMS Yeast Res.* 8, 979–995. doi: 10.1111/j.1567-1364.2008.00427.x

Franco-Luesma, E., and Ferreira, V. (2014). Quantitative analysis of free and bonded forms of volatile sulfur compounds in wine. Basic methodologies and evidences showing the existence of reversible cation-complexed forms. *J. Chromatogr. A.* 1359, 8–15. doi: 10.1016/j.chroma.2014.07.011

Harsch, M. J., and Gardner, R. C. (2013). Yeast genes involved in sulfur and nitrogen metabolism affect the production of volatile thiols from Sauvignon Blanc musts. *Appl. Microbiol. Biotechnol.* 97, 223–235. doi: 10.1007/s00253-012-4198-6

Lambrechts, M. G., and Pretorius, I. S. (2000). Yeast and its importance to wine aroma - a review. *S. Afr. J. Enol. Vitic.* 21, 97–129.

Lilly, M., Lambrechts, M. G., and Pretorius, I. S. (2000). Effect of increased yeast alcohol acetyltransferase activity on flavor profiles of wine and distillates. *Appl. Environ. Microbiol.* 66, 744–753. doi: 10.1128/AEM.66.2.744-753.2000

Liu, S. Q., and Pilone, G. (2000). An overview of formation and roles of acetaldehyde in winemaking with emphasis on microbiological implications. *Int. J. Food Sci. Tech.* 35, 49–61. doi: 10.1046/j.1365-2621.2000.00341.x

Mauriello, G., Capece, A., D'Auria, M., Garde-Cerdán, T., and Romano, P. (2009). SPME–GC method as a tool to differentiate VOC profiles in *Saccharomyces cerevisiae* wine yeasts. *Food Microbiol.* 26, 246–252. doi: 10.1016/j.fm.2009.01.003

Mestres, M., Busto, O., and Guasch, J. (2002). Application of headspace solid-phase microextraction to the determination of sulfur compounds with low volatility in wines. *J. Chromatogr. A* 945, 211–219. doi: 10.1016/S0021-9673(01)01521-7

Moreira, N., Guedes de Pinho, P., Santos, C., and Vasconcelos, I. (2010). Volatile sulphur compounds composition of monovarietal white wines. *Food Chem.* 123, 1198–1203. doi: 10.1016/j.foodchem.2010.05.086

Moreira, N., Mendes, F., Pereira, O., Guedes de Pinho, P., Hogg, T., and Vasconcelos, I. (2002). Volatile sulfur compounds in wines related to yeast metabolism and nitrogen composition of grape musts. *Anal. Chim. Acta* 458, 157–167. doi: 10.1016/S0003-2670(01)01618-X

Ndagijimana, M., Serrazanetti, D. I., Laghi, L., Perillo, L., and Guerzoni, M. E. (2010). "Rapid GC-MS-XCMS method for determination of Gamma-aminobutyric acid (GABA) produced by lactic acid bacteria in various media," in *22nd International ICFMH Symposium Food Micro 2010*, 30th Aug – 3rd Sept (Copenhagen).

Official Journal of the European Union (OJEU) (2010). *Information from European Union Institutions, Bodies, Offices and Agencies, European Commission*. Available online at: https://www.fsai.ie/uploadedfiles/legislation/fsai_-_legislation/2010/02_feb2010/cnotice_2010_c_43_01.pdf

Palomero, F., Morata, A., Benito, S., Gonzalez, M. C., and Suarez-Lepe, J. A. (2007). Conventional and enzyme-assisted autolysis during ageing over lees in red wines: influence on the release of polysaccharides from yeast cell walls and on wine monomeric anthocyanin content. *Food Chem.* 105, 838–846. doi: 10.1016/j.foodchem.2007.01.062

Pedersen, D. S., Capone, D. L., Skouroumounis, G. K., Pollnitz, A. P., and Sefton, M. A. (2003). Quantitative analysis of geraniol, nerol, linalool, and α-terpineol in wine. *Anal. Bioanal. Chem.* 375, 517–522. doi: 10.1007/s00216-002-1716-x

Rainieri, S., and Pretorius, I. S. (2000). Selection and improvement of wine yeasts. *Ann. Microbiol.* 50, 15–31.

Rainieri, S., Zambonelli, C., and Kaneko, Y. (2003). *Saccharomyces* sensu stricto: systematics, genetic diversity and evolution. *J. Biosci. Bioeng.* 96, 1–9. doi: 10.1016/S1389-1723(03)90089-2

Romano, A., Capozzi, V., Spano, G., and Biasioli, F. (2015). Proton transfer reaction–mass pectrometry: online and rapid determination of volatile organic compounds of microbial origin. *Appl. Microbiol. Biotech.* 99, 3787–3795. doi: 10.1007/s00253-015-6528-y

Rauhut, D. (1993). "Production of sulfur compounds," in *Wine Microbiology and Biotechnology*, ed G. H. Fleet (Switzerland: Harwood Academic Publishers), 183–242.

Sado Kamden, S. L., Ndagijimana, M., Vannini, L., and Guerzoni, M. E. (2007). Differentiation of fresh seafood products and storage time using an electronic nose:features selection for data analysis. *Italian J. Food Sci.* 393–399.

Styger, G., Prior, B., and Bauer, F. F. (2011). Wine flavor and aroma. *J. Ind. Microbiol. Biot.* 38, 1145–1159. doi: 10.1007/s10295-011-1018-4

Swiegers, J. H., Bartowsky, E. J., Henschke, P. A., and Pretorius, I. S. (2005). Yeast and bacterial modulation of wine aroma and flavour. *Aust. J. Grape Wine Res.* 11, 139–173. doi: 10.1111/j.1755-0238.2005.tb00285.x

Swiegers, J. H., and Pretorius, I. S. (2007). Modulation of volatile sulfur compounds by wine yeast. *Appl. Microbiol. Biotechnol.* 74, 954–960. doi: 10.1007/s00253-006-0828-1

Tominaga, T., Blanchard, L., Darriet, P., and Dubourdieu, D. (2000). A powerful aromatic volatile thiol, 2-furanmethanethiol, exhibiting roast coffee aroma in wines made from several *Vitis vinifera* grape varieties. *J. Agric. Food Chem.* 48, 1799–1802. doi: 10.1021/jf990660r

Tufariello, M., Chiriatti, M. A., Grieco, F., Perrotta, C., Capone, S., Rampino, P., et al. (2014). Influence of autochthonous *Saccharomyces cerevisiae* strains on volatile profile of Negroamaro wines. *LWT Food Sci. Technol.* 58, 35–48. doi: 10.1016/j.lwt.2014.03.016

Vararu, F., Moreno-García, J., Zamfir, C. I., Cotea, V. V., and Moreno, J. (2016). Selection of aroma compounds for the differentiation of wines obtained by fermenting musts with starter cultures of commercial yeast strains. *Food Chem.* 197, 373–381. doi: 10.1016/j.foodchem.2015.10.111

Vermeulen, C., and Gus, L. (2005). Sensorial contribution and formation pathways of thiols in foods: a review. *Food Rev. Int.* 21, 69–137. doi: 10.1081/FRI-200040601

Vernocchi, P., Ndagijimana, M., Serrazanetti, D. I., Chaves López, C., Fabiani, A., Gardini, F., et al. (2011). Use of *Saccharomyces cerevisiae* strains

endowed with β-glucosidase activity for the production of Sangiovese wine. *World J. Microbiol. Biotechnol.* 27, 1423–1433. doi: 10.1007/s11274-010-0594-1

Vernocchi, P., Patrignani, F., Ndagijimana, M., Chaves Lopez, C., Suzzi, G., Gardini, F., et al. (2015). Trebbiano wine produced by using *Saccharomyces cerevisiae* strains endowed with β-glucosidase activity. *Ann. Microbiol.* 65, 1565–1571. doi: 10.1007/s13213-014-0995-8

Vichi, S., Cortes-Francisco, and Caixach, J. (2015). Analysis of volatile thiols in alcoholic beverages by simultaneous derivatization/extraction and liquid chromatography-high resolution mass spectrometry. *Food Chem.* 175, 401–408. doi: 10.1016/j.foodchem.2014.11.095

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Diversity of *Saccharomyces cerevisiae* Strains Isolated from Two Italian Wine-Producing Regions

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Numerous studies, based on different molecular techniques analyzing DNA polymorphism, have provided evidence that indigenous *Saccharomyces cerevisiae* populations display biogeographic patterns. Since the differentiated populations of *S. cerevisiae* seem to be responsible for the regional identity of wine, the aim of this work was to assess a possible relationship between the diversity and the geographical origin of indigenous *S. cerevisiae* isolates from two different Italian wine-producing regions (Tuscany and Basilicata). For this purpose, sixty-three isolates from *Aglianico del Vulture* grape must (main *cultivar* in the Basilicata region) and from *Sangiovese* grape must (main *cultivar* in the Tuscany region) were characterized genotypically, by mitochondrial DNA restriction analysis and MSP-PCR by using $(GTG)_5$ primers, and phenotypically, by determining technological properties and metabolic compounds of oenological interest after alcoholic fermentation. All the *S. cerevisiae* isolates from each region were inoculated both in must obtained from *Aglianico* grape and in must obtained from *Sangiovese* grape to carry out fermentations at laboratory-scale. Numerical analysis of DNA patterns resulting from both molecular methods and principal component analysis of phenotypic data demonstrated a high diversity among the *S. cerevisiae* strains. Moreover, a correlation between genotypic and phenotypic groups and geographical origin of the strains was found, supporting the concept that there can be a microbial aspect to *terroir*. Therefore, exploring the diversity of indigenous *S. cerevisiae* strains can allow developing tailored strategies to select wine yeast strains better adapted to each viticultural area.

Keywords: *Saccharomyces cerevisiae*, wine, *terroir*, *Aglianico del Vulture*, *Sangiovese*, yeast diversity, genotyping, fermentation products

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INTRODUCTION

Traditionally, *Saccharomyces cerevisiae* is the predominant yeast species in spontaneous wine fermentations and thus it is the main responsible for the chemical and sensory properties of wines (Pretorius, 2000; Fleet, 2003; Romano et al., 2003; Cocolin et al., 2004; Camarasa et al., 2011). During the last decades, a large number of surveys, based on different molecular techniques analyzing DNA polymorphism, have demonstrated that this species is characterized by a high genetic diversity (Frezier and Dubourdieu, 1992; Querol et al., 1994; Guillamón et al., 1996; Sabate et al., 1998; Pramateftaki et al., 2000; Torija et al., 2001; Schuller et al., 2005; Agnolucci et al., 2007; Romano et al., 2008; Sun et al., 2009; Csoma et al., 2010; Mercado et al., 2011; Capece et al., 2013).

In spite of the occurrence of a high number of different *S. cerevisiae* strains at the beginning of the fermentation, it was pointed out that, usually, only few strains (from one to three) dominate the process in the latter stages. Some *S. cerevisiae* strains were isolated over several years in the same cellar as predominant microbiota in wine fermentations (Sabate et al., 1998; Gutièrrez et al., 1999; Augruso et al., 2006) so that the existence of a "winery effect" was suggested (Vezinhet et al., 1992). Alternatively, specific *S. cerevisiae* strains were widespread in different cellars of the same wine-producing region (Versavaud et al., 1995; Blanco et al., 2006) and they were considered representative of an oenological area (Guillamón et al., 1996; Torija et al., 2001). More recently, biogeographical characterization of *S. cerevisiae* wine yeasts carried out at scales above 100 km, has revealed the presence of regional population with specific genotype but no differentiation within the region (Knight and Goddard, 2015). These findings suggest that specific native strains could be associated with a *terroir*, a term that classically includes only grape variety, climate and soil as fundamental factors determining the typical nature of wines (Van Leeuwen and Seguin, 2006) and that might be revised including also a "microbial aspect" (Bokulich et al., 2014; Taylor et al., 2014). Since it is well established that chemical and sensory properties of some wines reflect their geographic origin (Villanova and Sieiro, 2006; Callejon et al., 2010) it was interesting to determine whether regionally defined *S. cerevisiae* genotypes actually exhibit specific metabolic profiles (or phenotypes) able to modulate the wine quality, thus contributing to *terroir*-associated wine characteristics. Indeed, in a recent study Knight et al. (2015) demonstrated significant correlation between the region of isolation of *S. cerevisiae* and aroma profile in wines. The evidence that certain regions have "signature" *S. cerevisiae* populations that can produce significantly different chemical and sensory profiles of wine is of relevance to the wine industry because it may link territory, environment, and final products for wine valorisation (Torija et al., 2001; Romano et al., 2003; Aa et al., 2006; Camarasa et al., 2011; Pretorius et al., 2012; Tofalo et al., 2013). For this reason, the demand of indigenous *S. cerevisiae*, which could be representative of a specific oenological area, is increasing (Orlić et al., 2010). In fact, each strain of *S. cerevisiae* is able to produce different types and quantities of secondary compounds, which are determinant on the desirable organoleptic characteristics of a wine (Pretorius, 2000; Romano et al., 2003; Barrajón et al., 2011; Scacco et al., 2012). Since to perform a better control of the alcoholic fermentation in the modern winemaking the use of yeast starter cultures is diffused, selecting the proper yeast strain can be critical for the development of the desired wine style. Moreover, by using these selected yeast starter cultures, that are better adapted to the environmental conditions, the must fermentation can occur in the correct way (Callejon et al., 2010). In this perspective, the goal of this study was to investigate a possible relationship between the diversity and the geographical origin of indigenous *S. cerevisiae* isolated from two different Italian wine-producing regions (Tuscany and Basilicata) considering two regional grape varieties usually used to produce Controlled Designation of Origin (DOC) wines. Such studies are of great interest in order to establish the existence of typical *S. cerevisiae* strains that would then be useful as inocula in the vinifications carried out in the specific oenological areas (Gutièrrez et al., 1999). The use of autochthonous yeast strains, besides assuring the maintenance of the typical sensory properties of the wines of any given region, can contribute to promote or retain the natural *S. cerevisiae* biodiversity.

MATERIALS AND METHODS

Yeast Strains

Sixty-three *Saccharomyces cerevisiae* isolates were used. The yeasts were previously isolated from spontaneously fermented grape musts of two varieties: "Aglianico del Vulture," Basilicata region (coded with R1-R33) and "Sangiovese," Tuscany region (coded with R34-R63). The isolates were maintained on YPD medium [1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose, 2% (w/v) agar].

Genotypic Characterization of *S. cerevisiae* Isolates

Differentiation between the 63 indigenous *S. cerevisiae* isolates was performed by two molecular methods: microsatellite-primed PCR (MSP-PCR) by using the synthetic oligonucleotide $(GTG)_5$ (Orlić et al., 2010) and mitochondrial DNA restriction analysis (mtDNA-RFLP) by using the restriction endonucleases *Rsa*I according to Granchi et al. (2003). Genomic DNA was extracted using a synthetic resin (Instagene Bio-Rad Matrix), following the protocol described in Capece et al. (2011). Amplification reactions were performed in a final volume of 50 μL containing 10 μL 5X Buffer (Promega), 4.0 μL of 25 mM $MgCl_2$ (Promega), 1 μL of 10mM dNTP (Promega), 5 μL of 5 μM primer, 0.25 μL (5 U/μL) of *Taq* DNA polymerase (Promega) and 5 μL of the extracted DNA, by adding sterile water until final volume. The thermal cycler was programmed as follows: initial denaturation at 95°C for 5 min, 35 cycles at 94°C for 1 min for denaturing, 1 min at 52°C, 2 min at 72°C for extension and a final step at 72°C for 5 min. PCR products were analyzed by electrophoresis in 1.2% (w/v) agarose gel. The obtained profiles were submitted to cluster analysis using "Complete Linkage" method with Pearson distance by FPQuest software v.4.5 (Bio-Rad).

DNA digestions were performed with the enzyme *Rsa*I and restriction DNA fragments were separated on 0.8% (w/v) agarose gels containing ethidium bromide (1 $\mu g\ mL^{-1}$) by electrophoresis in 1X·TBE buffer (90 mM Tris-borate, 2 mM, EDTA pH 8.0) at 4 V cm^{-1} for 6 h. The obtained patterns were submitted to pairwise comparison with the Dice coefficient (SD) (Sneat and Sokal, 1973) and cluster analysis with unweighted pair group method (UPGMA) by GelCompar 4.0 software (Applied Math, Kortrijk, Belgium).

Technological Characterization of *S. cerevisiae* Isolates

The 63 isolates were submitted to screening for some phenotypic properties, such as sulfur dioxide, ethanol and copper resistance and fermentative performance. The SO_2 and ethanol resistance

was evaluated on agarized grape must (pH 3.6), added with increasing doses of $K_2S_2O_5$ (100–300 mg L^{-1}) and ethanol (10–18% vol/vol), respectively. Copper resistance was evaluated on agarized synthetic medium, containing 6.7 g L^{-1} YNB (Yeast Nitrogen Base without amino acids and sulfate), 20 g L^{-1} glucose, added with increasing doses of $CuSO_4$ (50, 100, 200, 300, 400, and 500 $\mu molL^{-1}$). The strain resistance to the three compounds was evaluated on the basis of positive growth after incubation at 26°C for 24 h, in comparison to the control (the medium without the compound). The degree of resistance of each strain was reported as minimal dose of compounds allowing the growth. All the tests were carried out in duplicate.

Laboratory-Scale Fermentations

The fermentative performance of the 63 *S. cerevisiae* isolates was tested in inoculated fermentations in two different grape musts, "Aglianico del Vulture" and "Sangiovese" possessing, respectively, the following physico-chemicals characteristics: pH: 3.7 and 3.2; sugars (g L^{-1}): 227 and 214; yeast assimilable nitrogen (mgL^{-1}): (130 ± 1.4) and (120 ± 2.5). The fermentations were performed according to Capece et al. (2012): 130-mL Erlenmeyer flasks were filled with 100 mL of the two grape musts and added with 50 mg L^{-1} of SO_2. Each strain was inoculated in grape must at a concentration of 10^6 cells mL^{-1}, from a pre-culture grown for 48 h in the same must. The fermentation was performed at 26°C and the fermentative course was monitored by measuring weight loss, determined by carbon dioxide evolution during the process. At the end of the process, indicated by constant weight of the samples, the wine samples were refrigerated at 4°C to clarify the wine, racked and stored at −20°C until required for analysis. All the experiments were performed in duplicate. Fermentation vigor was measured as weight loss after 2 days of incubation at 26°C, whereas the fermentative power was defined as total weight loss, detected at the end of the process.

Chemical Analysis

In grape musts, α-amino acid and ammonium concentrations were determined by the NOPA procedure (Dukes and Butzke, 1998) and enzymatic assay according to the manufacturer's instructions (STEROGLASS s.r.l., Perugia), respectively. Glucose, fructose, ethanol, glycerol, 2,3-butanediol and acetic acid concentrations in experimental wines were determined by HPLC, according to Schneider et al. (1987) and Granchi et al. (1998), utilizing a MetaCarb H Plus Column (8 μm particle, 300 × 7.8 mm; Varian Inc.) and a Pro-star 210 chromatograph equipped with a Refractive Index Detector, in series (Varian Inc.). Higher alcohols (1-propanol, isobutanol, *n*-butanol, 2-methyl-1-butanol, 3-methyl-1-butanol), acetoin, diacetyl, acetaldehyde and ethyl acetate were analyzed by gas chromatography equipped with glass column (6.6% CW 20M BA 80/120 225, 2 m × 6 × 2 mm) as described by Romano et al. (1999).

Statistical Analysis

The raw data obtained by HPLC and GC analysis were subjected to Principal Component Analysis (PCA) and *t*-test by Statistica software (version 7, StatSoft, Tulsa, OK, USA).

RESULTS

Genotypic Characterization of *S. cerevisiae* Isolates

Genetic polymorphism in *S. cerevisiae* isolates from Aglianico del Vulture and Sangiovese grape musts was evaluated by MSP-PCR analysis with the $(GTG)_5$ primer and mt-DNA-RFLP. MSP-PCR profiles contained a variable number of bands, some of which were common to numerous isolates. The dendrogram resulting from MSP-PCR analysis (**Figure 1**), by considering a similarity coefficient of about 65%, revealed the presence of two clusters (A and B). For the majority of isolates, the distribution was related to yeast origin; in fact, the cluster A grouped isolates from Sangiovese grape must (except R28 and R29 strains), whereas the cluster B grouped all the isolates from Aglianico del Vulture grape must, except the Bs sub-cluster, which contains 9 *S. cerevisiae* isolates from Sangiovese grape must.

The mitochondrial DNA-RFLP analysis revealed the presence of 24 different patterns, i.e., 24 strains, among the 63 isolates analyzed, confirming the high polymorphism found in *S. cerevisiae* populations. The resulting dendrogram from UPGMA analysis of the patterns obtained with *Rsa*I, reported in **Figure 2**, indicated that *S. cerevisiae* isolates at 40% similarity grouped into four clusters, I, II, III, and IV. All the isolates from Aglianico del Vulture grape must, except for R6, were included in the clusters I, II, and III while all the isolates from Sangiovese grape must, except for R58, were comprised in the cluster IV. Therefore, the analysis pointed out a possible grouping of the assayed *S. cerevisiae* isolates according to their geographic origin.

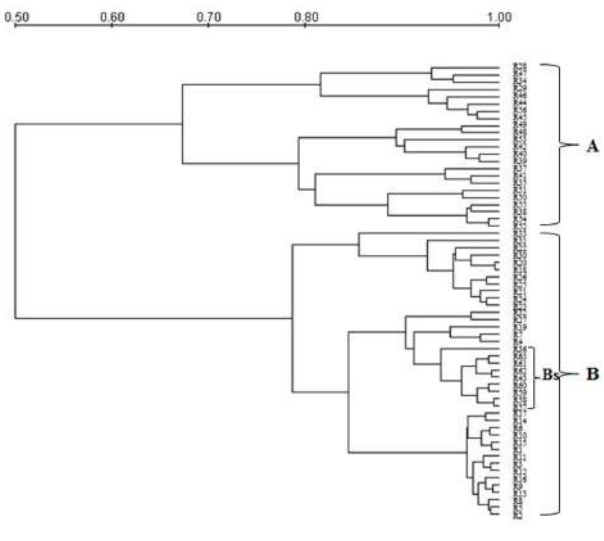

FIGURE 1 | Dendrogram from UPGMA clustering analysis, based on Pearson coefficient, of the profiles obtained by MSP-PCR of the *S. cerevisiae* isolates from the Aglianico del Vulture (cluster B) and Sangiovese (cluster A) grape must. Bs indicates a sub-cluster including 9 *S. cerevisiae* isolates from Sangiovese.

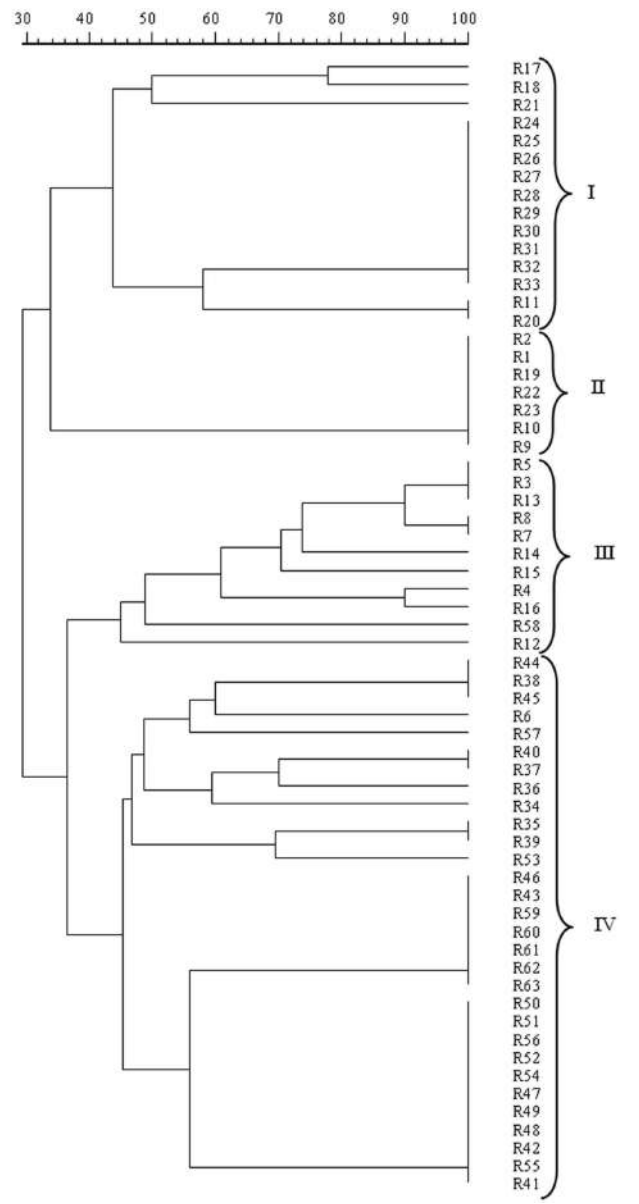

FIGURE 2 | Dendrogram from UPGMA clustering analysis, based on Dice coefficient of mtDNA *Rsa*I restriction patterns of the *S. cerevisiae* isolates from Aglianico del Vulture (clusters I, II, and III) and Sangiovese (cluster IV) grape must.

Technological Characterization of *S. cerevisiae* Isolates

As regards the evaluation of technological characters, the isolates exhibited a high tolerance to ethanol; almost all isolates tolerated 16% v/v of this compound (only few strains developed until 18% v/v of ethanol), whereas a certain variability was found for sulfur dioxide (**Figure 3A**) and copper (**Figure 3B**) resistance. Although the most of isolates exhibited a low tolerance to sulfur dioxide (100 mg L^{-1} of SO_2), all the yeasts developing to the highest tested doses of SO_2 were isolated from "Sangiovese" (except one). As regards copper tolerance, the isolates were distributed in different classes of resistance. A high number of Sangiovese isolates tolerated 200 μM $CuSO_4$, whereas the majority of "Aglianico" isolates developed between 200 and 400 μM $CuSO_4$. However, also for copper tolerance, the yeasts growing at the highest copper doses (500 μM $CuSO_4$) were mainly Sangiovese isolates.

Inoculated Fermentation at Laboratory-Scale

The wild strains were tested in inoculated fermentations at laboratory scale. Two different red musts, "Aglianico" (the isolation grape must of the R1-R33 isolates) and "Sangiovese" (the isolation grape must of the R34-R63 isolates), were used to evaluate strain fermentative performance. The data related to fermentative vigor of Aglianico isolates (**Figure 4A**) indicate that these isolates have shown a different vigor in the two musts; in particular, the isolates exhibited a lower vigor in Sangiovese must (mean value 2.76 g CO_2/100 mL) than in Aglianico (mean value 3.74 g CO_2/100 mL). Furthermore, these isolates showed higher variability in Aglianico than in Sangiovese must and the highest fermentative vigor was found in Aglianico isolates fermenting the same isolation grape must (8.24 g CO_2/100 mL, **Figure 4A**). Also Sangiovese isolates (**Figure 4B**) showed different fermentative vigor in the two grape musts. The results obtained for Sangiovese isolates were similar to data found in Aglianico yeasts, with highest vigor and variability in Aglianico fermentation, although the maximum fermentative vigor of Sangiovese isolates (5.48 g CO_2/100 mL, **Figure 4B**) was lower than maximum value showed by Aglianico isolates.

The data related to fermentative power, showed in **Figures 4C,D**, confirmed the results obtained for fermentative vigor, with different fermentative behavior in the two musts. As already found for fermentative vigor, Aglianico isolates (**Figure 4C**) showed higher fermentative power than Sangiovese isolates (**Figure 4D**), and both isolates groups showed the best performance in Aglianico grape must, with the highest value of 17.23 g CO_2/100mL for Aglianico isolates (**Figure 4C**) and 14.8 g CO2/100 mL for Sangiovese isolates (**Figure 4D**). Furthermore, the isolates exhibited higher variability for this parameter in Aglianico than in Sangiovese must (values ranging between 11.76–17.23 and 9.96–12.7 g CO_2/100 mL, respectively). Finally, the best value was exhibited in Aglianico must by strains isolated from this variety.

The content of 14 yeast metabolic compounds (ethanol, glycerol, acetic acid, acetaldehyde, 1-propanol, 3-methyl-isobutanol, *n*-butanol, 2-methyl-1-butanol, 3-methyl-1-butanol, diacetyl, acetoin, meso and racemic 2,3-butanediol, ethyl acetate) was determined in the experimental wines obtained at the end of the alcoholic fermentations of Aglianico and Sangiovese grape musts.

Statistical analysis of metabolites produced (**Table 1**) demonstrated that, independently of the grape must variety fermented (Aglianico or Sangiovese), some compounds showed significant differences which may be related to the different origin of the yeast strains carrying out the fermentative process.

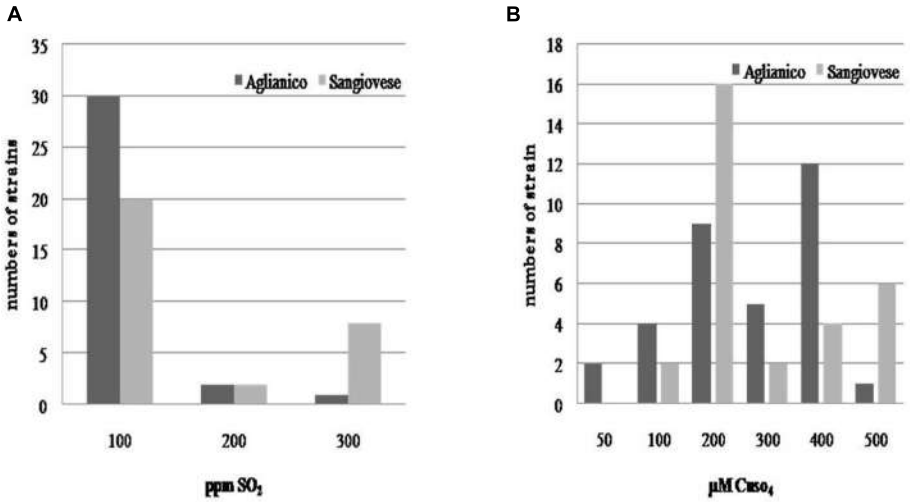

FIGURE 3 | Distribution of *S. cerevisiae* according to their resistance to sulfur dioxide (A) and copper (B).

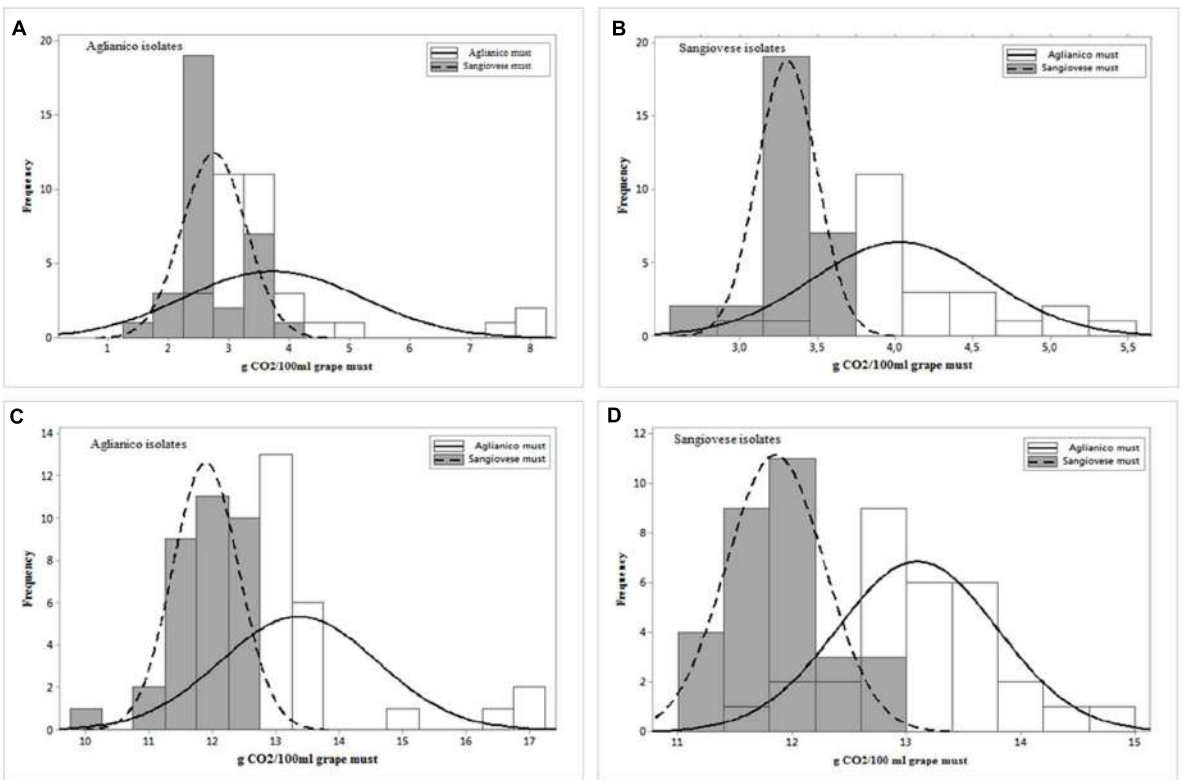

FIGURE 4 | Distribution of *S. cerevisiae* isolated from Aglianico del Vulture and Sangiovese according to fermentative vigor (g CO_2/100 mL after 2 days of fermentation) (A,B, respectively) and according to fermentative power (g CO_2/100 mL at the end of fermentation) (C,D, respectively) in Aglianico del Vulture and Sangiovese grape must.

In particular, *S. cerevisiae* isolates from Aglianico produced significantly higher amounts of acetic acid, acetaldehyde, acetoin, 1-butanol and 2,3-butanediols, while *S. cerevisiae* isolates from Sangiovese yielded higher concentrations of 2-methyl-1-butanol and 3-methyl-1-butanol (**Table 1**). The metabolic profiles obtained confirm the wide phenotypic variability within *S. cerevisiae* species, but, in any case, wine composition, independently of the grape variety, was markedly characterized by metabolites of the fermenting yeast strain.

TABLE 1 | Statistical analysis (*t*-test $p < 0.05$) of metabolites from fermentations of Aglianico del Vulture grape must (Ag fermented must) and Sangiovese grape must (Sg fermented must) carried out by *S. cerevisiae* isolates from Aglianico del Vulture (Ag) and Sangiovese (Sg), (Values as means ± SD) (different letters indicate significant differences among metabolites produced in the same must).

Compounds		Ag fermented must		Sg fermented must	
		Ag isolates	Sg isolates	Ag isolates	Sg isolates
1-propanol	mgL^{-1}	50.57 ± 15.40	48.27 ± 11.67	37.62^{b} ± 11.09	44.00^{a} ± 7.94
Isobutanol	mgL^{-1}	100.27^{b} ± 24.25	115.38^{a} ± 33.25	95.80 ± 28.75	91.54 ± 22.00
n-butanol	mgL^{-1}	6.17^{b} ± 3.00	3.28^{a} ± 1.25	3.25^{b} ± 2.10	1.59^{a} ± 0.54
2-methyl-1-butanol	mgL^{-1}	33.56^{b} ± 9.78	40.33^{a} ± 9.86	27.78^{b} ± 7.01	36.86^{a} ± 7.21
3-methyl-1-butanol	mgL^{-1}	275.19^{b} ± 81.82	316.71^{a} ± 74.89	227.33^{b} ± 64.35	286.67^{a} ± 34.47
Acetaldehyde	mgL^{-1}	105.31^{b} ± 39.75	79.28^{a} ± 20.57	173.27^{b} ± 87.16	102.32^{a} ± 41.42
Acetoin	mgL^{-1}	42.78^{b} ± 24.78	27.17^{a} ± 5.46	59.36^{b} ± 40.80	17.44^{a} ± 7.59
Diacetyl	mgL^{-1}	3.51^{b} ± 2.51	5.14^{a} ± 1.09	6.10^{b} ± 2.60	2.50^{a} ± 1.94
Ethyl acetate	mgL^{-1}	15.73^{b} ± 10.19	24.70^{a} ± 6.93	34.13^{b} ± 14.93	18.90^{a} ± 5.33
Glycerol	gL^{-1}	7.18 ± 0.74	7.39 ± 0.46	6.24^{b} ± 1.51	7.67^{a} ± 0.48
Acetic acid	gL^{-1}	0.37^{b} ± 0.11	0.28^{a} ± 0.10	0.52^{b} ± 0.20	0.23^{a} ± 0.07
2,3-butanediol meso	mgL^{-1}	261.79^{b} ± 90.02	149.95^{a} ± 49.67	268.37^{b} ± 114.69	127.00^{a} ± 56.95
2,3-butanediol racemic	mgL^{-1}	857.45^{b} ± 247.02	539.24^{a} ± 106.61	844.74^{b} ± 280.11	407.67^{a} ± 94.05
Ethanol	% v/v	11.89 ± 0.29	12.04 ± 0.35	11.98 ± 0.46	11.98 ± 0.41

Principal component analysis (PCA) was applied to the matrix of multivariate data comprising concentrations of the metabolic compounds. Residual sugars resulted lower than 2 gL^{-1} in all experimental fermented wines, with the exception for five Sangiovese wines obtained with the strains R2, R4, R16, R22, and R23 originating from Aglianico del Vulture and two Aglianico wines produced by the strains R2 and R53 (originating from Aglianico and from Sangiovese must, respectively). Therefore, these seven wine samples were not included in the PCA analysis.

Figures 5A,B show PCA scores and loadings biplots, respectively, for all the experimental wines deriving from both grape musts fermentation by the 56 *S. cerevisiae* strains. Examination of the data by PCA showed that PC1 and PC2 accounted for 54% of variation in the dataset. Along the first component, independently of the fermented must, most of the wine samples grouped into two clusters according to the geographic origin of the fermenting yeast strains. In particular, 95% of wines produced by *S. cerevisiae* isolates from Sangiovese grouped in a cluster on the right of the plot, whereas 70% of wines obtained by *S. cerevisiae* isolates from Aglianico del Vulture were grouped into a more scattered cluster on the left of the plot. The first principal component correlated positively with 2-methyl-1-butanol, 3-methyl-1-butanol and glycerol and negatively with 2,3-butanediols.

In addition, in order to evaluate whether *S. cerevisiae* isolates could group according to their geographic origin, all assayed oenological properties, including technological characters and by-products of alcoholic fermentations of both grape musts, were combined and analyzed by PCA. The biplot of the parameters considered, pointed out that along the first component all the Sangiovese isolates, except one, were positioned in the left quadrants while 88% of Aglianico isolates grouped on the right quadrants (**Figures 6A,B**). Therefore, a good relationship between *S. cerevisiae* isolates and their geographical origin was confirmed.

DISCUSSION

The concept of *terroir* for wine is classically considered as the result from the interaction between specific *Vitis vinifera* varieties and the local soils, geography, climate and agricultural practices (Van Leeuwen and Seguin, 2006). Recently, there is limited but increasing evidence showing that the microorganisms that influence vine growth, fermentation and wine style (as *S. cerevisiae* does) also exhibit regional differentiation (Lopandic et al., 2007; Gayevskiy and Goddard, 2012; Bokulich et al., 2014; Taylor et al., 2014; Knight and Goddard, 2015), supporting the concept that there could be a microbial aspect to *terroir*. In the present study, 63 *S. cerevisiae* isolates from two different grape musts (Aglianico del Vulture and Sangiovese) were characterized in order to assess the influence of geographic origin of these yeasts on their genetic and phenotypic patrimony. The results obtained by molecular fingerprinting using MSP-PCR with $(GTG)_5$ and RFLP-mtDNA methods confirmed applicability and sensitivity of these methodologies for identification of different *S. cerevisiae* strains (Schuller et al., 2007; Orlić et al., 2010). Furthermore, these techniques were able to detect genetic differences between "Aglianico" and "Sangiovese" strains, resulting suitable methods to differentiate *S. cerevisiae* isolates based on their provenience as most of the isolates grouped according to their origin of isolation. The high genetic polymorphism found in the yeasts analyzed using the MSP-PCR and RFLP-mtDNA could be a result of a constant adaptation to the ecological conditions they are exposed to. Studies based on genetic and microbiological analyses suggest that in *S. cerevisiae* a significant part of the mechanisms affecting this genetic polymorphism occur during the vegetative phase of its growth cycle, where meiosis is a rare event (Puig et al., 2000; Perez-Ortin et al., 2002; Aa et al., 2006). That is to say, if yeasts reproduce clonally and they are constantly adapting to their particular environment, there must be a link between the genetic similarity of the strains

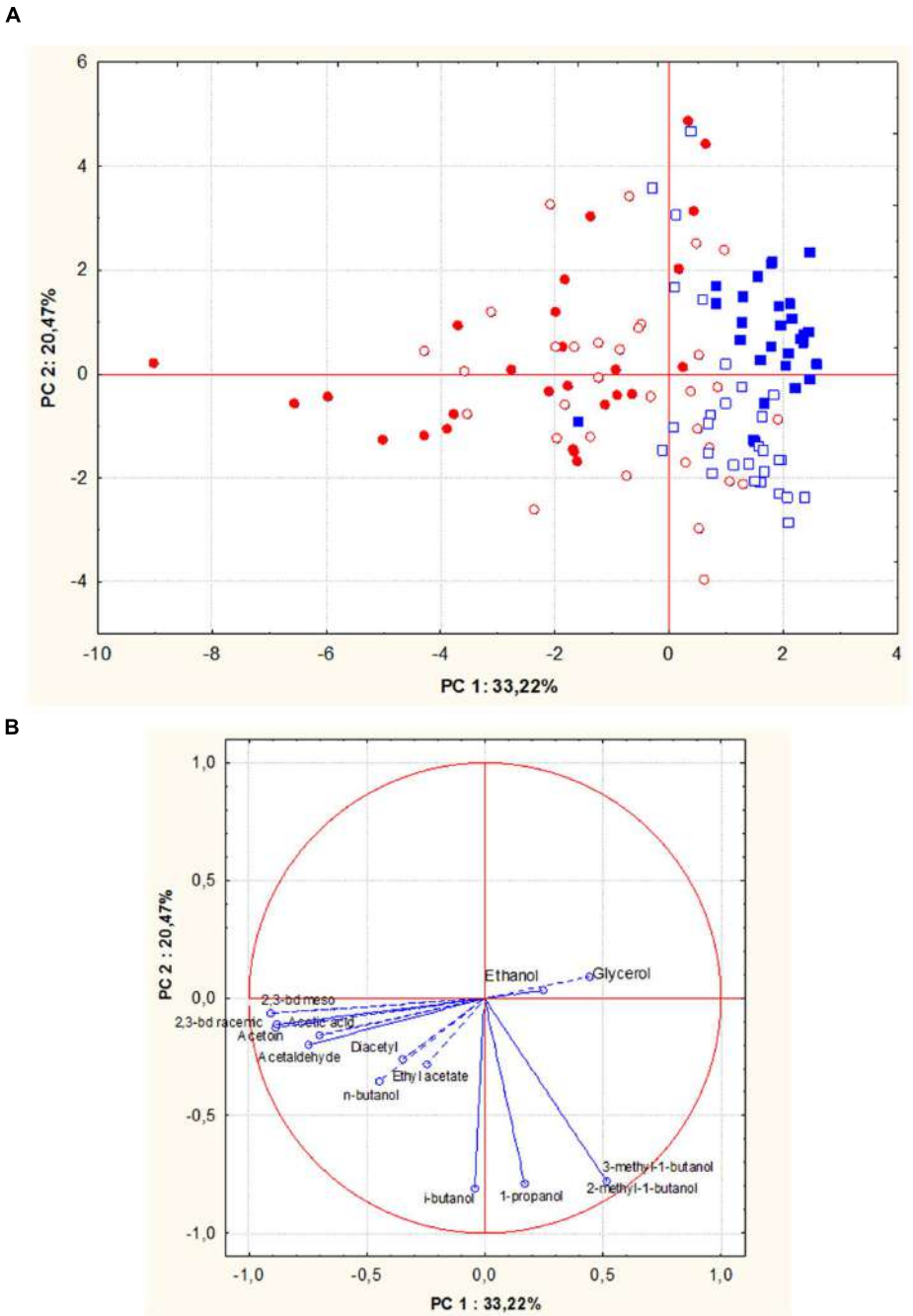

FIGURE 5 | Principal component analysis (PCA) plot based on by-products of alcoholic fermentations in Aglianico del Vulture and Sangiovese wine samples produced by *S. cerevisiae* isolates from Aglianico del Vulture and Sangiovese grape must. (Blue square = wines obtained by isolates from Sangiovese: open symbols indicate Aglianico wines and close symbols indicate Sangiovese wines; red circle = wines obtained by isolates from Aglianico: open symbols indicate Aglianico wines and close symbols indicate Sangiovese wines); the scores **(A)** and variable loadings **(B)** for the two first principal components.

and their geographic origin. It is well known that geographic or ecological isolation is one of the mechanisms involved in the process of speciation (Dobzhansky, 1951) as it creates a barrier for the genetic flux, so that strains coming from the same microenvironment will be more similar to each other than those from other geographic origin (Martínez et al., 2007). Our results, although based on the analysis of a small number of isolates, confirm that the wine production areas represent a reservoir of natural yeasts with peculiar genotypic profile, selected by the natural environment and by the interactions between yeasts and its environment (Guillamón et al., 1996; Martínez et al., 2007).

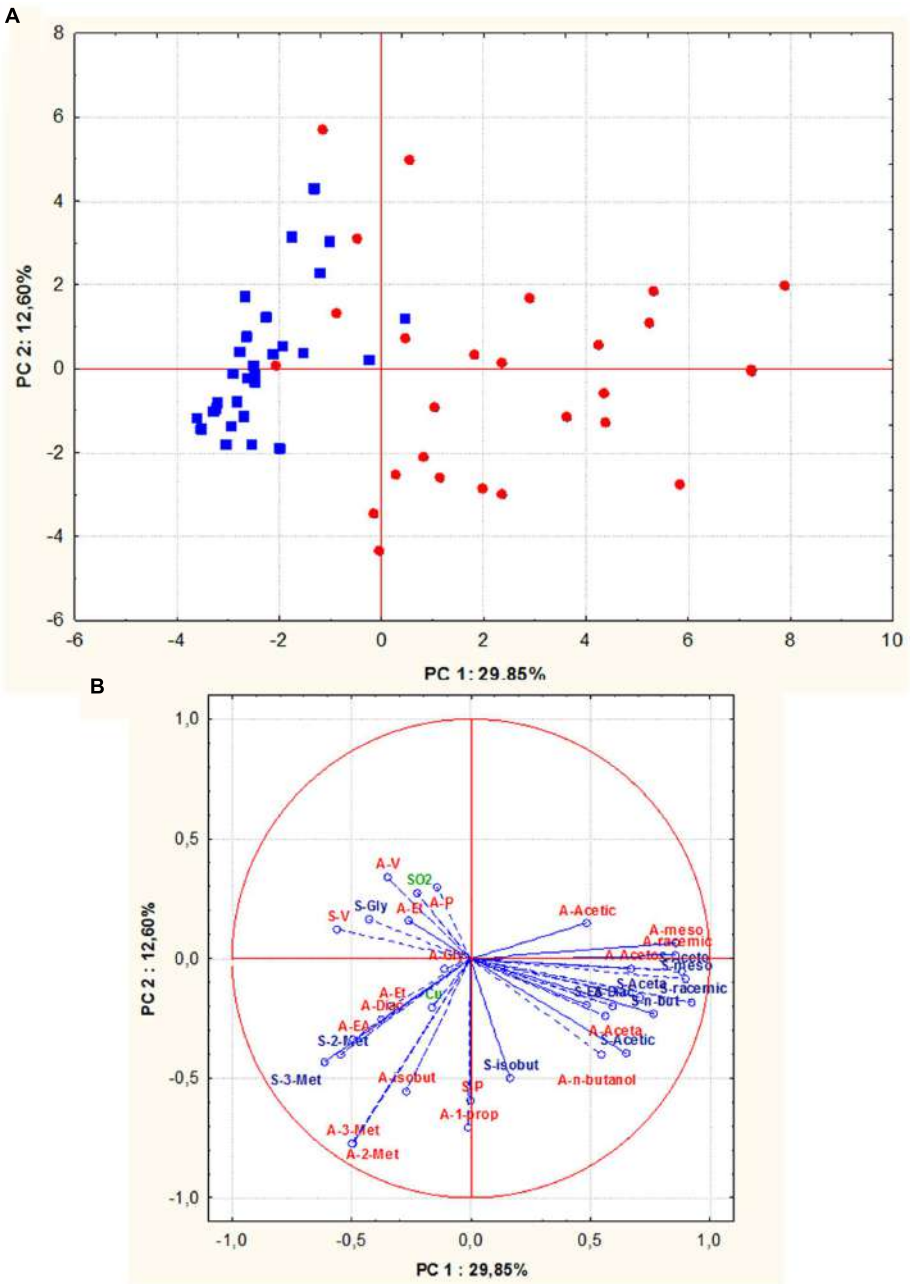

FIGURE 6 | Principal component analysis (PCA) of oenological properties (resistance to SO_2 and copper, fermentative vigor and power, by-products of alcoholic fermentation) of *S. cerevisiae* isolates (red circle = isolates from Aglianico del Vulture, blue square = isolates from Sangiovese), the scores (A) and variable loadings (B) for the two first principal components. Abbreviations for chemical measures in loadings are: red "A" refers to Aglianico del Vulture, blu "S" refers to Sangiovese, V, fermentative vigor; P, fermentative power; SO_2, SO_2 resistance; Cu, copper resistance; Et, ethanol; Acetic, acetic acid; meso, meso 2,3 butanediol; racemic, racemic 2,3 butanediol; Gly, glycerol; Aceta, acetaldehyde; Diac, diacetyl; Aceto, acetoin; EA, ethyl acetate; 3-met, 3- methyl 2 butanol; 2-met, 2- methyl 2 butanol; *n*-butanol, normal butanol; isobut, isobutanol; 1-prop, 1-propanol.

Further step of yeast characterization was addressed to identify potential relationships between strain origin and fermentation phenotype. Indeed, the yeast strain diversity might significantly affect the fermentation performance (Schuller et al., 2012; Tofalo et al., 2014). The data related to fermentative performance of the two groups of *S. cerevisiae* isolates, tested in the two isolation grape musts, seem to suggest that this parameter is influenced by both fermentation medium and source of yeast isolation. In fact, the isolates showed different fermentative performance in the two musts, with best results in Aglianico grape must, and Aglianico isolates exhibited higher fermentative performances than Sangiovese isolates (**Figures 4A–D**).

As regards the fermentation substrate, the different yeast behavior could be correlated to the different composition of grape must. It has been underlined that wine fermentation conditions represent a combination of various stresses (osmotic, ethanol, acidic, nutrient limitation) that accentuate the metabolic differences between strains. Our results showed a certain influence of isolation origin (the grapes variety, in our case) on strain performance during the fermentation and might support the hypothesis that the autochthonous yeast strains are better adapted to the ecological and technological features of their own winegrowing area. These relationships between the origin and the phenotypes of some yeast strains could be due to physiological and metabolic adaptations in response to specific environmental conditions (Camarasa et al., 2011). Probably, "in the isolation grape must the strains are able to express their own better characteristics because they are better adapted to metabolize the precursors present in this grape must" (Capece et al., 2012). Indeed, in Sangiovese must five *S. cerevisiae* isolates from Aglianico were unable to complete the alcoholic fermentation. Furthermore, our data indicate significant correlations between the geographic relatedness of *S. cerevisiae* isolates and their effect on content of some compounds in the resulting wines in agreement with the results obtained by Knight et al. (2015). The PCA of experimental wines obtained by inoculating the *S. cerevisiae* isolates in Aglianico and Sangiovese grape musts (**Figure 5**) revealed that the samples were mainly grouped according to the geographic origin of the yeast strains. To our knowledge, few researches reporting the correlation between strain origin and fermentation phenotype are available until now. A study, aimed to analyze the variability of 36 *S. cerevisiae* strains, isolated from different grape varieties and from two very distant Italian zones (Mauriello et al., 2009), demonstrated that production of volatile aromatic compounds (VOC) allowed to differentiate the yeasts in function of isolation area. Indeed, *S. cerevisiae* isolated from Southern Italy grapes were able to produce more volatile compounds than those from Northern Italy. In a study performed on regionally genetically differentiated population of *S. cerevisiae* in New Zealand, Knight et al. (2015) demonstrated that these populations differentially affected wine phenotype. By evaluating the correlation between *S. cerevisiae* genetic distance and volatile chemical profile of wines, obtained by inoculating these strains, the authors found that these factors are correlated, confirming "the significant relationship existing between the genetic relatedness of natural *S. cerevisiae* sub-populations and their effect on resulting wine phenotypes" (Knight et al., 2015). However, these authors found that the chemicals responsible for the differences between regions are not consistently from any particular class. On the contrary, our results show that the production level of some compounds is correlated with yeast origin, independently from fermentation substrate. In particular, in both grape musts tested, *S. cerevisiae* from Aglianico produced significantly higher amounts of acetic acid, acetoin, acetaldehyde, *n*-butanol and 2,3-butanediols, while experimental wines produced by inoculating *S. cerevisiae* isolates from Sangiovese contained higher concentrations of 2-methyl-1-butanol and 3-methyl-1-butanol (**Table 1**). These results might be correlated to genotypic characteristics of yeasts. In fact, it is reported that the types and concentrations of metabolites produced by *S. cerevisiae* are significantly influenced by yeast genotype (Camarasa et al., 2011; Pretorius et al., 2012; Richter et al., 2013), while not exclusively genetically determined.

CONCLUDING REMARKS

These findings support the need to unveil the indigenous *S. cerevisiae* population of specific areas and explore its natural biodiversity in order to produce valuable wines styles. The natural biodiversity of grape berries, grape juice, and winery environment, well correlated to each specific *terroir*, show a unique composition and represent great resources to winemaking. In fact, this work demonstrated that indigenous microorganisms are better adapted to the "chemical environment" of the grape must coming from a specific wine-producing area. The use of these strains as specific starter cultures can give distinct regional characteristics to wines. This suggests that safeguarding and exploiting natural biodiversity can allow the development of modern winemaking practices and the diversification of wine production, with tangible economic imperatives.

AUTHOR CONTRIBUTIONS

AC contributed to the design of the work, to the molecular characterization of yeasts, to the interpretation of data for the work, to draft the work and revising it; LG contributed to the design of the work, to the interpretation of data for the work, to draft the work and revising it; SG contributed to the molecular characterization of yeasts, to statistical analysis of data and to the interpretation of data for the work; SM contributed to chemical analysis of must and experimental wines, to statistical elaboration of data, RR contributed to the molecular characterization of yeasts, to the management of experimental fermentation, to the statistical elaboration of data; MV contributed to the design of the work, to the draft of the work and revising it, PR contributed to the design of the work, to the draft of the work and revising it, and ensured that that questions related to the accuracy or integrity of any part of the work were appropriately investigated and resolved.

REFERENCES

Aa, E., Townsend, J. P., Adams, R. I., Nielsen, K. M., and Taylor, J. W. (2006). Population structure and gene evolution in *Saccharomyces cerevisiae*. *FEMS Yeast Res.* 6, 702–715. doi: 10.1111/j.1567-1364.2006.00059.x

Agnolucci, M., Scarano, S., Santoro, S., Sassano, C., Toffanin, A., and Nuti, M. (2007). Genetic and phenotypic diversity of autochthonous *Saccharomyces* spp. strains associated to natural fermentation of 'Malvasia delle Lipari'. *Lett. Appl. Microbiol.* 45, 657–662. doi: 10.1111/j.1472-765X.2007.02244.x

Augruso, S., Granchi, L., and Vincenzini, M. (2006). Biodiversità intraspecifica di *Saccharomyces cerevisiae* e ambiente vitivinicolo. *VQ* 7, 38–45.

Barrajón, N., Capece, A., Arévalo-Villena, M., Briones, A., and Romano, P. (2011). Co-inoculation of different *Saccharomyces cerevisiae* strains and influence on volatile composition of wines. *Food Microbiol.* 28, 1080–1086. doi: 10.1016/j.fm.2011.02.016

Blanco, P., Ramilo, A., Cerdeira, M., and Orriols, I. (2006). Genetic diversity of wine *Saccharomyces cerevisiae* strains in an experimental winery from Galicia (NW Spain). *Antonie Van Leeuwenhoek* 89, 351–357. doi: 10.1007/s10482-005-9038-6

Bokulich, N. A., Thorngate, J. H., Richardson, P. M., and Mills, D. A. (2014). Microbial biogeography of wine grapes is conditioned by cultivar, vintage, and climate. *Proc. Natl. Acad. Sci. U.S.A.* 111, E139–E148. doi: 10.1073/pnas.1317377110

Callejon, R. M., Clavijo, A., Ortigueira, P., Troncoso, A. M., Paneque, P., and Morales, M. L. (2010). Volatile and sensory profile of organic red wines produced by different selected autochthonous and commercial *Saccharomyces cerevisiae* strains. *Anal. Chem. Acta* 660, 68–75. doi: 10.1016/j.aca.2009.09.040

Camarasa, C., Sanchez, I., Brial, P., Bigey, F., and Dequin, S. (2011). Phenotypic landscape of *Saccharomyces cerevisiae* during wine fermentation: evidence for origin-dependent metabolic traits. *PLoS ONE* 6:e25147. doi: 10.1371/journal.pone.0025147

Capece, A., Pietrafesa, R., and Romano, P. (2011). Experimental approach for target selection of wild wine yeasts from spontaneous fermentation of "Inzolia" grapes. *World J. Microbiol. Biotechnol.* 27, 2775–2783. doi: 10.1007/s11274-011-0753-z

Capece, A., Romaniello, R., Siesto, G., and Romano, P. (2012). Diversity of *Saccharomyces cerevisiae* yeasts associated to spontaneously fermenting grapes from an Italian "heroic vine-growing area." *Food Microbiol.* 31, 159–166. doi: 10.1016/j.fm.2012.03.010

Capece, A., Siesto, G., Romaniello, R., Lagreca, V. M., Pietrafesa, R., Calabretti, A., et al. (2013). Assessment of competition in wine fermentation among wild *Saccharomyces cerevisiae* strains isolated from Sangiovese grapes in Tuscany region. *LWT - Food Sci. Technol.* 54, 485–492. doi: 10.1016/j.lwt.2013.07.001

Cocolin, L., Pepe, V., Comitini, F., Comi, G., and Ciani, M. (2004). Enological and genetic traits of *Saccharomyces cerevisiae* isolated from former and modern wineries. *FEMS Yeast Res.* 5, 237–245. doi: 10.1016/j.femsyr.2004.08.005

Csoma, H., Zakany, N., Capece, A., Romano, P., and Sipiczki, M. (2010). Biological diversity of *Saccharomyces* yeasts of spontaneously fermenting wines in four wine regions: comparative genotypic and phenotypic analysis. *Int. J. Food Microbiol.* 140, 239–248. doi: 10.1016/j.ijfoodmicro.2010.03.024

Dobzhansky, T. (1951). *Genetics and the Origin of Species*. New York: Columbia University Press.

Dukes, B. C., and Butzke, C. E. (1998). Rapid determination of primary amino acids in grape juice using an o-phthaldialdehyde /N-acetyl-Lcysteine spectrophotometric assay. *Am. J. Enol. Vitic.* 49, 125–134.

Fleet, G. H. (2003). Yeast interactions and wine flavor. *Int. J. Food Microbiol.* 86, 11–22. doi: 10.1016/S0168-1605(03)00245-9

Frezier, V., and Dubourdieu, D. (1992). Ecology of yeast strain *Saccharomyces cerevisiae* during spontaneous fermentation in a Bordeaux winery. *Am. J. Enol. Vitic.* 53, 375–380.

Gayevskiy, V., and Goddard, M. R. (2012). Geographic delineations of yeast communities and populations associated with vines and wines in New Zealand. *ISME J.* 6, 1281–1290. doi: 10.1038/ismej.2011.195

Granchi, L., Ganucci, D., Messini, A., Rosellini, D., and Vincenzini, M. (1998). Dynamics of yeast populations during the early stages of natural fermentation for the production of Brunello di Montalcino wines. *Food Technol. Biotechnol.* 36, 313–318.

Granchi, L., Ganucci, D., Viti, C., Giovannetti, L., and Vincenzini, M. (2003). *Saccharomyces cerevisiae* biodiversity in spontaneous commercial fermentations of grape musts with adequate and inadequate assimilable-nitrogen content. *Lett. Appl. Microbiol.* 36, 54–58. doi: 10.1046/j.1472-765X.2003.01263.x

Guillamón, J. M., Barrio, E., and Querol, A. (1996). Characterization of Wine Yeast Strains of the *Saccharomyces* genus on the basis of molecular markers: relationships between genetic distance and geographic or ecological origin. *Syst. Appl. Microbiol.* 19, 122–132. doi: 10.1016/S0723-2020(96)80019-1

Gutièrrez, A. R., Santamarìa, R., Epifanio, S., Garijo, P., and Lopez, R. (1999). Ecology of spontaneous fermentation in one winery during 5 consecutive years. *Lett. Appl. Microbiol.* 29, 411–415. doi: 10.1046/j.1472-765X.1999.00657.x

Knight, S., and Goddard, M. R. (2015). Quantifying separation and similarity in a *Saccharomyces cerevisiae* metapopulation. *ISME J.* 9, 361–370. doi: 10.1038/ismej.2014.132

Knight, S., Klaere, S., Fedrizzi, B., and Goddard, M. R. (2015). Regional microbial signatures positively correlate with differential wine phenotypes: evidence for a microbial aspect to terroir. *Sci. Rep.* 5:14233. doi: 10.1038/srep14233

Lopandic, K., Gangl, H., Wallner, E., Tscheik, G., Leitner, G., Querol, A., et al. (2007). Genetically different wine yeasts isolated from Austrian vine-growing regions influence wine aroma differently and contain putative hybrids between *Saccharomyces cerevisiae* and *Saccharomyces kudriavzevii*. *FEMS Yeast Res.* 7, 953–965. doi: 10.1111/j.1567-1364.2007.00240.x

Martínez, C., Cosgaya, P., Vásquez, C., Gac, S., and Ganga, A. (2007). High degree of correlation between molecular polymorphism and geographic origin of wine yeast strains. *J. Appl. Microbiol.* 103, 2185–2195. doi: 10.1111/j.1365-2672.2007.03493.x

Mauriello, G., Capece, A., D'Auria, M., Garde-Cerdán, T., and Romano, P. (2009). SPME-GC method as a tool to differentiate VOC profiles in *Saccharomyces cerevisiae* wine yeasts. *Food Microbiol.* 26, 246–252. doi: 10.1016/j.fm.2009.01.003

Mercado, L., Sturm, M. E., Rojo, M. C., Ciklic, I., Martínez, C., and Combina, M. (2011). Biodiversity of *Saccharomyces cerevisiae* populations in Malbec vineyards from the "Zona Alta del Río Mendoza" region in Argentina. *Int. J. Food Microbiol.* 151, 319–326. doi: 10.1016/j.ijfoodmicro.2011.09.026

Orlić, S., Vojvoda, T., Babić, K. H., Arroyo-López, F. N., Jeromel, A., Kozina, B., et al. (2010). Diversity and oenological characterization of indigenous *Saccharomyces cerevisiae* associated with Zilavka grapes. *World J. Microbiol. Biotechnol.* 26, 1483–1489. doi: 10.1007/s11274-010-0323-9

Perez-Ortin, J. E., Querol, A., Puig, S., and Barrio, E. (2002). Molecular characterization of a chromosomal rearrangement involved in the adaptive evolution of yeast strains. *Genome Res.* 12, 1533–1539. doi: 10.1101/gr.436602

Prameteftaki, P. V., Lanaridis, P., and Typas, M. A. (2000). Molecular identification of wine yeasts at species or strain level: a case study with strains from two vine-growing areas of Greece. *J. Appl. Microbiol.* 89, 236–248. doi: 10.1046/j.1365-2672.2000.01102.x

Pretorius, I. S. (2000). Tailoring wine yeast for the new millennium: novel approaches to the ancient art of winemaking. *Yeast* 16, 675–729. doi: 10.1002/1097-0061(20000615)16:8<675::AID-YEA585>3.0.CO;2-B

Pretorius, I. S., Curtin, C. D., and Chambers, P. J. (2012). The winemaker's bug. *Winemakers Bug* 3, 149–158. doi: 10.4161/bbug.19687

Puig, S., Querol, A., Barrio, E., and Pérez-Ortin, J. (2000). Mitotic recombination and genetic change in *Saccharomyces cerevisiae* during wine fermentation. *Appl. Environ. Microbiol.* 66, 2057–2061. doi: 10.1128/AEM.66.5.2057-2061.2000

Querol, A., Barrio, E., and Ramon, D. (1994). Population dynamics of wine yeast strains in natural fermentations. *Int. J. Food Microbiol.* 21, 315–323. doi: 10.1016/0168-1605(94)90061-2

Richter, C. L., Dunn, B., Sherlock, G., and Pugh, T. (2013). Comparative metabolic footprinting of a large number of commercial wine yeast strains in Chardonnay fermentations. *FEMS Yeast Res.* 13, 394–410. doi: 10.1111/1567-1364.12046

Romano, P., Capece, A., Serafino, V., Romaniello, R., and Poeta, C. (2008). Biodiversity of wild strains of *Saccharomyces cerevisiae* as tool to complement and optimize wine quality. *World J. Microbiol. Biotechnol.* 24, 1797–1802. doi: 10.1007/s11274-008-9672-z

Romano, P., Fiore, C., Paraggio, M., Caruso, M., and Capece, A. (2003). Function of yeast species and strains in wine flavour. *Int. J. Food Microbiol.* 86, 169–180. doi: 10.1016/S0168-1605(03)00290-3

Romano, P., Marchese, R., Laurita, C., Salzano, G., and Turbanti, L. (1999). Biotechnological suitability of *Saccharomycodes ludwigii* for fermented beverages. *World J. Microbiol. Biotechnol.* 15, 451–545. doi: 10.1023/A:1008948623024

Sabate, J., Cano, J., Querol, A., and Guillamon, J. M. (1998). Diversity of *Saccharomyces* strains in wine fermentations: analysis for two consecutive years. *Lett. Appl. Microbiol.* 26, 452–455. doi: 10.1046/j.1472-765X.1998.00369.x

Scacco, A., Oliva, D., Di Maio, S., Polizzotto, G., Genna, G., Tripodi, G., et al. (2012). Indigenous *Saccharomyces cerevisiae* strains and their influence on the quality of Cataratto, Inzolia and Grillo white wines. *Food Res. Int.* 46, 1–9. doi: 10.1016/j.foodres.2011.10.038

Schneider, A., Gerbi, V., and Redoglia, M. (1987). A rapid HPLC method for separation and determination of major organic acids in grape musts and wines. *Am. J. Enol. Vitic.* 38, 151–155.

Schuller, D., Alves, H., Dequin, S., and Casal, M. (2005). Ecological survey of *Saccharomyces cerevisiae* strains from vineyards in the Vinho Verde Region of Portugal. *FEMS Microbiol. Ecol.* 51, 167–177. doi: 10.1016/j.femsec.2004.08.003

Schuller, D., Cardoso, F., Sousa, S., Gomes, P., Gomes, A. C., Santos, M. A. S., et al. (2012). Genetic diversity and population structure of *Saccharomyces cerevisiae* strains isolated from different grape varieties and winemaking regions. *PLoS ONE* 7:e32507. doi: 10.1371/journal.pone.0032507

Schuller, D., Pereira, L., Alves, H., Cambon, B., Dequin, S., and Casal, M. (2007). Genetic characterization of commercial *Saccharomyces cerevisiae* isolates recovered from vineyard environments. *Yeast* 24, 625–636. doi: 10.1002/yea.1496

Sneat, P. H. A., and Sokal, R. R. (ed.). (1973). *Numerical Taxonomy. The Principles and Practice of Numerical Classification*. San Francisco, CA: W. H. Freeman and Company.

Sun, H., Ma, H., Hao, M., Pretorius, I. S., and Chen, S. (2009). Identification of yeast population dynamics of spontaneous fermentation in Beijing wine region, China. *Ann. Microbiol.* 59, 69–76. doi: 10.1007/BF03175601

Taylor, M. W., Tsai, P., Anfang, N., Ross, H. A., and Goddard, M. R. (2014). Pyrosequencing reveals regional differences in fruit-associated fungal communities. *Environ. Microbiol.* 16, 2848–2858. doi: 10.1111/1462-2920.12456

Tofalo, R., Perpetuini, G., Fasoli, G., Schirone, M., Corsetti, A., and Suzzi, G. (2014). Biodiversity study of wine yeasts belonging to the "terroir" of Montepulciano d'Abruzzo "Colline Teramane" revealed *Saccharomyces cerevisiae* strains exhibiting atypical and "unique 5.8S-ITS restriction patterns". *Food Microbiol.* 39, 7–12. doi: 10.1016/j.fm.2013.10.001

Tofalo, R., Perpetuini, G., Schirone, M., Fasoli, G., Aguzzi, I., Corsetti, A., et al. (2013). Biogeographical characterization of *Saccharomyces cerevisiae* wine yeast by molecular methods. *Front. Microbiol.* 4:166. doi: 10.3389/fmicb.2013.00166

Torija, M., Rozès, N., Poblet, M., Guillamón, J. M., and Mas, A. (2001). Yeast population dynamics in spontaneous fermentations: comparison between two different wine-producing areas over a period of three years. *Antonie van Leeuwenhoek* 79, 345–352. doi: 10.1023/A:1012027718701

Van Leeuwen, C., and Seguin, G. (2006). The concept of terroir in viticulture. *J. Wine Res.* 17, 1–10. doi: 10.1080/09571260600633135

Versavaud, A., Courcoux, P., Roulland, C., Dulau, L., and Hallet, J. N. (1995). Genetic diversity and geographical distribution of wild *Saccharomyces cerevisiae* strains from the wine-producing area of Charentes, France. *Appl. Environ. Microbiol.* 61, 3521–3529.

Vezinhet, F., Hallet, J. N., Valade, M., and Poulard, A. (1992). Ecological survey of wine strains by molecular methods of identification. *Am. J. Enol. Vitic.* 43, 83–86.

Villanova, M., and Sieiro, C. (2006). Contribution by *Saccharomyces cerevisiae* yeast to fermentative flavor compounds in wines from cv. Albariño. *J. Ind. Microbiol. Biotechnol.* 33, 929–933. doi: 10.1007/s10295-006-0162-8

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REVIEW
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Flor Yeast: New Perspectives Beyond Wine Aging

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The most important dogma in white-wine production is the preservation of the wine aroma and the limitation of the oxidative action of oxygen. In contrast, the aging of Sherry and Sherry-like wines is an aerobic process that depends on the oxidative activity of flor strains of *Saccharomyces cerevisiae*. Under depletion of nitrogen and fermentable carbon sources, these yeast produce aggregates of floating cells and form an air–liquid biofilm on the wine surface, which is also known as velum or flor. This behavior is due to genetic and metabolic peculiarities that differentiate flor yeast from other wine yeast. This review will focus first on the most updated data obtained through the analysis of flor yeast with *-omic* tools. Comparative genomics, proteomics, and metabolomics of flor and wine yeast strains are shedding new light on several features of these special yeast, and in particular, they have revealed the extent of proteome remodeling imposed by the biofilm life-style. Finally, new insights in terms of promotion and inhibition of biofilm formation through small molecules, amino acids, and di/tri-peptides, and novel possibilities for the exploitation of biofilm immobilization within a fungal hyphae framework, will be discussed.

Keywords: flor yeast, wine, biofilm, -omic tools, immobilization, biofilm management, biocapsules

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INTRODUCTION

Saccharomyces cerevisiae flor yeast are responsible for the biological aging of Sherry and Sherry-like wines. The main feature of these yeast is that at the end of alcoholic fermentation, when they are under nitrogen and sugar depletion, they shift from fermentative to oxidative metabolism (i.e., the diauxic shift) and rise to the wine surface to form multicellular aggregates. This aggregation leads to the build-up of a biofilm, or velum or flor (Esteve-Zarzoso et al., 2001; Aranda et al., 2002; Alexander, 2013).

Biofilm formation is strongly dependent on the nutritional status of the wine. It is well known that biofilm starts when the concentration of any fermentable carbon source is imperceptible or null (Martínez et al., 1997a). In addition, the presence of other carbon sources, such as glycerol and ethyl acetate, can induce biofilm formation (Zara et al., 2010). Thus, biofilm formation is not limited to aerobic growth on ethanol, but occurs also on other reduced non-fermentable carbon sources that provide sufficient energy input. Moreover, biofilm formation is affected by the availability of nitrogen. It has been shown that in wine lacking nitrogen sources, the flor yeast do not form a

biofilm, and that the addition of amino acids to the medium does not induce biofilm formation (Mauricio et al., 2001; Berlanga et al., 2006). Zara et al. (2011) reported that biofilm formation is favored by addition of 37.5 mM ammonium sulfate, while when these concentrations exceed 150 mM, biofilm formation is prevented.

During biofilm growth, the lack of fermentable carbon sources and the availability of oxygen induce cells to maintain aerobic metabolism, which results in important changes to the wine sensorial and aromatic properties, and to its chemical composition. These changes include a reduction of the volatile acidity due to the metabolism of acetic acid, and production of acetaldehyde at the expense of ethanol. Moreover, acetaldehyde by-products provide the distinctive flavor of Sherry and Sherry-like wines, such as 1,1-diethoxyethane and sotolon (Dubois et al., 1976; Guichard et al., 1992; Moreno et al., 2005; Zea et al., 2015).

Oxidative metabolism is essential to allow flor strains to remain at the wine surface; indeed, Jiménez and Benítez (1988) demonstrated that flor *petite* mutants cannot form biofilm and are more sensitive to ethanol. Furthermore, sensitivity to ethanol is inversely correlated with rate of biofilm formation, where the less resistant strains produce the biofilm more rapidly (Martínez et al., 1997b).

The ability of *S. cerevisiae* to adapt to environmental and nutritional changes depends on the activation of metabolic pathways that induce the expression of specific genes. For biofilm formation, expression of the *FLO11* gene has been shown to be the key event. Indeed, the increased expression of *FLO11* during the diauxic shift results in higher cell-surface hydrophobicity. This encourages the formation of multicellular aggregates that entrap CO_2 bubbles deriving from the fermentation of the residual sugar, thus providing the buoyancy to the aggregates, and therefore promoting biofilm formation (Zara et al., 2005) (**Figure 1**). Activation of *FLO11* depends on three specific pathways: the cAMP-protein kinase A (PKA) pathway; the mitogen-activated protein kinase (MAPK) pathway; and the TOR pathway (Braus et al., 2003; Vinod et al., 2008). It has been shown that in biofilm-inducing media, biofilm formation and *FLO11* transcription can be significantly reduced by the addition of rapamycin, which is a well-known inhibitor of the TOR pathway, and the deletion of *RAS2,* which regulates the PKA and MAPK pathways (Zara et al., 2011). Finally, the expansion of minisatellites within the central domain of *FLO11* contributes to increased protein glycosylation and hydrophobicity of the Flo11 glycoprotein (Flo11p) of flor yeast (Reynolds and Fink, 2001; Zara et al., 2005; Fidalgo et al., 2006).

As well as the role of *FLO11* in the rising of cells and in their hydrophobicity, biofilm formation appears to be dependent on increased cell buoyancy. This is influenced by their lipid content and composition, as flor strains have greater chain lengths and unsaturation levels of their fatty-acid residues than those shown by non-biofilm-forming strains of *S. cerevisiae* (Farris et al., 1993; Zara et al., 2009). Addition of cerulenin, which is an antibiotic that inhibits *de-novo* fatty-acid biosynthesis, results in a dramatic reduction in *FLO11* transcription levels and biofilm weight of flor yeast grown in biofilm-inducing media (Zara et al., 2012). Inositol availability also affects biofilm formation, possibly due to its key role in the assembly of the glycosylphosphatidylinositol anchor of Flo11p, and in the regulation of lipid biosynthetic genes, such as *ACC1* (Zara et al., 2012).

Due to their metabolic and genetic peculiarities, flor strains can overcome stress caused by high ethanol and acetaldehyde contents in Sherry and Sherry-like wines (Budroni et al., 2005). It has been hypothesized that this adaptive ability is related to DNA mutations caused by acetaldehyde, such as double-strand breaks (Ristow et al., 1995). These mutations are considered to be responsible for mitochondrial DNA polymorphism (Castrejon et al., 2002) and for gross chromosomal rearrangements in flor yeast (Infante et al., 2003). The complexity and specificity of the flor yeast genome make these strains an interesting model for studies into speciation of *S. cerevisiae* and into adaptive evolution based on mutations in the *FLO11* gene (Fidalgo et al., 2006).

Considering that comparative genomics, proteomics and metabolomics of flor and wine yeast strains are shedding new light on several features of these special yeast, in this review we will discuss the most recent data obtained through the analysis of flor yeast with *-omic* tools. Moreover, we will report on new insights in terms of promotion and inhibition of biofilm formation through small molecules, amino acids and di/tri-peptides, and on novel possibilities for the exploitation of yeast immobilization within a fungal hyphae framework.

GENETIC DIVERSITY INDICATES THAT MOST FLOR YEAST SHARE THE SAME ORIGIN

Biological aging is performed traditionally in several countries in Europe, including Hungary (Tokaj-Hegyalja) to produce Szamorodni, Italy (Sardinia) to produce Vernaccia di Oristano, Spain (Jerez area) to produce Xeres, and France (Jura) to produce Vin Jaune. Flor yeast isolated from the biofilms of these different wines have long been considered as specific varieties of *S. cerevisiae* given their unique behavior, although until recently we had no knowledge whether the strains in these different countries are related or not.

The first attempt to differentiate flor strains based on their ability to metabolize sugars (i.e., galactose, dextrose, lactose, maltose, melibiose, raffinose, sucrose) classified flor yeast into four varieties: *Saccharomyces cerevisiae var. beticus*, *Saccharomyces cerevisiae var. cheresiensis*, *Saccharomyces cerevisiae var. montuliensis* and *Saccharomyces cerevisiae var. rouxii* (Martínez et al., 1997b). Strains of these four varieties were also detected among Jura flor strains (Charpentier et al., 2009). More recent molecular studies have revealed that despite the high diversity detected for mitochondrial restriction fragment length polymorphism profiles, Spanish Sherry wine yeast share a specific 24-bp deletion in ITS1, which suggests a single family for Spanish Sherry yeast (Esteve-Zarzoso et al., 2001), while another allele of the ITS1 region has been detected among French flor strains (Charpentier et al., 2009). Given the different geographic origins and the genetic specificities of flor yeast, the question of the origin of flor yeast can be investigated further.

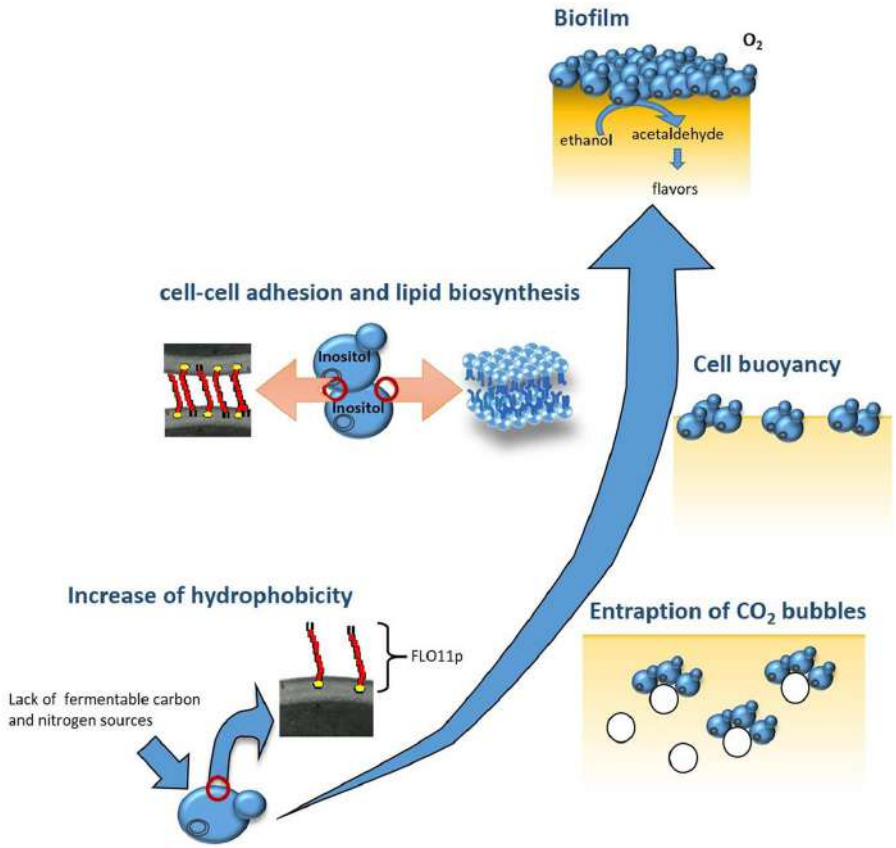

FIGURE 1 | Building of the biofilm by flor yeast. At the end of fermentation, *Saccharomyces cerevisiae* flor yeast adapt to the lack of fermentable carbon and nitrogen sources by triggering specific metabolic pathways: cAMP-protein kinase A (PKA), mitogen-activated protein kinase (MAPK), and TOR. These, in turn, activate the transcription of *FLO11*, which codes for a hydrophobic protein. The higher cell surface hydrophobicity encourages formation of multicellular aggregates that entrap CO_2 bubbles and float toward the wine surface. In biofilm cells overexpressing *FLO11*, inositol is used for the assembly of the glycosylphosphatidylinositol anchor of Flo11p. The decrease in the intracellular concentration of inositol and the availability of oxygen on the wine surface activate the expression of genes regulated by inositol–choline responsive elements, such as ACC1, which results in the *de-novo* biosynthesis of unsaturated fatty acids and in increased cell buoyancy. The different cell layers that constitute the mature biofilm protect the wine from direct exposure to oxygen, and induce important changes in the organoleptic, aromatic and chemical composition of the wine.

The genetic analysis of flor strains with microsatellite typing revealed that, surprisingly, most flor strains of Spain, Italy, France and Hungary belong to the same genetic group of *S. cerevisiae* (Legras et al., 2014), with sub-clustering that corresponds to the strains from each of these countries (**Figure 2**). Of note, this sub-clustering might be related to differences in the ability to produce a biofilm. Some strains in the main group of Jura flor strains differ in terms of the length of the *FLO11* gene and the presence of a 111-bp deletion in *ICR1*, the long, non-coding RNA that regulates the expression of *FLO11*. Jura flor strains with this deletion produce thicker biofilms, whereas Jura strains with a longer *FLO11* allele and a wild-type version of *ICR1* produce thinner biofilms (Legras et al., 2014). Interestingly, two isolates from Hungary have a heterozygote version of the promoter: one wild-type and one that has the deletion. The presence of a single cluster of flor strains from different countries attests that they share a unique origin and indicates that flor yeast have migrated within Europe, as has been shown for wine yeast all over the world (Legras et al., 2007). In agreement with this hypothesis, some isolates related to flor strains have also been isolated in Lebanon. This recent characterisation of flor strains from different countries into a single group demonstrates the ecological success of these flor strains, which occupy the specific niche of the wine surface. This suggests that there are genomic specificities associated to the adaptation to the wine biological aging environment, such as has been seen for *FLO11*, and will be very likely for other genes.

ADAPTATION OF FLOR YEAST AND COPY-NUMBER VARIATIONS

Comparative genome hybridization provided the first insight into the adaptation of wine (Dunn et al., 2005) and flor (Infante et al., 2003; Legras et al., 2014) yeast to their environment. Aneuploidies are frequently involved in adaptation to changing environments, as has been observed in adaptive evolution experiments (Dunham et al., 2002; Gresham et al., 2008).

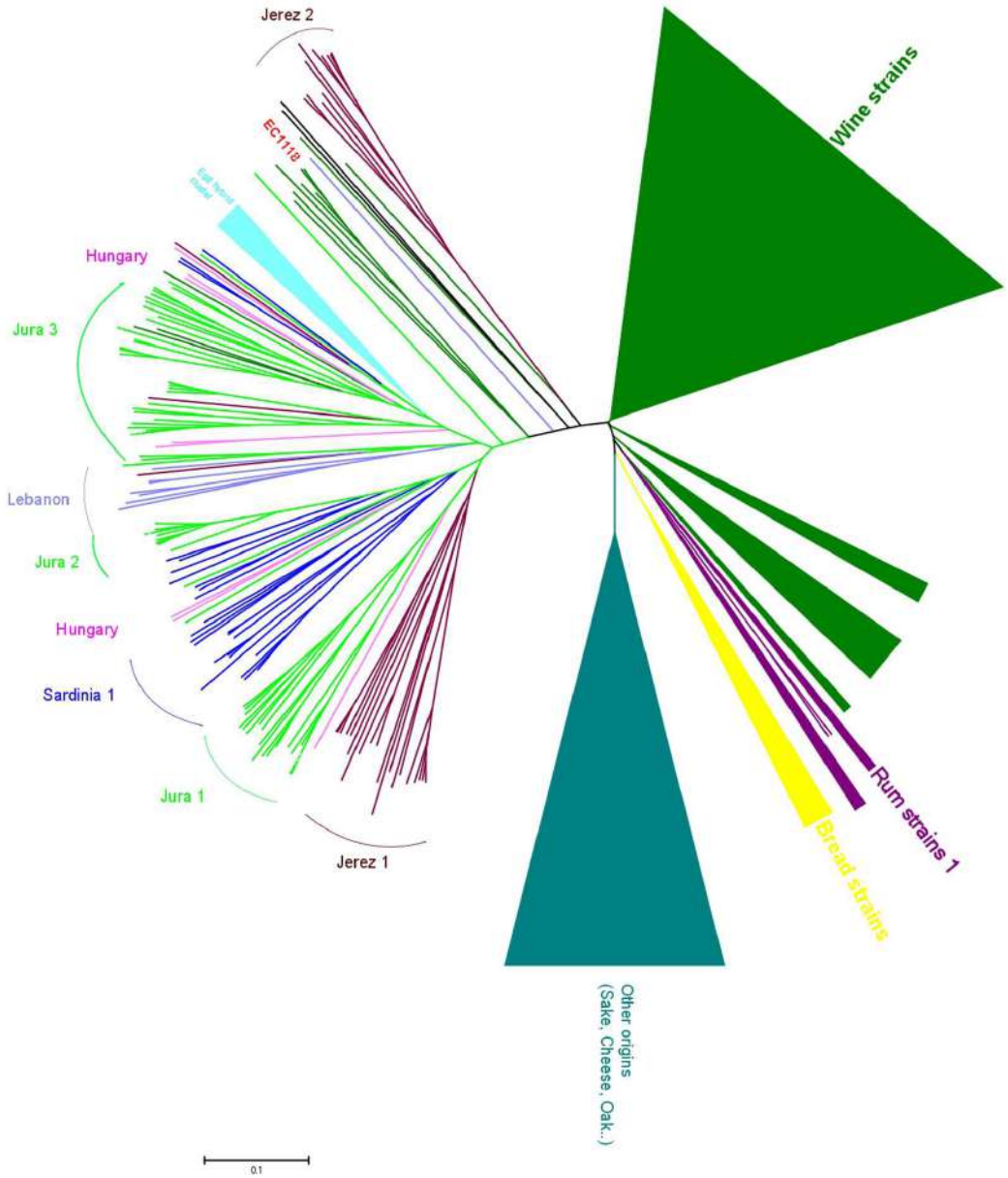

FIGURE 2 | Genomic diversity of flor strains. Neighbor-joining tree built by evaluating the flor strains at 12 microsatellite loci, in comparison with strains of other origins. The tree was built from the Dc chord distance and drawn with MEGA5.22. Clusters of wine strains and other origins have been condensed due to their large size (modified from Legras et al., 2014).

Sequences contained inside gross chromosomal rearrangements can be amplified, which leads to greater expression of some genes, and the first comparison of two flor strains was believed to indicate flor-strain peculiarities (Infante et al., 2003). However, the comparative genome hybridization profiles of six flor strains from Spain, Hungary, France and Italy, compared to those of wine strains and using S288C as a reference, did not reveal such complex aneuploidy profiles (Legras et al., 2014). This global comparison revealed a drop in the hybridisation signal in the sub-telomeric regions, which suggested missing or divergent genes in these regions. However, when looking for amplified genes, only three genes were detected: *FRE2*, *MCH2*, and *YKL222C* (Legras et al., 2014). *MCH2* is annotated as a putative monocarboxylic-acid transporter, although its involvement in monocarboxylic acid transport has not been shown experimentally (Makuc et al., 2001). *MCH2* is important for yeast survival during the second phase of alcoholic fermentation (i.e., alcohol accumulation) (Novo et al., 2013), and has been shown to be induced under vanillin stress and to confer vanillin resistance (Park et al., 2015). These results show that copy-number variations solely cannot explain the adaptation of flor yeast to their environment, as has been

proposed previously (Infante et al., 2003). However, *MCH2* and *YKL222C* are promising targets, the roles of which need to be evaluated. Further studies, such as population genomics, have to be performed to unravel the genetics basis of flor-yeast adaptation.

SPECIFICITIES OF FLOR YEAST: FURTHER INSIGHTS FROM PROTEOMICS AND METABOLOMICS

In recent years, proteomics and metabolomics have been applied to the study of flor yeast metabolism and their responses to environmental conditions. Moreno-García et al. (2014) performed a proteome analysis during biofilm formation that focussed on elucidation of the role of the mitochondria, which are the essential organelle for oxidative metabolism, for elaboration of several stress responses, and for the formation of biofilms (van Loon et al., 1986; Costa et al., 1993, 1997; Piper, 1999; Reinders et al., 2006; Ma and Liu, 2010; Fierro-Risco et al., 2013; Vandenbosch et al., 2013). From this proteome analysis, a number of mitochondrion-localized proteins that might be responsible for flor yeast behavior were highlighted. These included proteins involved in carbohydrate oxidative metabolism, biofilm formation, apoptosis, and responses to stresses typical of biological aging; e.g., ethanol, acetaldehyde, and reactive oxygen species (Fierro-Risco et al., 2013). Also, proteins associated with non-fermentable carbon uptake, glyoxylate and the TCA cycle, cellular respiration, and inositol metabolism are more expressed in yeast growing under biofilms than under fermentative conditions (Moreno-García et al., 2015b). Ino1p, which participates in inositol biosynthesis, was five-fold more expressed under biofilm conditions (Moreno-García et al., 2015b). Accordingly, Zara et al. (2012) reported that under biofilm-forming conditions, flor yeast show greater expression of genes involved in inositol biosynthesis.

The presence of proteins involved in cell-wall biosynthesis and protein glycosylation, which are important for cell-cell adhesion and hence for biofilm formation, has also been reported (Moreno-García et al., 2015b). Through the combination of proteomics and innovative metabolomics techniques that were aimed at quantifying minor volatile compounds under exhaustively controlled biofilm conditions, 33 proteins were shown to be directly involved in the metabolism of glycerol, ethanol and 17 aroma compounds (Moreno-García et al., 2015a). Although proteome analyses for oenological purposes have expanded substantially in recent years, particularly in terms of fermentative yeast (Zuzuarregui et al., 2006; Salvado et al., 2008; Rossignol et al., 2009), the relationships between changes in the yeast proteome and exometabolome and the influence of such changes on the organoleptic properties of wine still remained to be explored. The application of comparative *-omic* disciplines to flor yeast has provided novel knowledge on several features of these yeast, and has revealed the extent of the proteome remodeling that is imposed by the biofilm life-style. Under fermentative conditions, flor and wine yeast have comparable metabolism, although some differences have been revealed. For instance, unlike other fermentative strains, flor yeast increases the concentrations of some higher alcohols with their respective acetic acid esters and ethyl esters of C6 and C8 acids (Moreno et al., 1991). Furthermore, during the fermentation process and biofilm formation, flor strains yield higher levels of lactones than other non-flor strains (Zea et al., 1995). The intracellular accumulation and consequent excretion of terpenic compounds during fermentation, as well as during biofilm formation, was also shown by Zea et al. (1995).

Yeast growth under biofilm-forming conditions and wine biological aging are accompanied by the production of specific wine aromas. Systematic studies have shown that acetaldehyde is the most important metabolite in terms of the different levels between biologically aged and unaged wines, and have also highlighted the decrease in volatile acidity and glycerol content in aged wines (Mauricio et al., 1997, 2001; Cortés et al., 1998, 1999; Berlanga et al., 2004; Muñoz et al., 2005, 2007 and Moreno-García et al., 2013). Among the 35 aroma compounds quantified by Muñoz et al. (2005, 2007), acetaldehyde, 1,1-diethoxyethane, 2,3-butanediol (levo + meso forms), isoamyl alcohols, ethyl and isoamyl acetates, butanoic acid, 2,3-methylbutanoic acids, and 4-butyrolactone, were defined as the most active odorant compounds. Each of these showed odor activity values (OAVs) >0.8 in the biologically aged wines. Only 1-butanol, 2-butanol, isobutyl acetate, furanmethanol, and neral were present at levels 10-fold below their odor perception thresholds. The remaining compounds showed OAVs between 0.1 and 0.8. Compounds with OAVs >1 are considered as important contributors to the aroma of beverages, although there are exceptions when odorants with high OAVs are suppressed and compounds with lower OAVs are revealed as important contributors (Grosch, 2001). All of the 35 compounds studied here showed significant differences after biological aging, in relation to the initial control non-aerated wine (Cortés et al., 1998, 1999; Zea et al., 2001).

These data were confirmed and enriched by Muñoz et al. (2005, 2007) when they studied the effects of periodic aeration on metabolites such as acetaldehyde and its derivatives, and higher alcohols, their acetic-acid esters, and 3-(methylthio)-1-propanol, all of which increased in content as a consequence of the flor yeast growing under biofilm forming conditions. In contrast, the acids of 4, 5, and 6 carbon atoms showed lower concentrations in aged wines, and levels close to zero were obtained for 2-butanol, pantolactone, Z-whisky lactone, 4-ethylguaiacol, furanmethanol, 3-ethoxy-1-propanol and neral, after the same time of aging under biofilm forming conditions. Concentration changes obtained for other important aroma compounds, such as Z-whisky lactone and 4-ethylguaiacol, can only be explained because of the aging process carried out in contact with oak barrels.

The link between the intracellular proteins and metabolites excreted by yeast that are strongly related to sensorial properties constitutes a new and interesting advance in biological information systems. The knowledge generated can be considered as useful information for innovation in fermentative, winemaking and biotechnological-based industries in the near future.

FLOR YEAST AS A BIOLOGICAL MODEL FOR THE STUDY OF SMALL MOLECULES THAT INHIBIT OR PROMOTE BIOFILM FORMATION

Microbial biofilms are tenacious structures that can be difficult to eradicate and to treat with the current arsenal of antifungal agents. This is mainly due to a lack of guidelines for biofilm management, and to difficulties in their diagnosis and identification. In contrast, many microbial biofilms are beneficial for a plethora of biotechnological processes, like cleaning up hazardous waste sites, filtering biofuels and wastewaters, and forming bio-barriers to protect soil and groundwater from contamination (Ashraf et al., 2014). Similarly for many food processes, such as maturation of cheese (Licitra et al., 2007) and biological aging of Sherry wines (Zara et al., 2005).

Problems related to biofilm eradication motivate current efforts to find compounds that can alter cell-surface hydrophobicity, typically through interactions with cell-wall components, and mainly the cell-wall mannoproteins, thus counteracting biofilm formation. Antimicrobial peptides are lead compounds in this approach. Many antimicrobial peptides have been shown to modulate adhesion and biofilm formation of some yeast and fungi due to hydrophobic and electrostatic interactions. For example, histidine-rich glycoproteins greatly inhibit biofilm formation by *Candida albicans* by binding and rupturing cell-wall components (Rydengard et al., 2008). In contrast, the antimicrobial peptides histatin-5 and LL-37 are antagonized by the cell-wall mucin Msb2 of *C. albicans*, which enhances resistance toward such compounds (Szafranski-Schneider et al., 2012).

In addition to antimicrobial peptides, other small molecules are currently being assessed for anti-biofilm activity. Zhao and Liu (2010) have shown that N-acetyl cysteine has anti-bacterial properties toward *Pseudomonas aeruginosa* and might mediate detachment of *P. aeruginosa* biofilms. A recent study reported that when mixed with other amino acids and nisin, L-cysteine prevents biofilm formation by *Streptococcus mutans* (Tong et al., 2014). Other studies on the effects of amino acids are controversial. Sanchez et al. (2013) reported that D-amino acids inhibit biofilm formation in *P. aeruginosa*, while Sarkar and Pires (2015) showed that they have no effect on *Bacillus subtilis*, *Staphylococcus aureus*, or *Staphylococcus epidermidis*. Moreover, a report of promising anti-biofilm activity of D-amino acids on *B. subtilis* strains (Kolodkin-Gal et al., 2010) was recently retracted (Hofer, 2014).

Nitrogen is a fundamental nutrient in living cells, and its metabolism is involved in major developmental decisions in *S. cerevisiae* (Forsberg and Ljungdahl, 2001). According to Homann et al. (2005), clinical and vineyard isolates of *S. cerevisiae* can grow on a wide range of nitrogen sources, with respect to laboratory strains. Through phenotype microarray analysis, Bou Zeidan et al. (2014) showed that flor yeast can metabolize a wide range of nitrogen sources, including different dipeptides. The presence of *FOT* genes that code for oligopeptide transporters and were acquired by horizontal transfer from *Torulaspora microellipsoides* in wine strains, confers the ability to better use the nitrogen resource of grape must, which results in a competitive advantage (Damon et al., 2011; Marsit and Dequin, 2015; Marsit et al., 2016). As *FOT* genes have been shown for several flor strains (Marsit and Dequin, 2015), their presence might favor the adaptation of these strains to the nitrogen-limited environment during flor aging.

Remarkably, Bou Zeidan et al. (2014) observed that flor strains cannot metabolize dipeptides containing L-histidine, and showed a novel role of L-histidine in the dramatic reduction of biofilm formation and adhesion to polystyrene. Dose-response analysis in nutrient-rich medium showed that L-histidine reduces growth rates, delays the lag-phase, and finally inhibits the growth of the strains tested. Other studies have reported that L-carnosine, which is an L-histidine-containing dipeptide with potential antineoplastic effects (Letzien et al., 2014), can slow down cell growth rates and can kill yeast cells in fermentative metabolism (Cartwright et al., 2012). Interestingly, according to Letzien et al. (2014), L-histidine mimics the effects of L-carnosine, although it shows a stronger effect. Contrary to what was observed in glucose-rich medium, in ethanol medium, the presence of 10 mM L-histidine was sufficient to completely inhibit biofilm formation and adhesion to polystyrene, although these major inhibitory effects were not accompanied by any reduction in cell viability. Moreover, they did not correlate with the transcription level of *FLO11*, which was stable in the absence or presence of L-histidine. L-histidine is a cationic amino acid, with a unique imidazole ring as a side chain that shows high affinity for cationic metals, aromatic amino acids, and many other compounds (Shimba et al., 2003; Liao et al., 2013). By promoting non-specific physical interactions with embedded cell-wall components in general, and with the highly O-mannosylated cell-wall mannoprotein Flo11p in particular, these features might induce the loss of cell adhesion and the failure of air–liquid biofilm formation (**Figures 3A,B**).

Bou Zeidan et al. (2013) showed that a small peptide, PAF26, can promote biofilm formation. PAF26 is a short cationic and tryptophan-rich peptide with cell-penetrating and antifungal activities. It interacts with flor wine yeast without substantial cell death, and also promotes biofilm formation, thus indicating that the peptide interactions and cell death are not necessarily linked. The increased formation of biofilm in the presence of PAF26, and the absence of biofilm formation in the PAF26-treated Δ*flo*11 mutant, indicate that PAF26 requires the presence of Flo11p (**Figure 3C**). Flo11p is the main molecular target for PAF26 in ethanol-rich medium, but not in glucose-rich medium, possibly because with glucose-rich medium, the *FLO11* gene is induced solely during the stationary phase, when the cell concentration is high, and after PAF26 has completed its actions (Swinnen et al., 2006). Bou Zeidan and co-workers also observed that the enhancement of biofilm by PAF26 is independent of *FLO11* gene regulation, but requires expression of a functional *FLO11* gene. Therefore, the effects of PAF26 on biofilm is related to the enhancing of cell-to-cell aggregation by PAF26 under specific biofilm-forming conditions. Similar data were obtained in *C. albicans*, where the peptide LL-37 results in cell aggregation and prevention of cell adhesion (Ibeas et al., 2000).

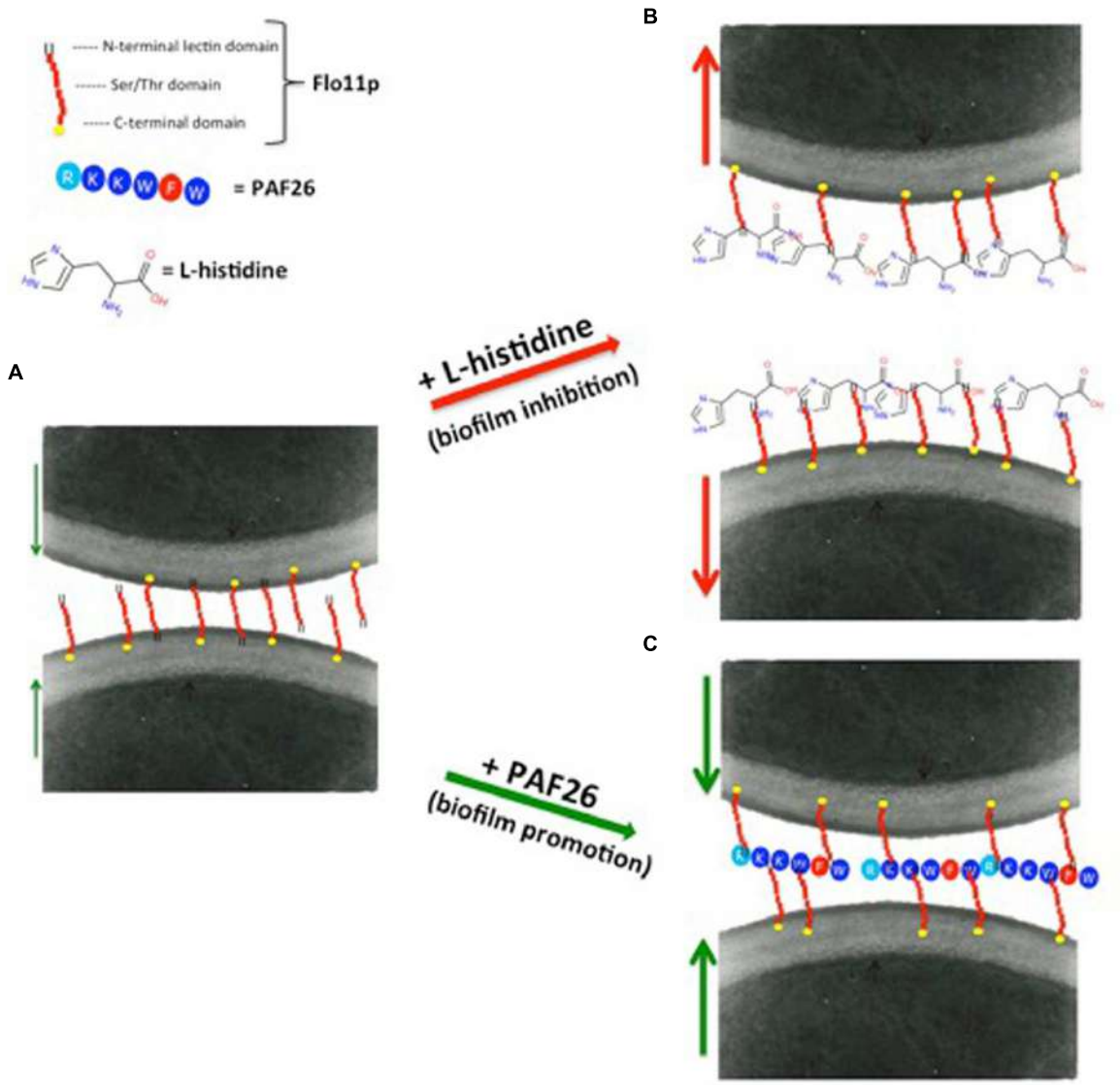

FIGURE 3 | Proposed models for promotion and inhibition of biofilm formation by flor yeast. (A) The large and highly glycosylated extracellular N-terminal domain of Flo11p confers high hydrophobicity and negative net charge to the yeast cell wall, and is responsible for cell-to-cell adhesion and biofilm formation. **(B)** Biofilm inhibition. Among the 20 naturally occurring amino acids, L-histidine is a cationic amino acid with a unique imidazole ring as a side chain. This feature of L-histidine might induce the loss of cell adhesion and biofilm formation of the flor strains, by providing non-specific physical interactions with the embedded cell-wall components in general, and with the highly *O*-mannosylated cell-wall mannoprotein Flo11p in particular. This would lead to the failure of air–liquid biofilm formation and cell adhesion. **(C)** Biofilm promotion. PAF26 is a highly hydrophobic and cationic peptide. Due to its properties, electrostatic and hydrophobic interactions can be established between PAF26 and Flo11p. Following this hypothesis, PAF26 would act by facilitating and bridging the Flo11p-mediated interactions between cells, and thus increasing biofilm formation. Red arrows indicate cell to cell repulsion; green arrows indicate cell to cell attraction.

BIOTECHNOLOGICAL APPLICATIONS OF FLOR YEAST

The potential applications of flor yeast in wine and other industries might be widened by their immobilization in rigid pla -tforms. Yeast immobilization provides a wide range of advantages compared to the use of free yeast; e.g., yield improvements, feasibility of continuous fermentation processing, and yeast reuse (Kourkoutasa et al., 2004).

Novel possibilities for the exploitation of flor yeast in other fermentative processes based on spontaneous immobilization within a fungal hyphae framework (*Penicillium chrysogenum*) have been recently attempted (Peinado et al., 2006). The higher immobilization efficiency of flor yeast *versus* non-flor yeast on filamentous fungi has been well demonstrated. Co-immobilization was carried out in a medium containing gluconic acid (as the carbon source for *P. chrysogenum*, and not for flor yeast) in the absence of physico-chemical

external support or chemical binders. The immobilization bodies thus obtained (i.e., yeast biocapsules) are hollow, smooth, elastic, strong, creamy-colored spheres of variable sizes, depending on the particular shaking rate and time in the co-immobilization medium (García-Martínez et al., 2011) (**Figure 4**). The biocapsule wall consists of yeast cells bound to fungal hyphae that are trapped. When biocapsules are placed in a medium containing fermentable sugars, the yeast cells colonize and invade all of the hyphae, thereby causing the fungus to die and thence to remain as a mere inert support for the yeast, which facilitates the subsequent reuse of the biocapsules.

Yeast-cell immobilization on *P. chrysogenum* and the suitability of the immobilized biocatalysts for sweet wine production was confirmed by the satisfactory operational stability during repeated batch fermentations of must of dried grapes (García-Martínez et al., 2015). The wines obtained by the fermentation of raisin musts contained greater amounts of volatile compounds. Successive reuse of the immobilized flor yeast revealed a gradual adaptation to the fermentation conditions and an increasingly uniform behavior, in terms of the fermentation kinetics and production of metabolites. Immobilized yeast cells produced higher concentrations of carbonyl compounds, esters and polyols than free yeast cells, and the opposite was true for higher alcohols. The nitrogen compounds (e.g., free amino acids, total aminic nitrogen, ammonium ions, urea) depended on the state of the cells (i.e., free or immobilized), and also on the number of times the yeast had been used.

Flor yeast immobilization might provide some advantages toward obtaining the desired ethanol levels by the easier removal of the yeast cells from the medium, or by reductions in the production costs in the inoculum preparation. Recently, different fungus–yeast combinations have also been attempted by using the Zygomycetes (i.e., *Rhizopus* sp.) (Nyman et al., 2013) as well as using non-flor forming yeast strains for different biotechnological purposes, such as for sparkling wine and sweet wine production (López de Lerma et al., 2012; García-Martínez et al., 2013; Puig-Pujol et al., 2013). The operational stability of the immobilization system proposed might enable its use at a commercial scale for the production of sweet wine (García-Martínez et al., 2015).

FIGURE 4 | Biocapsules of flor yeast. (A) Biocapsules of variable sizes obtained using an orbital shaker at 250, 200, and 150 rpm (left to right) for 7 days. **(B)** Biocapsules removed with a sterile strainer. **(C)** Biocapsule image under an optical microscope at 40× magnification. **(D)** Scanning electron micrograph showing immobilized yeast cells entrapped in the hyphae of the filamentous fungus.

CONCLUSION

Based on a survey of the most recent literature, flor yeast have emerged as a promising biological model for the study of yeast speciation and phylogenesis, of alternative life-styles in the microbial world, of management of microbial biofilms, and of biofilm industrial applications. The use of microsatellite genotyping has revealed that flor yeast are a group of *S. cerevisiae* strains close to wine strains, and given the contrasting life-styles of these two groups, this makes for an interesting model for the study of yeast adaptation to anthropic niches. Comparative genome hybridization only revealed two genes amplified in the genome of flor strains, which implies that other sources of allelic variations, such as single nucleotide polymorphisms, might explain the specific properties of flor strains and should be explored through population genomics strategies.

The exploitation of other comparative *-omic* tools has provided novel knowledge on several features of flor yeast, and has revealed that proteome remodeling under biofilm-forming conditions might also be related to the production of aroma-properties-related metabolites. Transcriptomic analysis associated to genetic quantitative analysis might deepen this knowledge, and also help to decipher the complex regulatory networks associated with flor aging. The use of flor yeast as a biological model for the study of the management of biofilms is very promising considering that the control of biofilm formation through the use of small molecules is of great interest not only in the biomedical field, but also for practical applications in industrial settings. For example, by using plastic coatings that release small inhibitory molecules, it might be possible to prevent biofilm formation. On the contrary, the use of small molecules that promote biofilm formation can be beneficial to enhance the biological maturation and aging of different foods and beverages. The use of bio-immobilization systems will certainly widen the spectrum of possible applications of flor yeast, which will open new perspectives for fermentation processes, with substantial technical and economic advantages over traditional fermentation methods based on free yeast cells (García-Martínez et al., 2012). Future insights into the role of the *FLO11* gene in flor yeast will also help to improve cell-immobilization technologies (Nedovic et al., 2015).

AUTHOR CONTRIBUTIONS

JMG, TG-M, JCM, JM wrote "Specificities of flor yeast: further insights from proteomic and metabolomics" and "Biotechnological applications of flor yeast"; J-LL, SD wrote "Genetic diversity indicates that most flor yeast share the same origin" and "Adaptation of flor yeast and copy-number variations"; SZ, GZ, IM, ALC, MBZ, MB wrote "Introduction", "Flor yeast as a biological model for the study of small molecules that inhibit or promote biofilm formation" and "Conclusion". MB coordinated the work and all authors critically revised the manuscript before submission.

REFERENCES

Alexander, H. (2013). Flor yeasts of *Saccharomyces cerevisiae*-Their ecology, genetics and metabolism. *Int. J. Food Microbiol.* 167, 269–275. doi: 10.1016/j.ijfoodmicro.2013.08.021

Aranda, A., Querol, A., and del Olmo, M. (2002). Correlation between acetaldehyde and ethanol resistance and expression of HSP genes in yeast strains isolated during the biological aging of Sherry wines. *Arch. Microbiol.* 177, 304–312. doi: 10.1007/s00203-001-0391-1

Ashraf, M. A., Ullah, S., Ahmad, I., Qureshi, A. K., Balkhair, K. S., and Abdur Rehman, M. (2014). Green biocides, a promising technology: current and future applications to industry and industrial processes. *J. Sci. Food Agric.* 94, 388–403. doi: 10.1002/jsfa.6371

Berlanga, T. M., Millàn, C., Mauricio, J. C., and Ortega, J. M. (2006). Influence of nitrogen on the biological aging of Sherry wine. *J. Sci. Food Agric.* 86, 2113–2118. doi: 10.1002/jsfa.2584

Berlanga, T. M., Peinado, R., Millan, C., and Ortega, J. M. (2004). Discriminant analysis of Sherry wine during biological aging. *Am. J. Enol. Vitic.* 55, 407–411.

Bou Zeidan, M., Carmona, L., Zara, S., and Marcos, J. F. (2013). FLO11 gene is involved in the interaction of flor strains of *Saccharomyces cerevisiae* with a biofilm-promoting synthetic hexapeptide. *Appl. Environ. Microbiol.* 79, 6023–6032. doi: 10.1128/AEM.01647-13

Bou Zeidan, M., Zara, G., Viti, C., Decorosi, F., Mannazzu, I., Budroni, M., et al. (2014). L-histidine inhibits biofilm formation and FLO11-associated phenotypes in *Saccharomyces cerevisiae* flor yeasts. *PLoS ONE* 9:e112141. doi: 10.1371/journal.pone.0112141

Braus, G. H., Grundmann, O., Bruckner, S., and Mosch, H. U. (2003). Amino-acid starvation and Gcn4p regulate adhesive growth and FLO11 gene expression in *Saccharomyces cerevisiae*. *Mol. Biol. Cell* 14, 4272–4284.

Budroni, M., Zara, S., Zara, G., Pirino, G., and Mannazzu, I. (2005). Peculiarities of flor strains adapted to Sardinian Sherry-like wine ageing conditions. *FEMS Yeast Res.* 5, 951–958. doi: 10.1016/j.femsyr.2005.04.002

Cartwright, S. P., Bill, R. M., and Hipkiss, A. R. (2012). L-carnosine affects the growth of *Saccharomyces cerevisiae* in a metabolism-dependent manner. *PLoS ONE* 7:e45006. doi: 10.1371/journal.pone.0045006

Castrejon, F., Codon, A. C., Cubero, B., and Benìtez, T. (2002). Acetaldehyde and ethanol are responsible for mitochondrial DNA (mtDNA) restriction fragment length polymorphism (RFLP) in flor yeasts. *Syst. Appl. Microbiol.* 25, 462–467. doi: 10.1078/0723-2020-00127

Charpentier, C., Colin, A., Alais, A., and Legras, J. L. (2009). French Jura flor yeasts: genotype and technological diversity. *Antonie Van Leeuwenhoek* 95, 263–273. doi: 10.1007/s10482-009-9309-8

Cortés, M. B., Moreno, J., Zea, L., Moyano, L., and Medina, M. (1998). Changes in aroma compounds of Sherry wines during their biological aging carried out by *Saccharomyces cerevisiae* races bayanus and capensis. *J. Agric. Food Chem.* 46, 2389–2394. doi: 10.1021/jf970903k

Cortés, M. B., Moreno, J., Zea, L., Moyano, L., and Medina, M. (1999). Response of the aroma fraction in Sherry wines subjected to accelerated biological aging. *J. Agric. Food Chem.* 47, 3297–3302. doi: 10.1021/jf9900130

Costa, V., Amorim, M. A., Reis, E., Quintanilha, A., and Moradas-Ferreira, P. (1997). Mitochondrial superoxide dismutase is essential for ethanol tolerance of *Saccharomyces cerevisiae* in the post-diauxic phase. *Microbiology* 143, 1649–1656. doi: 10.1099/00221287-143-5-1649

Costa, V., Reis, E., Quintanilha, A., and Moradas-Ferreira, P. (1993). Acquisition of ethanol tolerance in *Saccharomyces cerevisiae*: the key role of the mitochondrial superoxide dismutase. *Arch. Biochem. Biophys.* 300, 608–614. doi: 10.1006/abbi.1993.1084

Damon, C., Vallon, L., Zimmermann, Z., Haider, M. Z., Galeote, V., Dequin, S., et al. (2011). A novel fungal family of oligopeptide transporters identified by functional metatranscriptomics of soil eukaryotes. *ISME J.* 5, 1871–1880. doi: 10.1038/ismej.2011.67

Dubois, P., Rigaud, J., and Dekimpe, J. (1976). Identification de la di-méthyl-4, 5 tétrahydrafuranedione-2, 3 dans le "Vin Jaune" du Jura. *Lebenson. Wiss. Technol.* 9, 366–368.

Dunham, M. J., Badrane, H., Ferea, T., Adams, J., Brown, P. O., Rosenzweig, F., et al. (2002). Characteristic genome rearrangements in experimental evolution of *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. U.S.A.* 99, 16144–16149. doi: 10.1073/pnas.242624799

Dunn, B., Levine, R. P., and Sherlock, G. (2005). Microarray karyotyping of commercial wine yeast strains reveals shared, as well as unique, genomic signatures. *BMC Genomics* 16:53. doi: 10.1186/1471-2164-6-53

Esteve-Zarzoso, B., Peris-Torà, M. J., Garcìa-Maiquez, E., Uruburu, F., and Querol, A. (2001). Yeast population dynamics during the fermentation and biological aging of Sherry wines. *Appl. Environ. Microbiol.* 67, 2056–2061. doi: 10.1128/AEM.67.5.2056-2061.2001

Farris, G. A., Sinigaglia, M., Budroni, M., and Elisabetta, M. (1993). Cellular fatty acid composition in film-forming strains of two physicological races of *Saccharomyces cerevisiae*. *Lett. Appl. Microbiol.* 17, 215–219. doi: 10.1111/j.1472-765X.1993.tb01450.x

Fidalgo, M., Barrales, R. R., Ibeas, J. I., and Jimenez, J. (2006). Adaptative evolution by mutation in the FLO11 gene. *Proc. Natl. Acad. Sci. U.S.A.* 103, 11228–11233. doi: 10.1073/pnas.0601713103

Fierro-Risco, J., Rincón, A. M., Benítez, T., and Codón, A. C. (2013). Overexpression of stress-related genes enhances cell viability and velum formation in Sherry wine yeasts. *Appl. Microbiol. Biotechnol.* 97, 6867–6881. doi: 10.1007/s00253-013-4850-9

Forsberg, H., and Ljungdahl, P. O. (2001). Sensors of extracellular nutrients in *Saccharomyces cerevisiae*. *Curr. Genet.* 40, 91–109. doi: 10.1007/s002940100244

García-Martínez, T., López de Lerma, N., Moreno, J., Peinado, R. A., Millán, M. C., and Mauricio, J. C. (2013). Sweet wine production by two osmotolerant *Saccharomyces cerevisiae* strains. *J. Food Sci.* 78, 874–879. doi: 10.1111/1750-3841.12124

García-Martínez, T., Moreno, J., Mauricio, J. C., and Peinado, R. (2015). Natural sweet wine production by repeated use of yeast cells immobilized on *Penicillium chrysogenum*. *LWT Food Sci. Technol.* 61, 503–509. doi: 10.1016/j.lwt.2014.12.029

García-Martínez, T., Peinado, R. A., Moreno, J., García-García, I., and Mauricio, J. C. (2011). Co-culture of *Penicillium chrysogenum* and *Saccharomyces cerevisiae* leading to the immobilization of yeast. *J. Chem. Technol. Biotechnol.* 86, 812–817. doi: 10.1002/jctb.2593

García-Martínez, T., Puig-Pujol, A., Peinado, R. A., Moreno, J., and Mauricio, J. C. (2012). Potential use of wine yeasts immobilized on *Penicillium chrysogenum* for ethanol production. *J. Chem. Technol. Biotechnol.* 87, 351–359. doi: 10.1002/jctb.2725

Gresham, D., Desai, M. M., Tucker, C. M., Jenq, H. T., Pai, D. A., Ward, A., et al. (2008). The repertoire and dynamics of evolutionary adaptations to controlled nutrient-limited environments in yeast. *PLoS Genet.* 4:e1000303. doi: 10.1371/journal.pgen.1000303

Grosch, W. (2001). Evaluation of the key odorants of foods by dilution experiments, aroma models and omission. *Chem. Senses* 26, 533–545. doi: 10.1093/chemse/26.5.533

Guichard, E., Etievant, P., Henry, R., and Mosandl, A. (1992). Enantiomeric ratios of pantolaetone, solerone, 4-carboethoxy-4-hydroxy-butyrolactone and of sotolon, a flavour impact compound of flor-Sherry and botrytized wines. *Z. Lebensm. Unters. Forsch.* 195, 540–544. doi: 10.1007/BF01204559

Hofer, U. (2014). Biofilm disassembly revisited. *Nat. Rev. Microbiol.* 12, 234–235. doi: 10.1038/nrmicro3233

Homann, O. R., Cai, H., Becker, J. M., and Lindquist, S. L. (2005). Harnessing natural diversity to probe metabolic pathways. *PLoS Genet.* 1:e80. doi: 10.1371/journal.pgen.0010080

Ibeas, J. I., Lee, H., Damsz, B., Prasad, D. T., Pardo, J. M., Hasegawa, P. M., et al. (2000). Fungal cell wall phosphomannans facilitate the toxic activity of a plant PR-5 protein. *Plant J.* 23, 375–383. doi: 10.1046/j.1365-313x.2000.00792.x

Infante, J. J., Dombek, K. M., Rebordinos, L., Cantoral, J. M., and Young, E. T. (2003). Genome wide amplifications caused by chromosomal rearrangements play a major role in the adaptive evolution of natural yeast. *Genetics* 165, 1745–1759.

Jiménez, J., and Benítez, T. (1988). Yeast cell viability under conditions of high temperature and ethanol concentrations depends on the mitochondrial genome. *Curr. Genet.* 13, 461–469. doi: 10.1007/BF02427751

Kolodkin-Gal, I., Romero, D., Cao, S., Clardy, J., Kolter, R., and Losick, R. (2010). D-amino acids trigger biofilm disassembly. *Science* 328, 627–629. doi: 10.1126/science.1188628

Kourkoutasa, I., Bekatoroua, A., Banatb, I. M., Marchantb, R., and Koutinasa, A. A. (2004). Immobilization technologies and support materials suitable in alcohol beverages production: a review. *Food Microbiol.* 21, 377–397.

Legras, J. L., Erny, C., and Charpentier, C. (2014). Population structure and comparative genome hybridization of European flor yeast reveal a unique group of *Saccharomyces cerevisiae* strains with few gene duplications in their genome. *PLoS ONE* 9:e108089. doi: 10.1371/journal.pone.0108089

Legras, J. L., Merdinoglu, D., Cornuet, J. M., and Karst, F. (2007). Bread, beer and wine: *Saccharomyces cerevisiae* diversity reflects human history. *Mol. Ecol.* 16, 2091–2102. doi: 10.1111/j.1365-294X.2007.03266.x

Letzien, U., Oppermann, H., Meixensberger, J., and Gaunitz, F. (2014). The antineoplastic effect of carnosine is accompanied by induction of PDK4 and can be mimicked by L-histidine. *Amino Acids* 46, 1009–1019. doi: 10.1007/s00726-014-1664-8

Liao, S. M., Du, Q. S., Meng, J. Z., Pang, Z. W., and Huang, R. B. (2013). The multiple roles of histidine in protein interactions. *Chem. Cent. J.* 7, 44. doi: 10.1186/1752-153X-7-44

Licitra, G., Ogier, J. C., Parayre, S., Pediliggieri, C., Carnemolla, T. M., and Falentin, H. (2007). Variability of bacterial biofilms of the "tina" wood vats used in the ragusano cheese-making process. *Appl. Environ. Microbiol.* 73, 6980–6987.

López de Lerma, N., García-Martínez, T., Moreno, J., Mauricio, J. C., and Peinado, R. A. (2012). Volatile composition of partially fermented wines elaborated from sun dried Pedro Ximénez grapes. *Food Chem.* 135, 2445–2452. doi: 10.1016/j.foodchem.2012.07.058

Ma, M., and Liu, Z. L. (2010). Mechanisms of ethanol tolerance in *Saccharomyces cerevisiae*. *Appl. Microbiol. Biotechnol.* 87, 829–845. doi: 10.1007/s00253-010-2594-3

Makuc, J., Paiva, S., Schauen, M., Krämer, R., André, B., Casal, M., et al. (2001). The putative monocarboxylate permeases of the yeast *Saccharomyces cerevisiae* do not transport monocarboxylic acids across the plasma membrane. *Yeast* 18, 1131–1143. doi: 10.1002/yea.763

Marsit, S., and Dequin, S. (2015). Diversity and adaptive evolution of *Saccharomyces* wine yeast: a review. *FEMS Yeast Res.* 15, 1–12. doi: 10.1093/femsyr/fov067

Marsit, S., Sanchez, I., Galeote, V., and Dequin, S. (2016). Horizontally acquired oligopeptide transporters favor adaptation of *Saccharomyces cerevisiae* wine yeast to enological environment. *Environ. Microbiol.* doi: 10.1111/1462-2920.13117 [Epub ahead of print].

Martínez, P., Pérez Rodriguez, L., and Benítez, T. (1997a). Factors which affected velum formation by flor yeasts isolated from Sherry wine. *Syst. Appl. Microbiol.* 20, 154–157. doi: 10.1016/S0723-2020(97)80060-4

Martínez, P., Pérez Rodriguez, L., and Benítez, T. (1997b). Velum formation by flor yeasts isolated from Sherry wine. *Am. J. Enol. Vitic.* 48, 55–62.

Mauricio, J. C., Moreno, J. J., and Ortega, J. M. (1997). In vitro specific activities of alcohol and aldehyde dehydrogenases from two flor yeasts during controlled wine ageing. *J. Agric. Food Chem.* 45, 1967–1971. doi: 10.1021/jf960634i

Mauricio, J. C., Valero, E., Millan, C., and Ortega, J. M. (2001). Changes in nitrogen compounds in must and wine during fermentation and biological aging by flor yeasts. *J. Agric. Food Chem.* 49, 3310–3315. doi: 10.1021/jf010005v

Moreno, J., Millan, C., Ortega, J. M., and Medina, M. (1991). Analytical differentiation of wine fermentations using pure and mixed yeast cultures. *J. Ind. Microbiol.* 7, 181–189. doi: 10.1007/BF01575881

Moreno, J. A., Zea, L., Moyano, L., and Medina, M. (2005). Aroma compounds as markers of the changes in Sherry wines subjected to biological ageing. *Food Control* 16, 333–338. doi: 10.1016/j.foodcont.2004.03.013

Moreno-García, J., García-Martínez, T., Millán, M. C., Mauricio, J. C., and Moreno, J. (2015a). Proteins involved in wine aroma compounds metabolism by a *Saccharomyces cerevisiae* flor-velum yeast strain grown in two conditions. *Food Microbiol.* 51, 1–9. doi: 10.1016/j.fm.2015.04.005

Moreno-García, J., García-Martínez, T., Moreno, J., and Mauricio, J. C. (2015b). Proteins involved in flor yeast carbon metabolism under biofilm formation conditions. *Food Microbiol.* 46, 25–33. doi: 10.1016/j.fm.2014.07.001

Moreno-García, J., García-Martínez, T., Moreno, J., Millán, M. C., and Mauricio, J. C. (2014). A proteomic and metabolomic approach for understanding the role

of the flor yeast mitochondria in the velum formation. *Int. J. Food Microbiol.* 172, 21–29. doi: 10.1016/j.ijfoodmicro.2013.11.030

Moreno-García, J., Raposo, R., and Moreno, J. (2013). Biological aging status characterization of Sherry type wines using statistical and oenological criteria. *Food Res. Int.* 54, 285–292. doi: 10.1016/j.foodres.2013.07.031

Muñoz, D., Peinado, R. A., Medina, M., and Moreno, J. (2005). Biological aging of Sherry wines using pure cultures of two flor yeast strains under controlled microaeration. *J. Agric. Food Chem.* 53, 5258–5264. doi: 10.1021/jf0478713

Muñoz, D., Peinado, R. A., Medina, M., and Moreno, J. (2007). Biological aging of Sherry wines under periodic and controlled microaerations with *Saccharomyces cerevisiae* var. capensis: effect on odorant series. *Food Chem.* 100, 1188–1195. doi: 10.1016/j.foodchem.2005.10.065

Nedovic, V., Gibson, B., Mantzouridou, T. F., Bugarski, B., Djordjevič, V., Kalušević, A., et al. (2015). Aroma formation by immobilized yeast cells in fermentation processes. *Yeast* 32, 173–216. doi: 10.1002/yea.3042

Novo, M., Mangado, A., Quirós, M., Morales, P., Salvadó, Z., and Gonzalez, R. (2013). Genome-wide study of the adaptation of *Saccharomyces cerevisiae* to the early stages of wine fermentation. *PLoS ONE* 8:e74086. doi: 10.1371/journal.pone.0074086

Nyman, J., Lacintra, M. G., Westman, J. O., Berglin, M., Lundin, M., Lennartsson, P. R., et al. (2013). Pellet formation of zygomycetes and immobilization of yeast. *New Biotechnol.* 30, 516–522. doi: 10.1016/j.nbt.2013.05.007

Park, S. D., Magee, D. A., McGettigan, P. A., Teasdale, M. D., Edwards, C. J., Lohan, A. J., et al. (2015). Genome sequencing of the extinct Eurasian wild aurochs, Bosprimigenius, illuminates the phylogeography and evolution of cattle. *Genome Biol.* 16, 234. doi: 10.1186/s13059-015-0790-2

Peinado, R. A., Moreno, J. J., Villalba, J. M., González-Reyes, J. A., Ortega, J. M., and Mauricio, J. C. (2006). Yeast biocapsules: a new immobilization method and their applications. *Enzyme Microb. Technol.* 40, 79–84. doi: 10.1016/j.enzmictec.2005.10.040

Piper, P. W. (1999). Yeast superoxide dismutase mutants reveal a pro-oxidant action of weak organic acid food preservatives. *Free Radic. Biol. Med.* 27, 1219–1227. doi: 10.1016/S0891-5849(99)00147-1

Puig-Pujol, A., Bertran, E., García-Martínez, T., Capdevila, F., Mínguez, S., and Mauricio, J. C. (2013). Application of a new organic yeast immobilization method for sparkling wine production. *Am. J. Enol. Vitic.* 64, 386–394. doi: 10.5344/ajev.2013.13031

Reinders, J., Zahedi, R. P., Pfanner, N., Meisinger, C., and Sickmann, A. (2006). Toward the complete yeast mitochondrial proteome: multidimensional separation techniques for mitochondrial proteomics. *J. Proteome Res.* 7, 1543–1554. doi: 10.1021/pr050477f

Reynolds, T. B., and Fink, G. R. (2001). Bakers' yeast, a model for fungal biofilm formation. *Science* 291, 878–881. doi: 10.1126/science.291.5505.878

Ristow, H., Seyfarth, A., and Lochman, E. R. (1995). Chromosomal damages by ethanol and acetaldehyde in *Saccharomyces cerevisiae* as studied by pulsed field gel electrophoreses. *Mutat. Res.* 326, 165–170. doi: 10.1016/0027-5107(94)00165-2

Rossignol, T., Kobi, D., Jacquet-Gutfreund, L., and Blondin, B. (2009). The proteome of a wine yeast strain during fermentation, correlation with the transcriptome. *J. Appl. Microbiol.* 107, 47–55. doi: 10.1111/j.1365-2672.2009.04156.x

Rydengard, V., Shannon, O., Lundqvist, K., Kacprzyk, L., Chalupka, A., Olsson, A. K., et al. (2008). Histidine-rich glycoprotein protects from systemic *Candida* infection. *PLoS Pathog.* 4:e1000116. doi: 10.1371/journal.ppat.1000116

Salvado, Z., Chiva, R., Rodríguez-Vargas, S., Rández-Gil, F., Mas, A., and Guillamón, J. M. (2008). Proteomic evolution of a wine yeast during the first hours of fermentation. *FEMS Yeast Res.* 8, 1137–1146. doi: 10.1111/j.1567-1364.2008.00389.x

Sanchez, Z., Tani, A., and Kimbara, K. (2013). Extensive reduction of cell viability and enhanced matrix production in *Pseudomonas aeruginosa* PAO1 flow biofilms treated with a D-amino Acid mixture. *Appl. Environ. Microbiol.* 79, 1396–1399. doi: 10.1128/AEM.02911-12

Sarkar, S., and Pires, M. M. (2015). D-amino acids do not inhibit biofilm formation in *Staphylococcus aureus*. *PLoS ONE* 10:e0117613. doi: 10.1371/journal.pone.0117613

Shimba, N., Serber, Z., Ledwidge, R., Miller, S. M., Craik, C. S., and Dötsch, V. (2003). Quantitative identification of the protonation state of histidines in vitro and in vivo. *Biochemistry* 42, 9227–9234. doi: 10.1021/bi0344679

Swinnen, E., Wanke, V., Roosen, J., Smets, B., Dubouloz, F., Pedruzzi, I., et al. (2006). Rim15 and the crossroads of nutrient signalling pathways in *Saccharomyces cerevisiae*. *Cell Div.* 1, 3. doi: 10.1186/1747-1028-1-3

Szafranski-Schneider, E., Swidergall, M., Cottier, F., Tielker, D., Román, E., Pla, J., et al. (2012). Msb2 shedding protects *Candida albicans* against antimicrobial peptides. *PLoS Pathog.* 8:e1002501. doi: 10.1371/journal.ppat.1002501

Tong, Z., Zhang, L., Ling, J., Jian, Y., Huang, L., and Deng, D. (2014). An in vitro study on the effect of free amino acids alone or in combination with nisin on biofilms as well as on planktonic bacteria of *Streptococcus mutans*. *PLoS ONE* 9:e99513. doi: 10.1371/journal.pone.0099513

van Loon, A. P., Pesold-Hurt, B., and Schatz, G. (1986). A yeast mutant lacking mitochondrial manganese-superoxide dismutase is hypersensitive to oxygen. *Proc. Natl. Acad. Sci. U.S.A.* 83, 3820–3824. doi: 10.1073/pnas.83.11.3820

Vandenbosch, D., De Canck, E., Dhondt, I., Rigole, P., Nelis, H. J., and Coenye, T. (2013). Genomewide screening for genes involved in biofilm formation and miconazole susceptibility in *Saccharomyces cerevisiae*. *FEMS Yeast Res.* 13, 720–730. doi: 10.1111/1567-1364.12071

Vinod, P. K., Neelanjan Sengupta, P. J., Bhat, K. V., and Venkatesh, K. V. (2008). Integration of global signaling pathways, cAMP- PKA, MAPK and TOR in the regulation of FLO11. *PLoS ONE* 3:e1663. doi: 10.1371/journal.pone.0001663

Zara, G., Angelozzi, D., Belviso, S., Bardi, L., Goffrini, P., Lodi, T., et al. (2009). Oxygen is required to restore flor strain viability and lipid biosynthesis under fermentative conditions. *FEMS Yeast Res.* 9, 217–225. doi: 10.1111/j.1567-1364.2008.00472.x

Zara, G., Budroni, M., Mannazzu, I., and Zara, S. (2011). Air-liquid biofilm formation is dependent on ammonium depletion in a *Saccharomyces cerevisiae* flor strain. *Yeast* 28, 809–814. doi: 10.1002/yea.1907

Zara, G., Goffrini, P., Lodi, T., Zara, S., Mannazzu, I., and Budroni, M. (2012). FLO11 expression and lipid biosynthesis are required for air-liquid biofilm formation in a *Saccharomyces cerevisiae* flor strain. *FEMS Yeast Res.* 12, 864–866. doi: 10.1111/j.1567-1364.2012.00831.x

Zara, S., Bakalinsky, A. T., Zara, G., Pirino, G., Demontis, M. A., and Budroni, M. (2005). FLO11-based model for air-liquid interfacial biofilm formation by *Saccharomyces cerevisiae*. *Appl. Environ. Microbiol.* 71, 2934–2939. doi: 10.1128/AEM.71.6.2934-2939.2005

Zara, S., Gross, M. K., Zara, G., Budroni, M., and Bakalinsky, A. T. (2010). Ethanol-independent biofilm formation by a flor wine yeast strain of *Saccharomyces cerevisiae*. *Appl. Environ. Microbiol.* 76, 4089–4091. doi: 10.1128/AEM.00111-10

Zea, L., Moreno, J., Ortega, J. M., Mauricio, J. C., and Medina, M. (1995). Comparative study of the gamma-butyrolactone and pantolactone contents in cells and musts during vinification by three *Saccharomyces cerevisiae* races. *Biotechnol. Lett.* 17, 1351–1356. doi: 10.1007/BF00189225

Zea, L., Moyano, L., Moreno, J., Cortes, B., and Medina, M. (2001). Discrimination of the aroma fraction of Sherry wines obtained by oxidative and biological ageing. *Food Chem.* 75, 79–84. doi: 10.1016/S0308-8146(01)00190-X

Zea, L., Serratosa, M. P., Mérida, J., and Moyano, L. (2015). Acetaldehyde as key compound for the authenticity of sherry wines: a study covering 5 decades. *Compr. Rev. Food Sci. Food Saf.* 14, 681–693. doi: 10.1111/1541-4337.12159

Zhao, T., and Liu, Y. (2010). N-acetylcysteine inhibits biofilms produced by *Pseudomonas aeruginosa*. *BMC Microbiol.* 10:140. doi: 10.1186/1471-2180-10-140

Zuzuarregui, A., Monteoliva, L., Gil, C., and del Olmo, M. (2006). Transcriptomic and proteomic approach for understanding the molecular basis of adaptation of *Saccharomyces cerevisiae*to wine fermentation. *Appl. Environ. Microbiol.* 72, 836–847. doi: 10.1128/AEM.72.1.836-847.2006

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Yeast Interactions in Inoculated Wine Fermentation

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The use of selected starter culture is widely diffused in winemaking. In pure fermentation, the ability of inoculated *Saccharomyces cerevisiae* to suppress the wild microflora is one of the most important feature determining the starter ability to dominate the process. Since the wine is the result of the interaction of several yeast species and strains, many studies are available on the effect of mixed cultures on the final wine quality. In mixed fermentation the interactions between the different yeasts composing the starter culture can led the stability of the final product and the analytical and aromatic profile. In the present review, we will discuss the recent developments regarding yeast interactions in pure and in mixed fermentation, focusing on the influence of interactions on growth and dominance in the process.

Keywords: *Saccharomyces cerevisiae*, non-*Saccharomyces*, yeast–yeast interactions, starter dominance, inoculated wine fermentation

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INTRODUCTION

During the winemaking process, various microorganisms coexist and interact influencing the dominance, the persistence of fermenting yeasts and the analytical profiles of wine. Although the predominance of *Saccharomyces cerevisiae* on other genera is widely reported (Bisson, 1999; Bauer and Pretorius, 2000), few studies on the competition between species of the same genera (Arroyo-López et al., 2011) and between strains of the same species (Barrajón et al., 2009; Capece et al., 2013; Perrone et al., 2013) are present in literature. On the other hand, as consequence of the re-evaluation of the role of non-*Saccharomyces* yeasts, there is an increasing interest on the use of different species in mixed inoculated fermentation where the yeast interactions play a fundamental role. In this review, we will refer on the recent development regarding the dominance and yeast interactions in inoculated fermentations.

S. cerevisiae/*S. cerevisiae* INTERACTIONS

The use of *S. cerevisiae* as starter culture is the most widespread practice in winemaking. However, the inoculation of musts using selected *Saccharomyces* strains does not ensure their dominance at the end of fermentation (Capece et al., 2010). In fact, although possessing high competition, commercial strains do not completely inhibit wild strains until several days after the process has started. The starter culture should compete with not only non-*Saccharomyces* yeasts, but also with indigenous *S. cerevisiae* strains, which theoretically adapt better to must conditions (Barrajón et al., 2011; Capece et al., 2011). The knowledge of the mechanism(s) responsible for interaction among *Saccharomyces* strains could be of particular importance in understanding the

observed persistence of these indigenous *S. cerevisiae* strains and the metabolic influence among *S. cerevisiae* strains composing mixed starter cultures. It has been hypothesized that *S. cerevisiae* strains can metabolically interact each other, by modifying fermentation products when grown in mixed culture. A compound, produced by a strain, could be taken up and used by other yeasts present. In this way, yeast interaction and sharing of metabolites could occur. Cheraiti et al. (2005) have demonstrated that redox interactions can occur between yeasts in co-culture, in particular acetaldehyde produced by one yeast was metabolized by the other. This observation provides an explanation as to why modulation of wine flavor in mixed culture cannot be replicated by blending wines together, as the modification arises from complex interactions, largely unknown until now, between strains included in mixed starters (Howell et al., 2006; King et al., 2008; Capece et al., 2013).

The competition degree of each strain is influenced by a number of abiotic factors (pH, temperature, ethanol, osmotic pressure, nitrogen, molecular sulfur dioxide, etc.) and biotic factors (microorganisms, killer factors, grape variety, etc.), which determine the capacity of one strain to out-compete another (**Figure 1**).

Abiotic Factors

Some winemaking practices, such as the amount of inoculum, rehydration conditions, or certain physical–chemical characteristics of the must, such as temperature, nutrients (nitrogen, vitamins; Rodriguez-Porrata et al., 2008), led to non-optimal physiological conditions of the starter for competing with the wild biota, causing its growth inhibition by other strains better adapted to a specific oenological environment. Barrajón et al. (2010) evaluated the influence of oenological practices, strain vitality, stress tolerance, and nitrogen requirements on the starters implantation during industrial fermentations. The implantation of commercial strains was generally better in white musts than in red ones, probably in consequence of maceration practice, that might determine an increase of indigenous yeasts in must competing with the starter at the beginning of the fermentation. Different results were obtained: some commercial yeasts competed with one or several dominant wild strains, in other musts the inoculated yeast was completely displaced by only one wild strain at mid-fermentation, for some fermentations a wide variety of wild yeasts was found, none of them dominating the process. Vigentini et al. (2014) have investigated the evolution of the yeast populations during controlled fermentation of Chardonnay musts in two Italian wineries that used the same commercial strain. In the first winery, where the oenologist carefully managed only one starter culture and did not make any spontaneous fermentation, the commercial strain always mastered the process; conversely, in the second winery, where the oenologist performed also spontaneous fermentation, the starter culture did not even take over the dominance and a continuous succession of indigenous strains overcame without one prevailed on the others. Recently, some authors (Duarte et al., 2013) have hypothesized that some oenological additives, such as tannins and fermentation activators, can affect the starter implantation. García-Ríos et al. (2014) carried out a preliminary approach in order to study the fitness advantages of four commercial wine yeast strains (PDM, ARM, RVA, and TTA) against some important oenological parameters, such as nitrogen concentration of grape must, fermentation temperature profile, and ethanol tolerance, which can exert strong stresses on the inoculated strain and determine its competitive advantage. A mathematical approach was used to model the hypothetical time needed for the control strain (PDM) to out-compete the other three strains in a theoretical mixed population. The theoretical values obtained were subsequently verified by competitive mixed fermentations in both synthetic and natural musts, which showed a good fit between the theoretical and experimental data. Specifically, the increase in nitrogen concentration and temperature values improved the fitness advantage of the PDM strain, whereas the presence of ethanol significantly reduced its competitiveness. However, the RVA strain proved to be the most competitive yeast for the three oenological parameters assayed.

Very little is known as fermentation temperature affects the dynamics of the *Saccharomyces* strain population. Torija et al. (2003) studied the influence of fermentation temperature (from 15 to 35°C) on a mixed population of *S. cerevisiae* strains, by evaluating the competition during alcoholic fermentation, at different temperatures, as a tool for testing the natural endurance of indigenous strains. They demonstrated that the temperature of fermentation could clearly affect the development of the different *Saccharomyces* strains: some strains were predominant at low temperatures, whereas others predominated at high ones. The usual growth curve was observed at 25 and 30°C, whereas at 35°C a high yeast mortality was found, which may have induced stuck fermentations with high residual sugar. In fact, these results agree with previous studies, reporting a decrease of yeast viability as the temperature increases (Casey et al., 1984), probably as a consequence of a greater accumulation of intracellular ethanol at higher temperatures, that determine cell toxicity and alter the structure of the membrane, decreasing its functionality (Lucero et al., 2000). On the contrary, at low temperatures there was no decline phase, but the stationary phase lasted until the end of fermentation. The percentage of the different *Saccharomyces* strains changed considerably during fermentation, probably in consequence of their sensitivity to ethanol toxicity. However, it is reported that the ethanol tolerance of some yeast species depends on the temperature (Gao and Fleet, 1988), and this could be the case also for some *Saccharomyces* strains. This may explain why the presence of some strains decreases at higher temperatures, but they are able to finish the fermentation at lower temperatures (Torija et al., 2003).

Biotic Factors

Ineffective starter implantation was also observed in some fermentation processes despite the use of correct winemaking practices. This means that other factors, i.e., biotic factors, like competition between microorganisms for space and nutrients, or production of toxic compounds (killer factors, medium-chain fatty acids, etc.), can affect starter dominance.

Among the biotic factors underlying the interactions between the different *Saccharomyces* strains during alcoholic

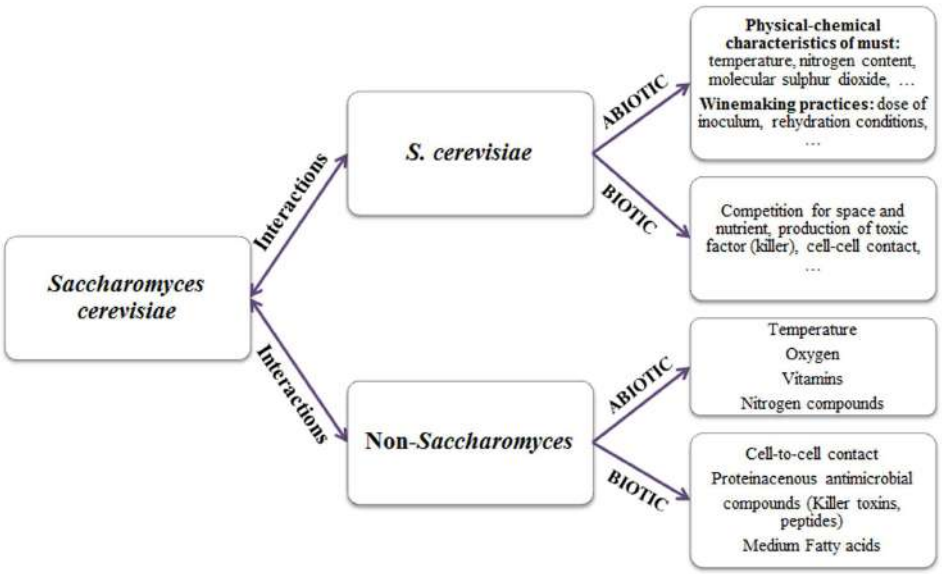

FIGURE 1 | Factors affecting yeast interactions in inoculated wine fermentation.

fermentation, the killer factor is the most studied. Both neutral and sensitive strains do not produce toxins, but the neutrals are resistant to their action. The use of selected *S. cerevisiae* strains with the killer factor may be effective in suppressing undesirable wild yeast strains or in avoiding stuck fermentations caused by indigenous killer yeasts. The magnitude of killer effect in wine fermentation depends on: the initial ratio of killer to sensitive strains, the presence of protein adsorbing substances, the environmental conditions and the growth phase of the sensitive cells, the presence of protective neutral yeasts, the susceptibility of sensitive strains to the killer toxins of different yeast strains, the inoculum size and nitrogen availability (Pérez et al., 2001). The killer phenotype seems to be linked to the execution of apoptosis, a form of active cell death, widely used by multicellular organisms, e.g., during development or as a mechanism to remove damaged and/or potentially cancerous cells. Apoptotic machinery has been also reported for *S. cerevisiae*. The finding of cell death with apoptosis-like features in yeast (Madeo et al., 1997) was unexpected, as a unicellular organism seems to have no advantages in committing suicide. As the exposure to killer toxins produced and secreted by concurring killer strains is another natural cell death situation for yeast, some authors (Reiter et al., 2005) investigated if killer toxins are able to induce the apoptotic process and if apoptosis is responsible for cell death in the presence of moderate or low toxin concentrations, closely reflecting the situation in the natural yeast habitat. The results showed that killer toxin action can trigger two modes of cell death. Under high toxin concentrations induction of apoptosis plays a minor role, whereas under moderate or low toxin doses, resembling the natural environment of toxin-secreting killer yeasts, induction of apoptosis might play an important role in efficient toxin-mediated cell killing. In this situation, it might be of general importance for a toxin-secreting yeast to induce apoptosis in competing yeast cells, in particular at toxin concentrations that are *per se* too low to kill via the toxin's primary mode of action.

Another biotic factor involved in the interaction among different yeasts is due to a cell-to-cell contact mechanism. Perrone et al. (2013) investigated *S. cerevisiae* intraspecies competition during wine fermentations, in which the cells of the different strains were mixed or kept separated. In co-fermentation, only the dominant strain was detected, whereas in bio-reactor, in which the cells from the two different strains were kept separate by a membrane and the strains did not sense each other, dominance did not take place. These authors postulated that growth arrest was due to cell-to-cell contact or microenvironment contact; in these conditions, cells compete for space when in high densities and in cell-to-cell contact.

NON-*Saccharomyces*/*Saccharomyces* INTERACTIONS

Controlled multistarter fermentations are characterized by complex interaction between non-*Saccharomyces* and *Saccharomyces* strains (Ciani et al., 2010; Ciani and Comitini, 2015). Although the physiological and biochemical basis for the overall antagonistic interactions among wine yeasts are still unclear, environmental factors, the production of bioactive yeast metabolites or yeast–yeast interaction could be involved (**Figure 1**). In this context, the management of mixed fermentations, such as cell concentration, inoculation modalities (pure or mixed fermentation), and timing of sequential fermentations, require more knowledge on environmental factors and metabolic activities influencing the yeast interactions.

TABLE 1 | Main killer toxins involved in wine making.

Killer yeast	Killer toxin	Sensitive strain	Applicative indications	Reference
Saccharomyces cerevisiae strain "Prise de mousse"	K2 type	*Saccharomyces cerevisiae*	Control of *S. cerevisiae* wild strains	Shimizu, 1993
Saccharomyces cerevisiae	K2 type	*Saccharomyces cerevisiae*	Enhance autolysis in Sparkling wine	Todd et al., 2000
Tetrapisispora phaffii	Kpkt	*Hanseniaspora/Kloeckera*	Control of "apiculate" yeast	Comitini and Ciani, 2010
Kluyveromyces wickerhamii	Kwkt	*Dekkera/Brettanomyces*	Anti-Brett activity	Comitini et al., 2004
Wickerhamomyces anomalus	Pikt	*Dekkera/Brettanomyces*	Anti-Brett activity	Comitini et al., 2004
Pichia membranifaciens	PMKT2	*Dekkera/Brettanomyces*	Anti-Brett activity	Santos et al., 2009
Torulaspora delbrueckii	Kbarr-1	*S. cerevisiae* killer strains	Broad anti-wine yeast activity	Ramírez et al., 2015
Torulaspora delbrueckii	TdKT	*Pichia* and *Brettanomyces/Dekkera*	Spoilage wine yeasts	Villalba et al., 2016

Management and Yeast Interactions

The management of mixed fermentation strongly influences the dominance and persistence of yeast species. Several investigations showed that in non-*Saccharomyces*/*S. cerevisiae* co-culture at ratio 1:1, the growth of *S. cerevisiae* was not affected by the co-inoculated yeast, that more or less quickly disappeared. However, at higher inoculation ratio (100:1), *Lachancea thermotolerans* and *Saccharomycodes ludwigii*, *Hanseniaspora uvarum,* and *H. guilliermondii* persisted for more time, while *Candida zemplinina* (synonym *Starmerella bacillaris*) showed a lower competitiveness, increasing its persistence only when the ratio was 10000:1 (Perez-Nevado et al., 2006; Comitini et al., 2011; Domizio et al., 2011). To enhance the competitiveness of non-*Saccharomyces* strains, the sequential fermentation is a useful inoculation modality. The timing of second inoculation, mimicked the spontaneous fermentation, allows to obtain a synergistic interaction between non-*Saccharomyces* and *S. cerevisiae* strains. Several works, investigating on sequential fermentation using various timing of second inoculation, highlighted the actual presence and contribution of several non-*Saccharomyces* species (Andorrà et al., 2012; Azzolini et al., 2012; Gobbi et al., 2013; Canonico et al., 2015).

Abiotic Factors

As generally recognized, the increasing concentration of ethanol during the fermentation process, is the main factor that determines the dominance of *S. cerevisiae* toward non-*Saccharomyces* yeasts (Pretorius, 2000). Indeed, *S. cerevisiae* strains possess a higher ethanol tolerance than non-*Saccharomyces* yeasts. On the other hand, the competition for nutrients, such as vitamins and nitrogen compounds, contributes to modulate the presence and dominance of yeasts species during wine fermentation (Liu et al., 2015). Oxygen availability affects growth and fermentation performance of wine yeasts having a selective action among the various yeast species (Ciani and Comitini, 2006; Brandam et al., 2013; Jolly et al., 2014; Taillander et al., 2014). Indeed, *S. cerevisiae* and non-*Saccharomyces* wine yeasts exhibit a different behavior in presence of a low oxygen content. In particular, in anaerobic conditions, *S. cerevisiae* is able to grow quickly (Hansen et al., 2001) while non-*Saccharomyces* yeasts belonging to *Hanseniaspora*, *Kloeckera,* and *Torulaspora* genera, grow poorly under the same conditions (Visser et al., 1990). The low competitiveness exhibited by *L. thermotolerans* and *Torulaspora delbrueckii* could be in part explained by their reduced tolerance to scarce oxygen availability (Nissen et al., 2004).

Another important nutrient factor, that could influence the behavior and the dominance of yeast strains in mixed fermentation, is the availability of nitrogen source and vitamins. In general, when non-*Saccharomyces* species grow early during wine fermentation (e.g., spontaneous fermentation), these species can consume amino acids and vitamins, thus limiting *S. cerevisiae* growth (Bisson, 1999; Fleet, 2003). A competition for nutrients was reported by Medina et al. (2012), while Taillander et al. (2014) reported a sluggish fermentation in 48h sequential fermentation of *T. delbrueckii*/*S. cerevisiae* due to nitrogen exhaustion by *T. delbrueckii*. In a recent work, Kemsawasd et al. (2015) indicated that different nitrogen sources had different impacts on the growth and fermentation behavior of *S. cerevisiae* and the other main non-*Saccharomyces* fermenting wine yeasts. On the other hand, non-*Saccharomyces* species and particularly yeast strains belonging to *Hanseniaspora* and *Metschnikowia* genera can contribute to enrichment of the medium as a nitrogen source by their proteolytic activity (Dizzy and Bisson, 2000).

Also the competition for other nutrients may influence the interactions between *S. cerevisiae* and non-*Saccharomyces*. In this context, several positive and negative interactions have been reported regarding substrate limitation or depletion (Ivey et al., 2013; Oro et al., 2014). Among the environmental factors, temperature has an important role in yeast interactions and dominance of the fermentation process. The high temperature in synergy with increasing ethanol concentration affects membrane permeability and integrity. In this contest, some works indicated that ethanol does not provide a clear advantage to *S. cerevisiae* at low temperature (<15°C). Indeed, the persistence and/or the dominance of non-*Saccharomyces* over *S. cerevisiae* at low temperature has been recognized (Gao and Fleet, 1988; Ciani and Comitini, 2006). A study on the interaction between co-inoculated *S. cerevisiae* and *L. thermotolerans* fermentation, showed that the antagonistic effect between these two yeasts were temperature dependent (Gobbi et al., 2013). A recent study, on the evolution of ecological dominance of yeast species, confirmed that temperature of fermentation plays an important role on the ability of *S. cerevisiae* to dominate high-sugar environments (Williams et al., 2015).

Biotic Factors

The metabolic activities, that influence the controlled multistarter fermentations, could be grouped in antimicrobial molecules and cell-to-cell contact mechanism. Albergaria et al. (2010), investigating on the nature of the toxic compounds produced by *S. cerevisiae* responsible of the early death of *H. guilliermondii* during mixed fermentations, found that the killing effect was due to proteinaceous compounds. In particular, the active proteinaceous compounds exhibited a very low molecular weight that ranged from 2 to 10 kDa and showed a wide antimicrobial spectrum against strains of *Kluyveromyces marxianus*, *L. thermotolerans,* and *T. delbrueckii*. Further investigations demonstrated that *S. cerevisiae* during alcoholic fermentation secretes antimicrobial peptides, corresponding to fragments of the glyceraldehyde 3-phosphate dehydrogenase enzyme, that are active against a wide spectrum of wine yeasts including *Dekkera bruxellensis* and the malolactic bacterium *Oenococcus oeni* (Branco et al., 2014, 2015). Among the antimicrobial compounds, killer toxins are certainly involved on the interactions in mixed fermentations. An example of yeast interaction during mixed fermentations non-*Saccharomyces*/*S. cerevisiae* yeasts due to the action of killer toxin was described by Comitini and Ciani (2010). Another application of non-*Saccharomyces* killer yeasts in sequential fermentation with *S. cerevisiae* starter strain was the use of *Wickerhamomyces anomalus* and *Kluyveromyces wickerhamii* to control *Dekkera*/*Brettanomyces* spoilage yeasts (Comitini et al., 2004). The main killer toxins involved in wine fermentation are showed in **Table 1**.

Together with proteinaceous antimicrobial compounds, medium fatty acids, produced during alcoholic fermentation above a given threshold, could exhibit inhibitory actions toward *S. cerevisiae* and/or other species (Viegas et al., 1989).

Cell-to-cell contact is the other mechanism that could influence the interaction among yeast strains. Nissen et al. (2003) demonstrated this phenomenon carrying out single- and mixed-culture fermentations using both *L. thermotolerans* and *T. delbrueckii* with *S. cerevisiae*.

Similarly, Renault et al. (2013), investigating on the interaction between *S. cerevisiae* and *T. delbrueckii* in a new double-compartment fermenter, found that physical contact between *S. cerevisiae* and *T. delbrueckii* induced a rapid death of the non-*Saccharomyces* yeast. In contrast, when physically separated from *S. cerevisiae*, *T. delbrueckii* maintained its viability and metabolic activity determining a marked impact on *S. cerevisiae* growth and viability. More recently, Kemsawasd et al. (2015) clarified the phenomenon of the early death of *L. thermotolerans* during anaerobic, mixed-culture fermentations with *S. cerevisiae*. They found that this phenomenon was caused by a combination of cell-to-cell contact and antimicrobial peptides.

CONCLUSION AND FUTURE PERSPECTIVES

Investigations on yeast interactions in pure and mixed inoculated fermentation in winemaking are in fast development. Further knowledge on yeast interactions needs to manage the inoculated fermentations, to assure the dominance of inoculated strain in pure fermentation and the contribution of each inoculated yeast in mixed fermentation. In addition, these studies on yeasts interactions will contribute to control undesirable or spoilage microflora avoiding or reducing the use of synthetic antimicrobial compounds, such as sulfur dioxide. As reported above, several features influence the yeast interactions in wine fermentation. To obtain a more complete picture on yeast interaction in inoculated fermentation (pure and mixed with non-*Saccharomyces*) a multifactorial approach using "omics" methodologies should be planned.

AUTHOR CONTRIBUTIONS

MC, AC, FC, and PR conceived the idea and outline of the review, LC and GS contributed to the graphical elaboration of data. All authors contributed to writing specific sections and approved the final version of the manuscript.

REFERENCES

Albergaria, H., Francisco, D., Gori, K., Arneborg, N., and Girio, F. (2010). *Saccharomyces cerevisiae* CCMI 885 secretes peptides that inhibit the growth of some non-*Saccharomyces* wine-related strains. *Appl. Microbiol. Biotechnol.* 86, 965–972. doi: 10.1007/s00253-009-2409-6

Andorrà, I., Berradre, M., Mas, A., Esteve-Zarzoso, B., and Guillamón, J. M. (2012). Effect of mixed culture fermentations on yeast populations and aroma profile. *LWT Food Sci. Technol.* 49, 8–13. doi: 10.1016/j.lwt.2012.04.008

Arroyo-López, F. N., Peréz-Través, L., Querol, A., and Barrio, E. (2011). Exclusion of *Saccharomyces* kudriavzevii from a wine model system mediated by *Saccharomyces cerevisiae*. *Yeast* 28, 423–435. doi: 10.1002/yea.1848

Azzolini, M., Fedrizzi, B., Tosi, E., Finato, F., Vagnoli, P., Scrinzi, C., et al. (2012). Effects of *Torulaspora delbrueckii* and *Saccharomyces cerevisiae* mixed cultures on fermentation and aroma of Amarone. *Eur. Food Res. Technol.* 235, 303–313. doi: 10.1007/s00217-012-1762-3

Barrajón, N., Arévalo-Villena, M., Rodríguez-Aragón, L. J., and Briones, A. (2009). Ecological study of wine yeast in inoculated vats from La Mancha region. *Food Control* 20, 778–783. doi: 10.1016/j.foodcont.2008.10.002

Barrajón, N., Arévalo-Villena, M., Úbeda, J., and Briones, A. (2010). "Competition between spontaneous and commercial yeasts in winemaking: study of possible factors involved," in *Communicating Current Research and Education Topics and Trends in Applied Microbiology and Microbiology Book Series* Vol. 2, ed. A. Mendez-Vilas (Badajoz: Formatex Research Center), 1035–1041.

Barrajón, N., Arévalo-Villena, M., Úbeda, J., and Briones, A. (2011). Enological properties in wild and commercial *Saccharomyces cerevisiae* yeasts: relationship with competition during alcoholic fermentation. *World J. Microbiol. Biotechnol.* 27, 2703–2710. doi: 10.1007/s11274-011-0744-0

Bauer, F. F., and Pretorius, I. S. (2000). Yeast stress response and fermentation efficiency: how to survive the making of wine. *South Afr. J. Enol. Vitic.* 21, 27–51.

Bisson, L. F. (1999). Stuck and sluggish fermentations. *Am J. Enol Vitic.* 50, 107–119.

Branco, P., Francisco, D., Chambon, C., Hébraud, M., Arneborg, N., and Almeida, M. G. (2014). Identification of novel GAPDH-derived antimicrobial

peptides secreted by *Saccharomyces cerevisiae* and involved in wine microbial interactions. *Appl. Microbiol. Biotechnol.* 98, 843–853. doi: 10.1007/s00253-013-5411-y

Branco, P., Viana, T., Albergaria, H., and Arneborg, N. (2015). Antimicrobial peptides (AMPs) produced by *Saccharomyces cerevisiae* induce alterations in the intracellular pH, membrane permeability and culturability of *Hanseniaspora guilliermondii* cells. *Int. J. Food Microbiol.* 16, 205–212. doi: 10.1016/j.ijfoodmicro.2015.04.015

Brandam, C., Lai, Q. P., Julien-Ortiz, A., and Taillandier, P. (2013). Influence oxygen of alcoholic fermentation by a wine strain of *Torulaspora delbrueckii*: kinetics and carbon mass balance. *Biosci. Biotechnol. Biochem.* 77, 1848–1853. doi: 10.1271/bbb.130228

Canonico, L., Comitini, F., and Ciani, M. (2015). Influence of vintage and selected starter on *Torulaspora delbrueckii*/*Saccharomyces cerevisiae* sequential fermentation. *Eur. Food Res. Technol.* 241, 827–833. doi: 10.1007/s00217-015-2507-x

Capece, A., Romaniello, R., Poeta, C., Siesto, G., Massari, C., Pietrafesa, R., et al. (2011). Control of inoculated fermentations in wine cellars by mitochondrial DNA analysis of starter yeast. *Ann. Microbiol.* 61, 49–56. doi: 10.1007/s13213-010-0087-3

Capece, A., Romaniello, R., Siesto, G., Pietrafesa, R., Massari, C., Poeta, C., et al. (2010). Selection of indigenous *Saccharomyces cerevisiae* strains for Nero d'Avola wine and evaluation of selected starter implantation in pilot fermentation. *Int. J. Food Microbiol.* 144, 187–192. doi: 10.1016/j.ijfoodmicro.2010.09.009

Capece, A., Siesto, G., Romaniello, R., Lagreca, V. M., Pietrafesa, R., Calabretti, A., et al. (2013). Assessment of competition in wine fermentation among wild *Saccharomyces cerevisiae* strains isolated from Sangiovese grapes in Tuscany region. *LWT- Food Sci. Technol.* 54, 485–492. doi: 10.1016/j.lwt.2013.07.001

Casey, G. P., Magnus, C. A., and Ingledew, W. M. (1984). High gravity brewing: effects of nutrition on yeast composition, fermentative ability and alcohol production. *Appl. Environ. Microbiol.* 48, 639–646.

Cheraiti, N., Guezenec, S., and Salmon, J.-M. (2005). Redox interactions between *Saccharomyces cerevisiae* and *Saccharomyces uvarum* in mixed culture under enological conditions. *Appl. Environ. Microbiol.* 71, 255–260. doi: 10.1128/AEM.71.1.255-260.2005

Ciani, M., and Comitini, F. (2006). Influence of temperature and oxygen concentration on the fermentation behavior of *Candida stellata* in mixed fermentation with *Saccharomyces cerevisiae*. *World J. Microbiol. Biotechnol.* 22, 619–623. doi: 10.1007/s11274-005-9080-6

Ciani, M., and Comitini, F. (2015). Yeast interactions in multi-starter wine fermentation. *Curr. Opin. Food Sci.* 1, 1–6. doi: 10.1016/j.cofs.2014.07.001

Ciani, M., Comitini, F., Mannazzu, I., and Domizio, P. (2010). Controlled mixed culture fermentation: a new perspective on the use of non-*Saccharomyces* yeasts in winemaking. *FEMS Yeast Res.* 10, 123–133. doi: 10.1111/j.1567-1364.2009.00579.x

Comitini, F., and Ciani, M. (2010). The zymocidial activity of *Tetrapisispora phaffii* in the control of *Hanseniaspora uvarum* during the early stages of winemaking. *Lett. Appl. Microbiol.* 50, 50–56. doi: 10.1111/j.1472-765X.2009.02754.x

Comitini, F., De Ingeniis, J., Pepe, L., Mannazzu, I., and Ciani, M. (2004). *Pichia anomala* and *Kluyveromyces wickerhamii* killer toxins as new tools against *Dekkera*/*Brettanomyces* spoilage yeasts. *FEMS Microbiol. Lett.* 238, 235–240. doi: 10.1111/j.1574-6968.2004.tb09761.x

Comitini, F., Gobbi, M., Domizio, P., Romani, C., Lencioni, L., Mannazzu, I., et al. (2011). Selected non-*Saccharomyces* wine yeasts in controlled multi starter fermentations with *Saccharomyces cerevisiae*. *Food Microbiol.* 28, 873–882. doi: 10.1016/j.fm.2010.12.001

Dizzy, M., and Bisson, L. F. (2000). Proteolytic activity of yeast strains during grape juice fermentation. *Am. J. Enol. Vitic.* 51, 155–167. doi: 10.1007/s10482-015-0578-0

Domizio, P., Romani, C., Lencioni, L., Comitini, F., Gobbi, M., Mannazzu, I., et al. (2011). Outlining a future for non-*Saccharomyces* yeasts: selection of putative spoilage wine strains to be used in association with *Saccharomyces cerevisiae* for grape juice fermentation. *Int. J. Food Microbiol.* 147, 170–180. doi: 10.1016/j.ijfoodmicro.2011.03.020

Duarte, F. L., Claudia Alves, A., Alemão, M. F., and Baleiras-Couto, M. M. (2013). Influence of red wine fermentation oenological additives on inoculated strain implantation. *World J. Microbiol. Biotechnol.* 29, 1139–1144. doi: 10.1007/s11274-013-1272-x

Fleet, G. H. (2003). Yeast interactions and wine flavor. *Int. J. Food Microbiol.* 86, 11–22. doi: 10.1016/S0168-1605(03)00245-9

Gao, C., and Fleet, G. H. (1988). The effects of temperature and pH on the ethanol tolerance of the wine yeasts, *Saccharomyces cerevisiae*, *Candida stellata* and *Kloeckera apiculata*. *J. Appl. Bacteriol.* 65, 405–410. doi: 10.1111/j.1365-2672.1988.tb01909.x

García-Ríos, E., Gutiérrez, A., Salvadó, Z., Arroyo-López, F. N., and Guillamon, M. (2014). The fitness advantage of commercial wine yeasts in relation to the nitrogen concentration, temperature, and ethanol content under microvinification conditions. *Appl. Environ. Microbiol.* 80, 704–713. doi: 10.1128/AEM.03405-13

Gobbi, M., Comitini, F., Domizio, P., Romani, C., Lencioni, L., Mannazzu, I., et al. (2013). *Lachancea thermotolerans* and *Saccharomyces cerevisiae* in simultaneous and sequential co-fermentation: a strategy to enhance acidity and improve the overall quality of wine. *Food Microbiol.* 33, 271–281. doi: 10.1016/j.fm.2012.10.004

Hansen, E. H., Nissen, P., Sommer, P., Nielsen, J. C., and Arneborg, N. (2001). The effect of oxygen on the survival of non-*Saccharomyces* yeasts during mixed culture fermentations of grape juice with *Saccharomyces cerevisiae*. *J. Appl. Microbiol.* 91, 541–547. doi: 10.1046/j.1365-2672.2001.01426.x

Howell, K. S., Cozzolino, D., Bartowsky, E. J., Fleet, G. H., and Henschke, P. A. (2006). Metabolic profiling as a tool for revealing *Saccharomyces* interactions during wine fermentation. *FEMS Yeast Res.* 6, 91–101. doi: 10.1111/j.1567-1364.2005.00010.x

Ivey, M., Massel, M., and Phister, T. G. (2013). Microbial interactions in food fermentations. *Annu. Rev. Food Sci. Technol.* 4, 141–162. doi: 10.1146/annurev-food-022811-101219

Jolly, N. P., Varela, C., and Pretorius, I. S. (2014). Not your ordinary yeast: non-*Saccharomyces* yeast in wine production uncovered. *FEMS Yeast Res.* 14, 215–237. doi: 10.1111/1567-1364.12111

Kemsawasd, V., Viana, T., Ardo, Y., and Arneborg, N. (2015). Influence of nitrogen sources on growth and fermentation performance of different wine yeast species during alcoholic fermentation. *Appl. Microbiol. Biotechnol.* 99, 10191–10207. doi: 10.1007/s00253-015-6835-3

King, E. S., Swiegers, J. H., Travis, B., Francis, I. L., Bastian, S. E. P., and Pretorius, I. S. (2008). Coinoculated fermentations using *Saccharomyces* yeasts affect the volatile composition and sensory properties of *Vitis vinifera* L. cv. Sauvignon Blanc wines. *J. Agric. Food Chem.* 56, 10829–10837. doi: 10.1021/jf801695h

Liu, Y., Rousseaux, S., Tourdot-Marèchal, R., Sadoudi, M., Gougeon, R., Schmitt-Kopplin, P., et al. (2015). Wine microbiome, a dynamic world of microbial interactions. *Crit. Rev. Food Sci. Nutr.* doi: 10.1080/10408398.2014.983591 [Epub ahead of print].

Lucero, P., Peñalver, E., Moreno, E., and Lagunas, R. (2000). Internal trehalose protects endocytosis from inhibition by ethanol in *Saccharomyces cerevisiae*. *Appl. Environ. Microbiol.* 66, 4456–4461. doi: 10.1128/AEM.66.10.4456-4461.2000

Madeo, F., Frohlich, E., and Frohlich, K. U. (1997). A yeast mutant showing diagnostic markers of early and late apoptosis. *J. Cell Biol.* 139, 729–734. doi: 10.1083/jcb.139.3.729

Medina, K., Boido, E., Dellacassa, E., and Carrau, F. (2012). Growth of non-*Saccharomyces* yeasts effects nutrient availability for *Saccharomyces cerevisiae* during wine fermentation. *Int. J. Food Microbiol.* 157, 245–250. doi: 10.1016/j.ijfoodmicro.2012.05.012

Nissen, P., Nielsen, D., and Arneborg, N. (2003). Viable *Saccharomyces cerevisiae* cells at high concentrations cause early growth arrest of non- *Saccharomyces* yeasts in mixed cultures by a cell-cell contact-mediated mechanism. *Yeast* 20, 331–341. doi: 10.1002/yea.965

Nissen, P., Nielsen, D., and Arneborg, N. (2004). The relative glucose uptake abilities of non-*Saccharomyces* yeasts play a role in their coexistence with *Saccharomyces cerevisiae* in mixed cultures. *Appl. Microbiol. Biotechnol.* 64, 543–550. doi: 10.1007/s00253-003-1487-0

Oro, L., Ciani, M., and Comitini, F. (2014). Antimicrobial activity of *Metschnikowia pulcherrima* on wine yeasts. *J. Appl. Microbiol.* 116, 1209–1217. doi: 10.1111/jam.12446

Pérez, F., Ramírez, M., and Regodón, J. A. (2001). Influence of killer strains of *Saccharomyces cerevisiae* on wine fermentation. *Antonie Van Leeuwenhoek* 79, 393–399. doi: 10.1023/A:1012034608908

Perez-Nevado, F., Albergaria, H., Hogg, T., and Girio, F. (2006). Cellular death of two non-*Saccharomyces* wine-related yeasts during mixed fermentation with *Saccharomyces cerevisiae*. *Int. J. Food Microbiol.* 108, 336–345. doi: 10.1016/j.ijfoodmicro.2005.12.012

Perrone, B., Giacosa, S., Rolle, L., Cocolin, L., and Rantsiou, K. (2013). Investigation of the dominance behavior of *Saccharomyces cerevisiae* strains during wine fermentation. *Int. J. Food Microbiol.* 165, 156–162. doi: 10.1016/j.ijfoodmicro.2013.04.023

Pretorius, I. S. (2000). Tailoring wine yeast for the new millennium: novel approaches to the ancient art of winemaking. *Yeast* 16, 675–729. doi: 10.1002/1097-0061(20000615)16:8<675::AID-YEA585>3.0.CO;2-B

Ramírez, M., Velázquez, R., Maqueda, M., López-Piñeiro, A., and Ribas, J. C. (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

Reiter, J., Herker, E., Madeo, F., and Schmitt, M. J. (2005). Viral killer toxins induce caspase-mediated apoptosis in yeast. *J. Cell. Biol.* 168, 353–358. doi: 10.1083/jcb.200408071

Renault, P. E., Albertin, W., and Bely, M. (2013). An innovative tool reveals interaction mechanisms among yeast populations under oenological conditions. *Appl. Microbiol. Biotechnol.* 97, 4105–4119. doi: 10.1007/s00253-012-4660-5

Rodriguez-Porrata, B., Novo, M., Guillamon, J., Rozes, N., Mas, A., and Otero, R. C. (2008). Vitality enhancement of the rehydrated active dry wine yeast. *Int. J. Food Microbiol.* 126, 116–122. doi: 10.1016/j.ijfoodmicro.2008.05.016

Santos, A., San Mauro, M., Bravo, E., and Marquina, D. (2009). PMKT2, a new killer toxin from, and its promising biotechnological properties for control of the spoilage yeast *Brettanomyces bruxellensis*. *Microbiology* 155, 624–634. doi: 10.1099/mic.0.023663-0

Shimizu, K. (1993). "Killer yeasts," in *Wine Microbiology and Biotechnology*, ed. G. H. Fleet (Chur: Harwood Academic), 243–264.

Taillander, P., Lai, Q. P., Julien-Ortiz, A., and Brandam, C. (2014). Interaction between *Torulaspora delbrueckii* and *Saccharomyces cerevisiae* in wine fermentation: influence of inoculation and nitrogen content. *World J. Microbiol. Biotechnol.* 30, 1959–1967. doi: 10.1007/s11274-014-1618-z

Todd, B. E., Fleet, G. H., and Henschke, P. A. (2000). Promotion of autolysis through the interaction of killer and sensitive yeasts: potential application in sparkling wine production. *Am. J. Enol. Vitic.* 51, 65–72. doi: 10.1023/A:1012027718701

Torija, M. J., Rozès, N., Poblet, M., Guillamón, J. M., and Mas, A. (2003). Effects of fermentation temperature on the strain population of *Saccharomyces cerevisiae*. *Int. J. Food Microbiol.* 80, 47–53. doi: 10.1016/S0168-1605(02)00144-7

Viegas, C. A., Rosa, M. F., Sá-Correia, I., and Novais, J. M. (1989). Inhibition of yeast growth by octanoic and decanoic acids produced during ethanolic fermentation. *Appl. Environ. Microbiol.* 55, 210–221.

Vigentini, I., Fabrizio, V., Faccincani, M., Picozzi, C., Comasio, A., and Foschino, R. (2014). Dynamics of *Saccharomyces cerevisiae* populations in controlled and spontaneous fermentations for Franciacorta D.O.C.G. base wine production. *Ann. Microbiol.* 64, 639–651. doi: 10.1007/s13213-013-0697-7

Villalba, M. L., Susana Sáez, J., del Monaco, S., Lopes, C. A., and Sangorrín, M. P. (2016). TdKT, a new killer toxin produced by *Torulaspora delbrueckii* effective against wine spoilage yeasts. *Int. J. Food Microbiol.* 217, 94–100. doi: 10.1016/j.ijfoodmicro.2015.10.006

Visser, W., Scheffers, W. A., Batenburg-Van Der Vegte, W. H., and Van Dijken, J. P. (1990). Oxygen requirements of yeasts. *Appl. Environ. Microbiol.* 56, 3785–3792.

Williams, K. M., Liu, P., and Fay, J. C. (2015). Evolution of ecological dominance of yeast species in high-sugar environments. *Evolution* 69, 2079–2095. doi: 10.1111/evo.12707

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Persistence of Two Non-*Saccharomyces* Yeasts (*Hanseniaspora* and *Starmerella*) in the Cellar

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Different genera and/or species of yeasts present on grape berries, in musts and wines are widely described. Nevertheless, the community of non-*Saccharomyces* yeasts present in the cellar is still given little attention. Thus it is not known if the cellar is a real ecological niche for these yeasts or if it is merely a transient habitat for populations brought in by grape berries during the winemaking period. This study focused on three species of non-*Saccharomyces* yeasts commonly encountered during vinification: *Starmerella bacillaris* (synonymy with *Candida zemplinina*), *Hanseniaspora guilliermondii* and *Hanseniaspora uvarum*. More than 1200 isolates were identified at the strain level by FT-IR spectroscopy (207 different FTIR strain pattern). Only a small proportion of non-*Saccharomyces* yeasts present in musts came directly from grape berries for the three species studied. Some strains were found in the must in two consecutive years and some of them were also found in the cellar environment before the arrival of the harvest of second vintage. This study demonstrates for the first time the persistence of non-*Saccharomyces* yeast strains from year to year in the cellar. Sulfur dioxide can affect yeast populations in the must and therefore their persistence in the cellar environment.

Keywords: non-*Saccharomyces* strains, persistence, cellar, *Hanseniaspora*, *Starmerella*

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INTRODUCTION

Fresh grape berries harbor a wide diversity of non-*Saccharomyces* yeasts (NS). The main genera or species isolated and identified have been (by decreasing order and in relative proportion of the genera/species detected): *Hanseniaspora uvarum*, *Aureobasidium pullulans*, *Candida*, *Issatchenkia*, *Metschnikowia,* and *Pichia* (Barata et al., 2012). The population density and diversity of these indigenous yeasts on grape berries are strongly linked to numerous factors such as geographic location, climatic condition, viticultural practices (vineyard management and fungicide treatment), vineyard age, grape variety, sanitary state and berry maturity (Hierro et al., 2006; Xufre et al., 2006; Nisiotou et al., 2007; Barata et al., 2008, 2012; Cadez et al., 2010; Cordero-Bueso et al., 2011a,b; Milanović et al., 2013). An even greater diversity of species has been detected in musts (Jolly et al., 2003) and non-*Saccharomyces* yeast levels can reach 10^6–10^7 CFU/ml (Fleet, 2003). The main genera usually found in the first stages of spontaneous fermentation are

Hanseniaspora, Candida, Metschnikowia, *Pichia* and, occasionally, *Brettanomyces*, *Issatchenkia, Kluyveromyces*, *Rhodotorula, Schizosaccharomyces*, *Torulaspora* and *Zygosaccharomyces* (Fleet et al., 1984; Heard and Fleet, 1986; Clemente-Jimenez et al., 2004; Zott et al., 2008; Tristezza et al., 2013; David et al., 2014; Pérez-Martín et al., 2014; Pinto et al., 2015; Wang et al., 2015).

It is now accepted that the yeasts involved in fermentation processes have two possible origins: grapes and the winery/cellar environment (Fleet and Heard, 1993; Mortimer and Polsinelli, 1999). However, the diversity of non-*Saccharomyces* yeast in the cellar has been given little attention. The few studies found in the literature report that the diversity, distribution and percentage of identified species vary depending on the cellar and also depending on the area of the cellar scanned (Sabate et al., 2002; Garijo et al., 2008; Ocón et al., 2010, 2013; Bokulich et al., 2013; Pérez-Martín et al., 2014). Most studies show a higher proportion of NS yeasts in the environment of the cellar compared to the population of *Saccharomyces*. But these percentages vary according to the cellar (Ocón et al., 2010), the different periods of the year (Bokulich et al., 2013; Ocón et al., 2013) and different parts of the cellar (Bokulich et al., 2013). Proportions of NS yeasts reported in the cellar air are variable (Ocón et al., 2013; Pérez-Martín et al., 2014) and high increases in the number and percentage of *Saccharomyces* were observed during the vinification period (Garijo et al., 2008). The main genera in the winery environment (equipment, soil, air) are *Aureobasidium*, *Bullera*, *Candida*, *Cryptococcus*, *Debaryomyces*, *Dekkera*, *Hanseniaspora*, *Kluyveromyces*, *Metschnikowia*, *Pichia*, *Rhodotorula*, *Sporidiobolus*, *Sporobolomyces*, *Torulaspora* and *Williopsis* (Sabate et al., 2002; Sangorrín et al., 2007; Ocón et al., 2010, 2013; Bokulich et al., 2013; Pérez-Martín et al., 2014).

However, the exact role of the winery environment on the microbiota involved in the fermentation, the transfer of yeast communities from the grape berry to the must and the persistence of these yeasts, are poorly understood. The same genus or the same species can be isolated on grape berries, in the must during alcoholic fermentation (AF) and in the winery environment. But at present, it is still difficult to prove the transfer of yeast strains between the vine, the wine and the cellar (soil, air equipment) and their potential persistence over time. To answer this question, it is first necessary to identify the NS yeasts isolated at the strain level and, secondly, to monitor the strains between the different compartments (vine, wine, cellar), as has already been done for *Saccharomyces cerevisiae* strains (Ciani et al., 2004; Le Jeune et al., 2006). Indeed, the existence of a cellar *Saccharomyces* flora has already been demonstrated. Sabate et al. (1998) isolated a large number of *S. cerevisiae* strains common to 2 years during AF. Moreover, the persistence of a commercial *S. cerevisiae* strain in the cellar and its participation in AF 2 years after its last use as a starter were highlighted by Santamaría et al. (2005). Thus some strains seem to persist in the cellar from 1 year to another and could reimplant in grape must during the next vintage. To our knowledge, no monitoring of non-*Saccharomyces* yeast strains has been conducted so far. This study had two objectives: (i) to determine the origin of non-*Saccharomyces* strains isolated in grape must: grape berries and/or cellar, and (ii) to demonstrate their persistence or not in the winery in two consecutive vintages. We selected two yeast genera often found in grape must and implicated in the fermentation process: *Starmerella* reclassified by Duarte et al. (2012) and *Hanseniaspora* for which discrimination at the strain level was possible by Fourier-Transform Infrared (FT-IR) spectroscopy.

MATERIALS AND METHODS

Grape Berry Sampling

Samples of grape berries were taken from a plot of Chardonnay planted in 1986 and located in Burgundy, France (46°18′32.2′′N, 4°44′17.9′′E, 258 m altitude). The sampling of grape berries or bunches of grapes were carried out six rows of the plot. 18 kg of ripe bunches of grapes were collected aseptically in sterile bags from 60 different vine plants distributed along the six rows (one cluster per plant plant) for the 2012 vintage. Ten berries from each vine plant of each row were collected aseptically for the 2013 vintages (1 kg). Grapes were pressed manually in sterile plastic bags and one sample of must was collected aseptically immediately afterward (noted Tberries). For the 2012 vintage, AF in aseptic conditions at 20°C (2 L erlens) was carried out to enable the development of fermentative genera present but in minority on the bunch. No commercial yeast starter was inoculated in the different musts. Samples corresponding to this enrichment step were noted Tenrich.

Grape Must Sampling

The harvest was collected manually and placed in 20 kg crates. The 2012 harvest provided 468 kg and the 2013 harvest 100 kg. The must obtained after pressing was left for one night at 10°C, and then distributed into six tanks for 2012 and four tanks for 2013. For each tank, a sample of 50 ml of must was then taken and noted T0 (sample grape must before starting of AF). Immediately after sampling, 30 mg/l of SO_2 was added in three of the six tanks for the 2012 vintage and in two of the four tanks for the 2013 vintage.

No commercial yeast starter was inoculated in the different musts. AF was monitored by enzymatic dosing of the ethanol produced (Bio-SenTec, France). Samples were taken during fermentation: 3 days after settling (T3), 6 days after settling (T6), 9 days after settling (T9) and at the end of fermentation, (Tf) (data not shown).

Winery Environment Sampling

For the 2013 vintage, samples were taken from the air, floor and the surface of the winery equipment before the arrival of the harvest. Samples of air (flow rate 100 l/min) were taken using a microbial air sampler, MAS-100 Eco (MBV, Stäfa, Switzerland) placed 1.50 m above the floor. For each sample, a dish with YPD medium (0.5% [w/v] yeast extract, 1% [w/v] peptone, 2% [w/v] glucose and 2% [w/v] agar supplemented with chloramphenicol at 200 ppm to inhibit the development of bacteria) was placed in the air sampler to isolate the yeasts. The volume of air analyzed for each agar gel was 500 l, with three repetitions per sample.

A total of 12 samples were taken from the floor and surface of the winery equipment using swabs. After having rubbed the different surfaces by streaking, each swab was placed in a tube containing 1 ml of water supplemented with NaCl (at 0.9% [w/v]) then vortexed for 5 min before analysis.

Yeast Isolation

Serial dilutions were performed from grape berries and must and 3 × 100 µl of each dilution was spread on the YPD medium described previously and incubated at 28°C. For the samples taken from the winery floor and the surfaces of the winery equipment, 3 × 100 µl of the NaCl solution in which the swab was placed were spread on the YPD medium and incubated at 28°C. For the air samples, the Petri dishes exposed were incubated at 28°C. For all the samples, according to the colonies present, 50 colonies per replicate were selected randomly, purified in YPD medium, then cultivated in liquid YPD medium and finally stored at −80°C in YPD medium supplemented with glycerol (20% [v/v] final concentration).

Yeast Identification by FT-IR

Identification of yeast isolates was performed by Fourier-Transform Infrared (FT-IR) spectroscopy using a Tensor^{TM} 27 spectrometer coupled with an HTS-XT unit (Bruker, Ettlingen, Germany), as described by Adt et al. (2010), Grangeteau et al. (2015, 2016).

Strain Typing

Typing of strains belonging to the genera *Hanseniaspora* and *Starmerella* was performed by a hierarchical cluster analysis of the spectra obtained by FT-IR. The dendrogram was calculated using the Average Linkage algorithm and correlation with normalization to reproducibility level. The algorithm is part of the OPUS software package and implemented under the "Cluster analysis" option. The second derivatives of the spectra were used. The frequency ranges were 3,032 cm^{-1} to 2,829 cm^{-1}, 1,351 cm^{-1} to 1,200 cm^{-1}, and 901 cm^{-1} to 699 cm^{-1}. Classification into sub-clusters was done by defining a spectral distance as a value for separation on the strain level. According to Kümmerle et al. (1998) and applied in previous works on strains of the genera *Starmerella* and *Hanseniaspora* (Grangeteau et al., 2015, 2016), all the branches above a spectral distance of 0.3 were sub-clusters, i.e., different strain patterns.

RESULTS AND DISCUSSION

During this study, 4049 yeasts were isolated from grape berries, the cellar environment and musts for the 2012 and 2013 vintages. We focused on two yeast genera often found in grape must and implicated in the fermentation process: *Hanseniaspora* and *Starmerella*. Thus, among these isolates, 214 yeasts were all identified as belonging to the species *Starmerella bacillaris*. 1078 isolates belonged to the genus *Hanseniaspora* of which 313 the species *H. guilliermondii* and 765 were the species *H. uvarum*. In spite of the high number of isolates obtained for these two genera, only two species were identified for the genus *Hanseniaspora* and only one for the genus *Starmerella*. On the contrary, 100 different strain patterns in the 765 isolates belonging to the species *H. uvarum* were identified by FT-IR and for the 313 isolates of the species *H. guilliermondii*, 74 different strain patterns were identified by hierarchical cluster analysis of the spectra obtained by FT-IR (Grangeteau et al., 2015). Using this method on FT-IR spectra of 214 isolates of the *S. bacillaris* species, 33 different patterns corresponding to 33 different strains were obtained. This high intraspecific diversity has recently been highlighted for the same species using the microsatellite method (Masneuf-Pomarede et al., 2015).

Distribution of the Species *Starmerella bacillaris*, *Hanseniaspora guilliermondii*, and *Hanseniaspora uvarum* during the 2012 Vintage

The distribution of three species isolated on berries, must and during AF is shown in **Figure 1**. In 2012, the species *S. bacillaris* was not isolated on berries (**Figure 1A**), while the species *H. guilliermondii* was isolated only once (**Figure 1B**) and the species *H. uvarum* represented only 11.5% of total isolates (**Figure 1C**). In spite of the enrichment step, *S. bacillaris* remained proportionally very low (2%) (**Figure 1A**). However, this step allowed isolating a higher number of yeasts belonging to two species of the genus *Hanseniaspora* (11 and 44% for *H. guilliermondii* and *H. uvarum,* respectively). The low presence in particular of *S. bacillaris* and *H. guilliermondii* was also observed by Li et al. (2010) for different grape varieties including Chardonnay. Compared to populations isolated on berries, the proportion of these three species isolated in must (T0), obtained after pressing and clarification, was higher: from 0% (berries) to 19% of isolates (must) for *S. bacillaris*, from 0.4 to 14% for *H. guilliermondii* and from 11.5 to 20% for *H. uvarum*. Their presence in must has already been shown in different studies (Xufre et al., 2006; Zott et al., 2008). Several hypotheses may explain the increase in the proportion of these species in must: the selection and modification of the distribution of species linked to changes in environmental conditions such as the modification of osmotic pressure (high concentration of sugars in grape must), pH or available oxygen (Sannino et al., 2013), or enrichment by exogenous yeasts present in the cellar environment (Ocón et al., 2010; Tello et al., 2012). In the absence of SO_2, during the first days of AF, the proportion of *S. bacillaris* fell considerably compared to T0, while remaining at a low percentage until T9 (1%, 1 and 3% at T3, T6 and T9, respectively) (**Figure 1A**). This species did not appear able to implant itself in the must, which may be explained by the strong presence of the genus *Hanseniaspora* in the same medium during the first days of AF. Indeed, this genus represented more than 90% of the population (**Figures 1B,C**) at T3 and T6, with the strong presence of the species *H. uvarum* (66% at T3 and 64% at T6) (**Figure 1C**). At T9, the proportion of the genus *Hanseniaspora* fell considerably, from 32 to 10% for *H. guilliermondii* and from 64 to 14% for *H. uvarum*. The low presence of *S. bacillaris* when that of the genus *Hanseniaspora* was substantial may result from antagonism between strains, as has already been

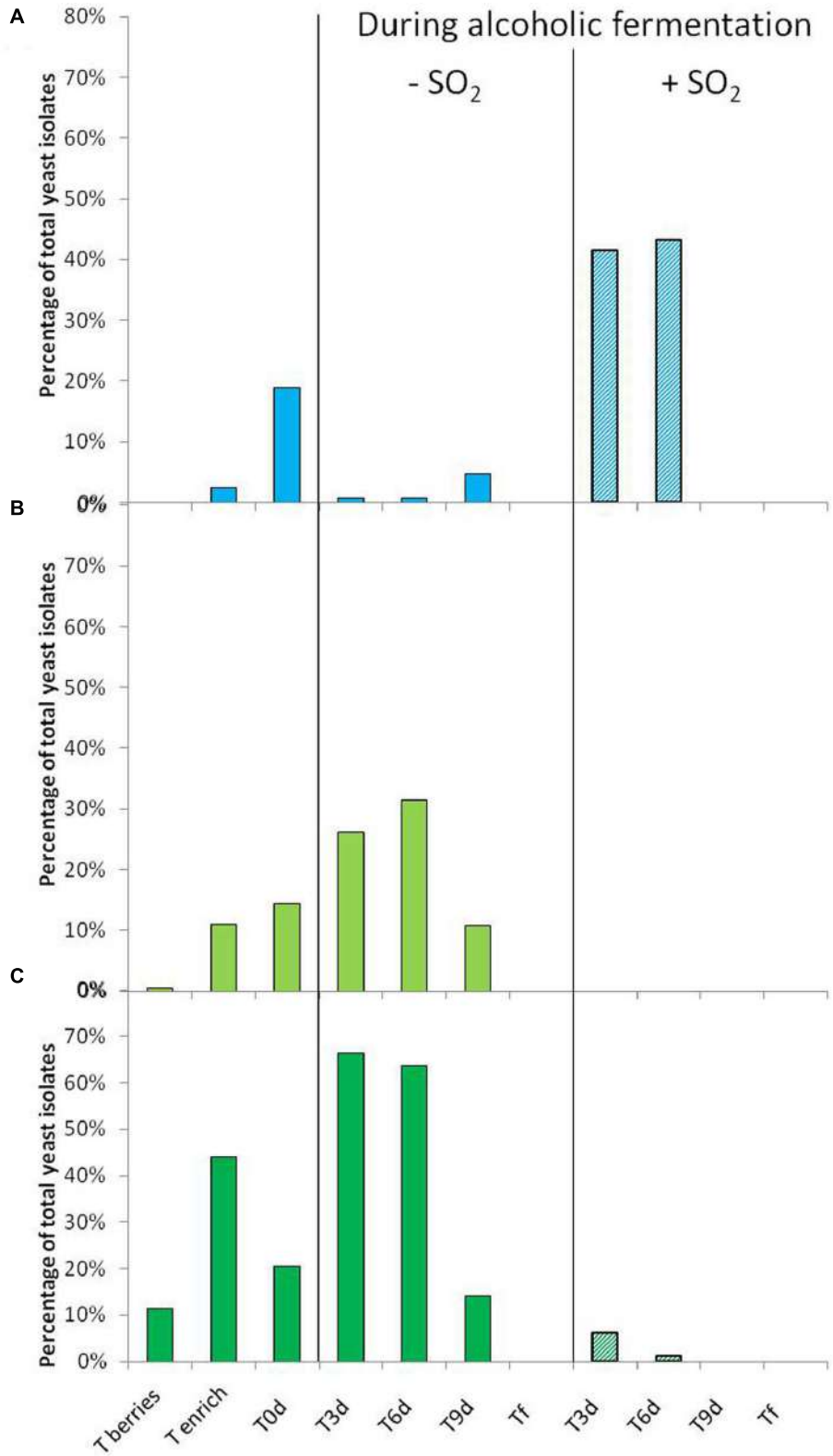

FIGURE 1 | Percentage of *Starmerella bacillaris* (A), *Hanseniaspora guilliermondii* (B) and *H. uvarum* (C) isolated from berries (T berries, T enrich), grape must (T0d) and during alcoholic fermentation (T3d, T6d, T9d, Tf) without (■) and with SO_2 (▩) for 2012 vintage. These percentages correspond to yeast belonging to the respective species compared to the total number of yeast isolates in the corresponding sample time.

shown for other yeast strains: between *Brettanomyces* and *Pichia* (Santos et al., 2009), between *Metschnikowia* and *Brettanomyces, Hanseniaspora* and *Pichia* (Oro et al., 2014). None of these three species (*S. bacillaris*, *H. guilliermondii,* and *H. uvarum*) was isolated at the end of AF (Tf). They were replaced by the indigenous species *S. cerevisiae* during AF (data not shown).

In the presence of SO_2, the behavior observed differed greatly according to yeast genus. During the first days of FA, the proportion of the species *S. bacillaris* increased strongly, continuing until T6 (41% and 46% of the population at T3 and T6, respectively). On the contrary, the proportion of the species *H. uvarum* decreased considerably during the first days of AF (6 and 1% of the population at T3 and T6, respectively) (**Figure 1B**) and the species *H. guilliermondii* was not isolated in the presence of SO_2 (**Figure 1C**). The implantation of the species *S. bacillaris* appeared to be facilitated following the addition of SO_2 given its known resistance to this antiseptic (Albertin et al., 2014). Besides its resistance to SO_2, the rapid and strong development of the species *S. bacillaris* could also have occurred to the detriment of the sparse implantation of the genus *Hanseniaspora,* inhibited by the presence of the antiseptic (Albertin et al., 2014), thereby freeing an ecological niche. Nonetheless, for the three species, the presence of SO_2 resulted in a rapid decrease in their proportion since they were not found after T9 (**Figure 1**). As for the total disappearance of the species *S. bacillaris* at T9, this may have been directly linked to its sensitivity to ethanol, as shown by Magyar and Tóth (2011). At T9, the content of ethanol in the medium was about 9%v/v whereas it was only 5%v/v at T6. Furthermore, as described by Henick-Kling et al. (1998), the presence of SO_2 favored the implantation of strains of *S. cerevisiae*, thus leading to faster production of ethanol in the medium. In our study, this implantation of *S. cerevisiae* was observed from T3 and reached 100% of the population at T9 (data not shown), possibly explaining the total disappearance the species *S. bacillaris* at T9.

The results obtained highlight an increase of the population of the three species studied in must compared to that isolated on berry. This increase may be linked either to the implantation of exogenous yeasts or to the preferential development of these species. To verify these hypotheses, the intraspecific biodiversity of the yeasts from grape berries, the grape must and the cellar environment was characterized by FT-IR spectroscopy for the three species studied. This study also allowed highlighting possible differences in resistance to SO_2 as a function of strain for the three species concerned.

Intraspecific Study of Populations of *Starmerella bacillaris* in 2012 and 2013

The results of the intra-specific study of *S. bacillaris* in 2012 are shown in **Figure 2**. The number of strain patterns detected in berries, even following enrichment, was very low (only three different strains: CF, CG and CP). However, high intraspecific diversity was observed in the must (T0, **Figures 2A,B**), since 19 different strain patterns were identified. No strain pattern was seen to be predominant. Only one strain pattern isolated in berries after enrichment (Tenrich) was found in the must at T0, i.e., strain pattern CP. In the absence of SO_2 (**Figure 2A**), no strain pattern isolated at T0 was isolated again during AF except strain CO isolated at T9. During AF, four new strain patterns (CL, CS, DC, and CQ) were detected but at only one time. These results confirm the low implantation of certain strains of the species *S. bacillaris* during AF. In the presence of SO_2 (**Figure 2B**), the three different strain patterns isolated on berries after enrichment did not implant during AF, except for strain pattern CP isolated at Tenrich, T0 and T3. The proportion of CP was 4% at T0, before reaching 9% at T3. However, it was no longer isolated afterward (**Figure 2B**). None of the five strain patterns (CO, CL, CS, DC and CQ) isolated during AF without SO_2 was found in the must fermented with SO_2. This could be due to the high sensitivity of these strains to SO_2. On the other hand, in the must fermented with SO_2, eight new strain patterns were isolated: CX, CY, CZ, DA and DB at T3 and CR, CM and CN at T6. Certain of these strains were present in high percentages of the total yeast population (26% for CX, 28% for CR and 16% for CM). These results highlight for the first time the implantation of strains of *S. bacillaris* stemming from the cellar environment (strains from the air, floor, wine-making equipment and other grape musts fermenting in the winery). The implantation of these eight strains was perhaps aided by the presence of SO_2 against which their resistance could be higher than the other strains. This implantation could also be due to the disappearance of other strains of *S. bacillaris* and to the disappearance of other yeast species or genera such as *Hanseniaspora* (as mentioned in the results in §3.1). As observed already for *S. cerevisiae* (Vezinhet and Hallet, 1992), the dynamics of the species *S. bacillaris* during AF corresponds to a succession of different strain patterns. Indeed, the five strain patterns isolated at T3 disappeared and then three other strain patterns were isolated at T6 before disappearing too, probably due to the ethanol content of the medium at that time (4–5%v/v) and competition between the yeasts during AF.

The strains found in the must during AF with and without SO_2 likely originated from the cellar since only one of these strains (CP) was found on the grape berry. In addition, despite the inability of *S. bacillaris* to persist in wine, we wanted to know whether certain strains isolated in 2012 could persist in the cellar environment for 1 year. Thus isolates were obtained before the arrival of the 2013 harvest in the cellar environment (air, floor, equipment). No yeast of this species was isolated. However, *S. bacillaris* had already been found in the cellar environment but in very low proportion and mainly on the type of equipment not used for the fermentations performed in this study (CO_2 suction line) (Bokulich et al., 2013). This was also the case for the genus *Starmerella* which was not isolated on the grapes harvested in 2013, in the must or during the AF of this vintage. The results therefore appear to indicate the low capacity of *S. bacillaris* strains to persist in the cellar environment from one vintage to the next. However, it is possible that certain strains persisted but that the conditions of the 2013 vintage did not prove propitious for their development and they therefore remained at levels below detection limits.

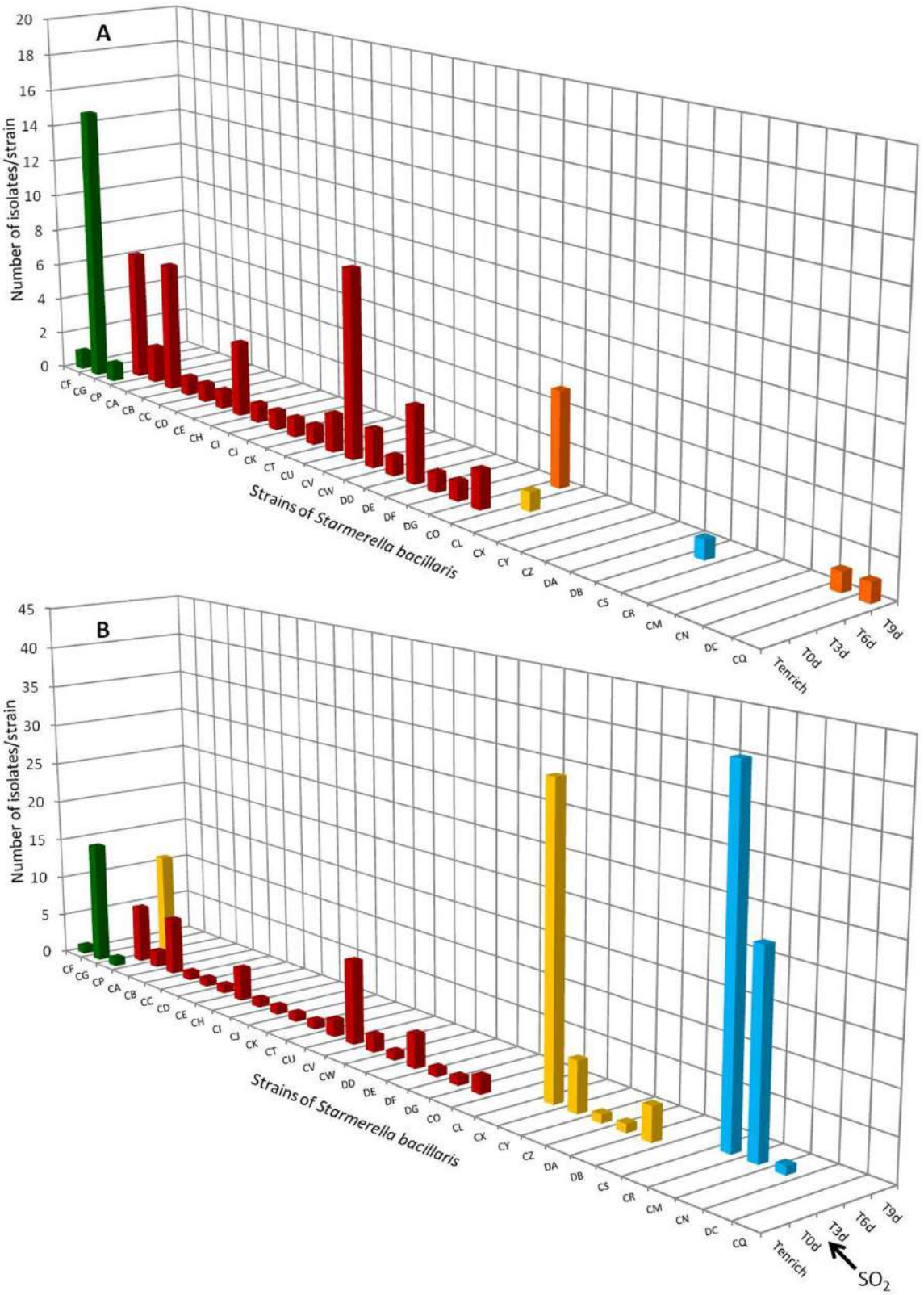

FIGURE 2 | Distribution of *Starmerella bacillaris* strain patterns during 2012 vintage isolated on berries (T enrich), in grape must (T0d) and during alcoholic fermentation (T3d, T6d, T9d, Tf) without SO_2 (A) and with SO_2 (B). No strain pattern was detected on grape berries without enrichment step.

Intraspecific Study of Populations of the Genus *Hanseniaspora* in 2012 and 2013

The results of the intraspecific study for *H. guilliermondii* and *H. uvarum* are presented in **Figures 3** and **4,** respectively. In 2012, very considerable diversity was observed for the population of *Hanseniaspora* present on berries following the enrichment step. Thus 24 different strain patterns were isolated for *H. guilliermondii* and 30 for *H. uvarum*. For these two species, the enrichment step proved very useful as it allowed significantly increasing the number of isolates and thus strains isolated (1 strain pattern on berries and 24 after enrichment for *H. guilliermondii*, 6 strain patterns on berries and 30 after enrichment for *H. uvarum*). On the contrary, it is noteworthy that four strain patterns of *H. uvarum* isolated on berries were not found after the enrichment step. Regarding this vintage, and contrary to what was observed for the species *S. bacillaris* (three strain patterns at Tenrich and 19 at T0), the number of different strain patterns was lower in the must at T0: 18 strain patterns for *H. guilliermondii* and 20 patterns for *H. uvarum* compared to the number of strain patterns identified after enrichment: 24 and 30 strain patterns for *H. guilliermondii* and *H. uvarum,* respectively. Of the 24 different strain patterns of *H. guilliermondii* and the 34 different patterns of *H. uvarum* from berries (Tberries and/or Tenrich), only 5 strain patterns (B, E, F, I, and J) for *H. guilliermondii* and three strain patterns (G′, T′, and X′) for *H. uvarum* were found in the grape must at T0. Several other strain patterns present on berries were also detected, not at T0 but during AF. These strain patterns were K (T6) for *H. guilliermondii* and Y′ and W′ present at T3 and U′, V′, and Z′ present at T6 for *H. uvarum*. This confirmed that part of the non-*Saccharomyces* yeasts present in the grape must have come from the vineyard. However, the major part of the strain patterns identified at T0 (13 for *H. guilliermondii* and 17 for *H. uvarum*) and during AF (30 for *H. guilliermondii* and 41 for *H. uvarum*) were not found on the berries and therefore likely came from the cellar environment. The strain patterns found at T0 implanted in the must during the pre-fermentation steps. We can therefore assume that the pressing and clarification steps lead to a selection of strains while favoring the implantation of strains better adapted to grape must conditions. The strains found in the must during fermentation were also certainly better adapted to the medium. In the absence of SO_2, the number of strain patterns of the species *H. guilliermondii* (**Figure 3**) decreased progressively during AF (18, 16, 14, 9, and 0 strain pattern at T0, T3, T6, T9 and Tf, respectively). Despite this decrease, it is noteworthy that the great majority of strain patterns identified at T3 (12) and all the strain patterns isolated at T6 (14) were not present at T0. Only two strain parttens, AS and AA, were isolated at T0 and at T3 and strain pattern K was isolated at Tenrich and T6. Thus most of the strain patterns found during AF appeared to have originated from the cellar environment. At T9, except for the strain patterns found in very low proportions (5), all the strain patterns present (B, F, and J) have been isolated previously during AF. Thus it appears that from T6, the selection of strain patterns was more related to the increased concentration of ethanol rather than to new implantations of strain patterns. We can observe different cases for these results: strain patterns present on the grape berries (Tberries and/or Tenrich) that persisted during AF (B, F, J, K), strain patterns present on berries and that were found only in the grape must and which did not implant during AF (E, I), strain patterns present on berries and that were never found again (19) and, finally, the large majority of strain patterns (29) probably stemming from the cellar environment and which were isolated once or possibly several times (AA, AP, and AS) during fermentation. Regarding the species *H. uvarum* (**Figure 4**), in 2012 and in the absence of SO_2, the number of strain patterns increased slightly at the beginning of AF (20 at T0 and 29 at T3). As from T6, the number of strain patterns decreased (20, 8, and 0 at T6, T9 and Tf, respectively). Of the strain patterns isolated throughout fermentation, only AQ′, BD′, CA′, CS′, D′, F′, G′, and X′ were also isolated in the must (T0). This leads to the assumption of strain patterns from outside. Indeed, certain strain patterns not isolated in the grape must at T3 or at T6 were found at T9 (3). Thus there was a succession of strain patterns during AF though much less obvious than that observed for *S. bacillaris.* In the same way as for *H. guilliermondii,* we observed different behaviors of strain patterns of *H. uvarum*: strain patterns present on grape berries (Tberries and/or Tenrich) that persisted during AF (G′, U′, V′, X′, Y′, W′ and Z′), strain patterns present on berries that were only found in grape must and which were not implanted during fermentation (T′), strain patterns present on berries but which were never found again (26), strain patterns probably stemming from the cellar environment isolated in the must (T0) and which persisted during FA (D′, F′, AQ′, BD′, CA′, CS′) or for the great majority of strain patterns (44) that were only isolated once or twice during fermentation.

SO_2 had a very strong effect on *H. guilliermondii* as this species was no longer detected in the medium after adding this antiseptic (**Figure 3**). These results confirm this species' low tolerance for SO_2. Regarding *H. uvarum*, a small number of strain patterns resisted the presence of SO_2; thus four strain patterns were isolated at T3 and other strain patterns at T6 (**Figure 4**). As with *S. bacillaris*, the strain patterns present during fermentation with SO_2 were not those that had been isolated at T0. Thus it is likely that the cellar environment contained strains particularly adapted to these fermentation conditions and which implanted and developed following the elimination of less well adapted strains. Resistance to SO_2 for the species *S. bacillaris* and *H. uvarum* could be strain dependent, as with *S. cerevisiae* (Divol et al., 2006). As observed for *S. bacillaris,* no strain belonging to the genus *Hanseniaspora* (**Figures 3** and **4**) was present at the end of AF whether with or without SO_2. In 2013, and contrary to *S. bacillaris,* different strain patterns of *H. guilliermondii* (six on the floor and three on the equipment) (**Figure 3**) and *H. uvarum* (seven on the floor, three on the equipment and one in the air) (**Figure 4**) were isolated in the winery before the arrival of the harvest. These results clearly confirm the presence of these species of non-*Saccharomyces* among others in the cellar environment already observed by different authors (Ocón et al., 2010; Bokulich et al., 2013). On the other hand, these results show the presence of different strains of the same species in the cellar environment for the first time. Of these strains in the cellar environment, strain patterns B and E for *H. guilliermondii* and C′, D′, F′, and G′ for *H. uvarum* had already been isolated in 2012. Furthermore,

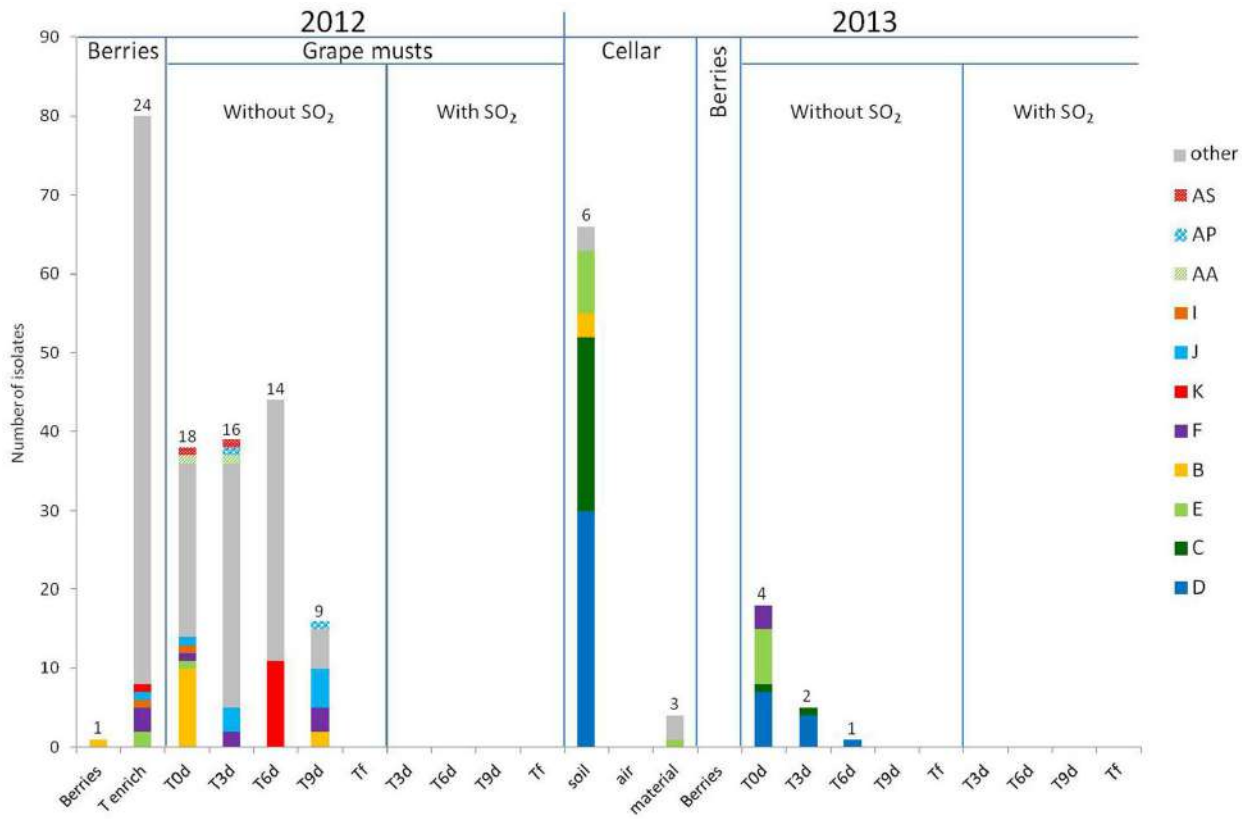

FIGURE 3 | Repartition of *H. guilliermondii* strain patterns isolated on berries, in grape must, during alcoholic fermentation (T3d, T6d, T9d, Tf) with and without SO_2 for 2012 and 2013 vintages and in cellar environment in 2013 before the arrival of harvest. Numbers on the top of the barplot correspond to the number of different strain patterns. The term "other" includes the strain patterns which were detected once.

strain patterns B and E (*H. guilliermondii*) and C′ and G′ (*H. uvarum*) came from the vineyard. Also demonstrated for the first time was the capacity of certain strains of *H. guilliermondii* and *H. uvarum* to persist from one vintage to another in the cellar environment. The species *S. cerevisiae* (Sabate et al., 1998; Santamaría et al., 2005) is also known to persist in the same environment, which raises the question whether yeasts of the genus *Hanseniaspora* can implant in musts after staying in the cellar environment for a year in the same way as strains of *S. cerevisiae*.

The most probable source of the *Hanseniaspora* yeasts isolated in the must for this vintage was the cellar environment since no other yeast belonging to the genus *Hanseniaspora* was isolated on berries in 2013. Three strain patterns of *H. guilliermondii* (C, D, and E) (**Figure 3**) and 6 of *H. uvarum* (C′, D′, E′, F′, G′, I′) (**Figure 4**), isolated in musts in 2013 at T0 were found again in the environment before the arrival of the harvest. Among these strain, strain patterns B and E of the species *H. guilliermondii* and D′, F′ and G′ of the species *H. uvarum* had already been isolated in the musts of 2012. They therefore survived for a year in the cellar environment before reimplanting in the musts of the following year. Among the strains that had remained in the cellar environment between 2012 and 2013 only strain pattern B (**Figure 3**) was not isolated in the musts of 2013. These results show the considerable capacity for implantation of these strains after 1 year in the cellar environment. What is more, strain pattern C′ (**Figure 4**) isolated in the vineyard in 2012 but not found again in the musts of 2012 was isolated in the cellar environment before the arrival of the harvest and in the musts in 2013 and at several times (T0, T3, and T6). This strain could have been introduced in the cellar in 2012 with our harvest without having developed sufficiently to be detected. This strain could also have been introduced by the harvests and the later AF performed in the same winery. Lastly, this strain could have been present in the cellar environment during several vintages but not implanted and developed sufficiently to be detected in 2012. This case had already been observed for *Saccharomyces* by Santamaría et al. (2005) who isolated certain strains in one vintage, but not in several succeeding ones, and then found the same strain again. In addition, our results highlighted two strain patterns D′ and F′, not isolated on berries in 2012 but present at every stage of AF (from T0 to T9 for F′ and up to T6 for D′), that persisted in the cellar environment (floor and/or equipment) and which were isolated in the must (T0) and during AF (T3 and T6) in 2013. These strain patterns appeared to be particularly well-adapted to the wine-making environment and the conditions imposed by the wine medium (except for the addition of SO_2).

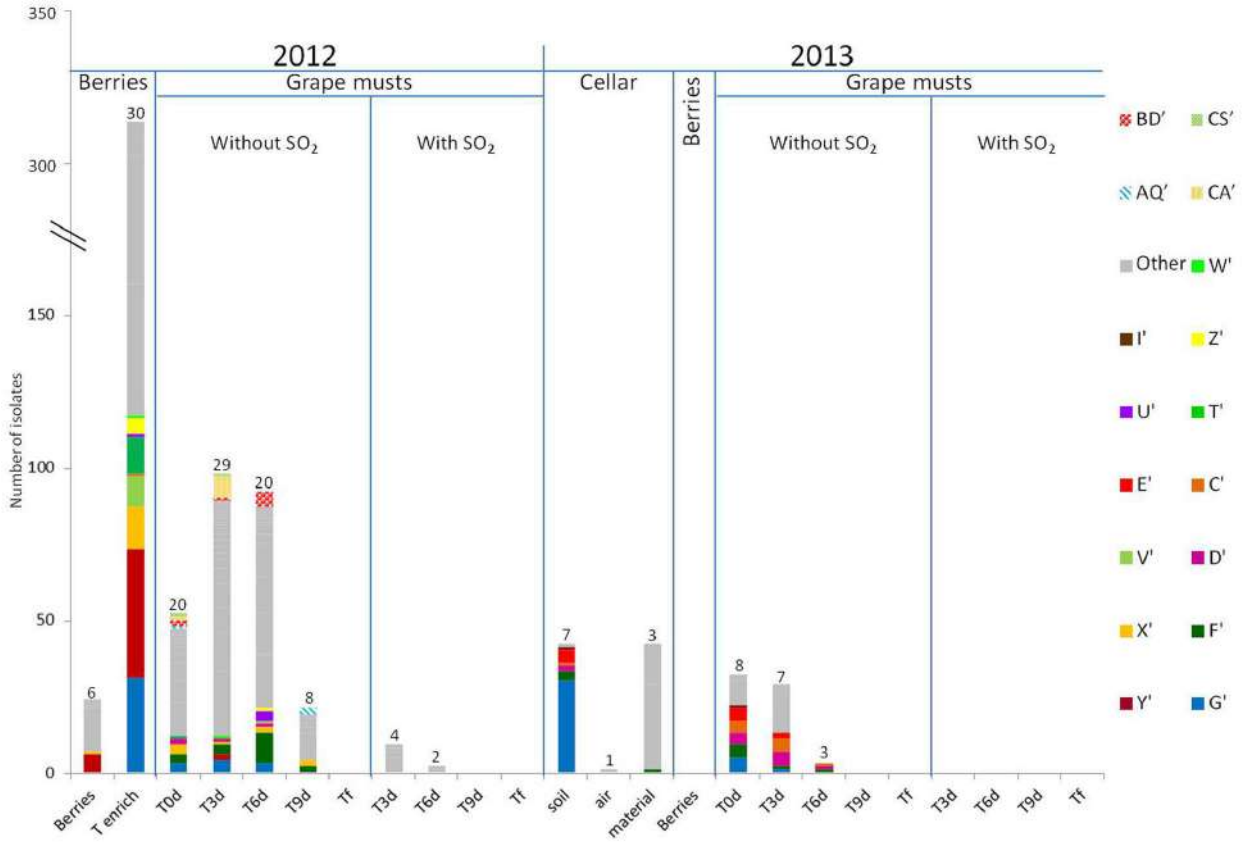

FIGURE 4 | Repartition of *H. uvarum* strain patterns isolated on berries, in grape must, during alcoholic fermentation (T3d, T6d, T9d, Tf) with and without SO_2 for 2012 and 2013 vintages and in cellar environment in 2013 before the arrival of the harvest. Numbers on the top of the barplot correspond to the number of different strain patterns. The term "other" includes the strain patterns which were detected once.

Much lower intraspecific diversity was observed for the two species of *Hanseniaspora* in the musts in 2013 (four and eight strain patterns for *H. guilliermondii* and *H. uvarum*, respectively) in comparison to 2012 (18 and 20 strain patterns for *H. guilliermondii* and *H. uvarum*, respectively). This low diversity could be due to the absence of strain patterns stemming from grape and to a lower volume of musts linked to a less abundant harvest in 2013. In the absence of SO_2, the number of strain patterns of *H. guilliermondii* fell as from the first days of FA. 4 strain patterns were isolated at T0, 2 at T3, 1 at T6 and none from T9. Strain pattern F isolated at T0 was the only pattern not to have been isolated in the cellar environment but which was present in the musts (T0, T3, and T9) and on the berries (Tenrich) in 2012. Regarding the species *H. uvarum*, all the strain patterns except I′, isolated at T0 were still present at T3. At T6, three strain patterns were still isolated and none were from T9 onward. Thus the best adapted strains had undergone selection since the three strain patterns C′, D′ and F′ were present from the start until T6.

In the presence of SO_2, no strain belonging to the genus *Hanseniaspora* was isolated in the must during fermentation in 2013 whereas strains of the species *H. uvarum* were isolated at T3 and T6 in 2012. Nonetheless, none of the strains that had resisted SO_2 in 2012 appeared to have subsisted in the cellar environment between 2012 and 2013. Furthermore, several studies have shown that the yeasts of the genus *Hanseniaspora* are quite sensitive to the presence of SO_2 (Cocolin and Mills, 2003; Albertin et al., 2014). The capacity to resist this antiseptic is undoubtedly a rare characteristic among the strains of the genus *Hanseniaspora*. This was also confirmed in this study by the small number of strains persisting in the presence of SO_2 in 2012 (only six strain patterns). Lastly, during the 2013 vintage, the species *S. cerevisiae* was present as from T0 whereas it was only detected from T3 in 2012 (data not shown). This initial presence coupled with that of SO_2 which favors an increase in the proportion of *Saccharomyces* (Henick-Kling et al., 1998) could be detrimental to strains of *Hanseniaspora* in comparison to those of *S. cerevisiae* and explain their disappearance from the beginning of AF (Nissen et al., 2003; Pérez-Nevado et al., 2006).

This is the first time populations of non-*Saccharomyces* yeasts have been studied at the intraspecific level in the vineyard, the cellar environment and grape musts during AF for two consecutive vintages. In spite of the low interspecific diversity for the two genera studied here (a single species for the genus *Starmerella* and 2 for the genus *Hanseniaspora*), high intraspecific diversity was demonstrated for the three species identified: (74 strain patterns for *H. guilliermondii*, 100 strain patterns for

H. uvarum and 33 strain patterns for *Starmerella bacillaris*). Monitoring these strain patterns in musts during AF showed that, whatever the species considered, there was no really predominant species but rather a succession of different strain patterns, as was observed for the species *S. cerevisiae*.

Furthermore, this study confirmed that using sulfur dioxide eliminates the strains of the genus *Hanseniaspora* and thus permits the development of the species *Starmeralla bacillaris* which is more resistant to this antiseptic. Intraspecific differences regarding resistance to SO_2 lead, at species level, to the elimination of the most sensitive strains, thereby permitting the development and/or implantation of more resistant strains from the cellar environment.

Lastly, this study showed for the first time the persistence in the cellar environment of strains of non-*Saccharomyces* yeasts capable of reimplantation during the following vintage. Thus, the cellar is not only a transient habitat. However, this capacity is not shared between every yeast species since only two species of the genus *Hanseniaspora* were isolated in the cellar environment during the second vintage. This concerns a limited number of strains: five strain patterns (one for *H. guilliermondii* and four for *H. uvarum*). As described for *S. cerevisiae*, we highlighted for the first time that the non-*Saccharomyces* flora of the cellar appeared to predominate in comparison to the grape flora. The capacity of species and strains to persist in the cellar therefore influences yeast biodiversity in musts. But an opposite hypothesis could be proposed, namely that yeast biodiversity in must influences the capacity of strains residing in the cellar to implant the must.

AUTHOR CONTRIBUTIONS

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

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REFERENCES

Adt, I., Kohler, A., Gognies, S., Budin, J., Sandt, C., Belarbi, A., et al. (2010). FTIR spectroscopic discrimination of *Saccharomyces cerevisiae* and *Saccharomyces bayanus* strains. *Can. J. Microbiol.* 56, 793–801. doi: 10.1139/w10-062

Albertin, W., Miot-Sertier, C., Bely, M., Marullo, P., Coulon, J., Moine, V., et al. (2014). Oenological prefermentation practices strongly impact yeast population dynamics and alcoholic fermentation kinetics in Chardonnay grape must. *Int. J. Food Microbiol.* 178, 87–97. doi: 10.1016/j.ijfoodmicro.2014.03.009

Barata, A., Malfeito-Ferreira, M., and Loureiro, V. (2012). The microbial ecology of wine grape berries. *Int. J. Food Microbiol.* 153, 243–259. doi: 10.1016/j.ijfoodmicro.2011.11.025

Barata, A., Seborro, F., Belloch, C., Malfeito-Ferreira, M., and Loureiro, V. (2008). Ascomycetous yeast species recovered from grapes damaged by honeydew and sour rot. *J. Appl. Microbiol.* 104, 1182–1191. doi: 10.1111/j.1365-2672.2007.03631.x

Bokulich, N. A., Ohta, M., Richardson, P. M., and Mills, D. A. (2013). Monitoring seasonal changes in winery-resident microbiota. *PLoS ONE* 8:e66437. doi: 10.1371/journal.pone.0066437

Cadez, N., Zupan, J., and Raspor, P. (2010). The effect of fungicides on yeast communities associated with grape berries. *FEMS Yeast Res.* 10, 619–630. doi: 10.1111/j.1567-1364.2010.00635.x

Ciani, M., Mannazzu, I., and Marinangeli, P. (2004). Contribution of winery-resident *Saccharomyces cerevisiae* strains to spontaneous grape must fermentation. *Antonie Van Leeuwenhoek* 85, 159–164. doi: 10.1023/B:ANTO.0000020284.05802.d7

Clemente-Jimenez, J. M., Mingorance-Cazorla, L., Martı̀nez-Rodrı̀guez, S., Heras-Vázquez, F. J. L., and Rodrı̀guez-Vico, F. (2004). Molecular characterization and oenological properties of wine yeasts isolated during spontaneous fermentation of six varieties of grape must. *Food Microbiol.* 21, 149–155. doi: 10.1016/S0740-0020(03)00063-7

Cocolin, L., and Mills, D. A. (2003). Wine yeast inhibition by sulfur dioxide: a comparison of culture-dependent and independent methods. *Am. J. Enol. Vitic.* 2, 125–130.

Cordero-Bueso, G., Arroyo, T., Serrano, A., Tello, J., Aporta, I., Vélez, M. D., et al. (2011a). Influence of the farming system and vine variety on yeast communities associated with grape berries. *Int. J. Food Microbiol.* 145, 132–139. doi: 10.1016/j.ijfoodmicro.2010.11.040

Cordero-Bueso, G., Arroyo, T., Serrano, A., and Valero, E. (2011b). Remanence and survival of commercial yeast in different ecological niches of the vineyard. *FEMS Microbiol. Ecol.* 77, 429–437. doi: 10.1111/j.1574-6941.2011.01124.x

David, V., Terrat, S., Herzine, K., Claisse, O., Rousseaux, S., Tourdot-Maréchal, R., et al. (2014). High-throughput sequencing of amplicons for monitoring yeast biodiversity in must and during alcoholic fermentation. *J. Ind. Microbiol. Biotechnol.* 41, 811–821. doi: 10.1007/s10295-014-1427-2

Divol, B., Miot-Sertier, C., and Lonvaud-Funel, A. (2006). Genetic characterization of strains of *Saccharomyces cerevisiae* responsible for "refermentation" in *Botrytis*-affected wines. *J. Appl. Microbiol.* 100, 516–526. doi: 10.1111/j.1365-2672.2005.02818.x

Duarte, F. L., Pimentel, N. H., Teixeira, A., and Fonseca, A. (2012). *Saccharomyces bacillaris* is not a synonym of *Candida stellata*: reinstatement as *Starmerella bacillaris* comb. nov. *Antonie Van Leeuwenhoek* 102, 653–658. doi: 10.1007/s10482-012-9762-7

Fleet, G. (2003). "Yeasts in fruit and fruit products," in *Yeasts in Food: Beneficial and Detrimental Aspects*, eds T. Boekhout and V. Robert (Boca Raton, FL: CRC Press Publishers), 13–54.

Fleet, G. H., and Heard, G. (1993). "Yeasts-growth during fermentation," in *Wine Microbiology and Biotechnology*, ed. G. H. Fleet (London: CRC Press), 27–54.

Fleet, G. H., Lafon-Lafourcade, S., and Ribereau-Gayon, P. (1984). Evolution of yeasts and lactic acid bacteria during fermentation and storage of Bordeaux wines. *Appl. Environ. Microbiol.* 48, 1034–1038.

Garijo, P., Santamaría, P., López, R., Sanz, S., Olarte, C., and Gutiérrez, A. R. (2008). The occurrence of fungi, yeasts and bacteria in the air of a Spanish winery during vintage. *Int. J. Food Microbiol.* 125, 141–145. doi: 10.1016/j.ijfoodmicro.2008.03.014

Grangeteau, C., Gerhards, D., Rousseaux, S., von Wallbrunn, C., Alexandre, H., and Guilloux-Bénatier, M. (2015). Diversity of yeast strains of the genus *Hanseniaspora* in the winery environment: what is their involvement in grape must fermentation? *Food Microbiol.* 50, 70–77. doi: 10.1016/j.fm.2015.03.009

Grangeteau, C., Gerhards, D., Terrat, S., Dequiedt, S., Alexandre, H., Guilloux-Benatier, M., et al. (2016). FT-IR spectroscopy: a powerful tool for studying the inter- and intraspecific biodiversity of cultivable non-*saccharomyces* yeasts isolated from grape must. *J. Microbiol. Methods* 121, 50–58. doi: 10.1016/j.mimet.2015.12.009

Heard, G. M., and Fleet, G. H. (1986). Evaluation of selective media for enumeration of yeasts during wine fermentation. *J. Appl. Microbiol.* 60, 477–481. doi: 10.1111/j.1365-2672.1986.tb01086.x

Henick-Kling, T., Edinger, W., Daniel, P., and Monk, P. (1998). Selective effects of sulfur dioxide and yeast starter culture addition on indigenous yeast populations and sensory characteristics of wine. *J. Appl. Microbiol.* 84, 865–876. doi: 10.1046/j.1365-2672.1998.00423.x

Hierro, N., González, A., Mas, A., and Guillamón, J. M. (2006). Diversity and evolution of non-*Saccharomyces* yeast populations during wine fermentation: effect of grape ripeness and cold maceration. *FEMS Yeast Res.* 6, 102–111. doi: 10.1111/j.1567-1364.2005.00014.x

Jolly, N. P., Augustyn, O. P. H., and Pretorius, I. S. (2003). The occurrence of non-*Saccharomyces cerevisiae* yeast species over three vintages in four vineyards and grape musts from four production regions of the Western Cape, South Africa. *S. Afr. J. Enol. Vitic.* 24, 63–69.

Kümmerle, M., Scherer, S., and Seiler, H. (1998). Rapid and reliable identification of food-borne yeasts by Fourier-transform infrared spectroscopy. *Appl. Environ. Microbiol.* 64, 2207–2214.

Le Jeune, C., Erny, C., Demuyter, C., and Lollier, M. (2006). Evolution of the population of *Saccharomyces cerevisiae* from grape to wine in a spontaneous fermentation. *Food Microbiol.* 23, 709–716. doi: 10.1016/j.fm.2006.02.007

Li, S. S., Cheng, C., Li, Z., Chen, J. Y., Yan, B., Han, B. Z., et al. (2010). Yeast species associated with wine grapes in China. *Int. J. Food Microbiol.* 138, 85–90. doi: 10.1016/j.ijfoodmicro.2010.01.009

Magyar, I., and Tóth, T. (2011). Comparative evaluation of some oenological properties in wine strains of *Candida stellata*, *Candida zemplinina*, *Saccharomyces uvarum* and *Saccharomyces cerevisiae*. *Food Microbiol.* 28, 94–100. doi: 10.1016/j.fm.2010.08.011

Masneuf-Pomarede, I., Juquin, E., Miot-Sertier, C., Renault, P., Laizet, Y., Salin, F., et al. (2015). The yeast *Starmerella bacillaris* (synonym *Candida zemplinina*) shows high genetic diversity in winemaking environments. *FEMS Yeast Res.* 15, 1–11. doi: 10.1093/femsyr/fov045

Milanović, V., Comitini, F., and Ciani, M. (2013). Grape berry yeast communities: influence of fungicide treatments. *Int. J. Food Microbiol.* 161, 240–246. doi: 10.1016/j.ijfoodmicro.2012.12.019

Mortimer, R., and Polsinelli, M. (1999). On the origins of wine yeast. *Res. Microbiol.* 150, 199–204. doi: 10.1016/S0923-2508(99)80036-9

Nisiotou, A., Spiropoulos, A. E., and Nychas, G. J. (2007). Yeast community structures and dynamics in healthy and *Botrytis*-affected grape must fermentations. *Appl. Environ. Microbiol.* 73, 6705–6713. doi: 10.1128/AEM.01279-07

Nissen, P., Nielsen, D., and Arneborg, N. (2003). Viable *Saccharomyces cerevisiae* cells at high concentrations cause early growth arrest of non-*Saccharomyces* yeasts in mixed cultures by a cell – cell contact-mediated mechanism. *Yeast* 20, 331–341. doi: 10.1002/yea.965

Ocón, E., Garijo, P., Sanz, S., Olarte, C., Santamaría, P., and Gutiérrez, A. R. (2013). Analysis of airborne yeast in one winery over a period of one year. *Food Control* 30, 585–589. doi: 10.1016/j.foodcont.2012.07.051

Ocón, E., Gutiérrez, A. R., Garijo, P., López, R., and Santamaría, P. (2010). Presence of non-*Saccharomyces* yeasts in cellar equipment and grape juice during harvest time. *Food Microbiol.* 27, 1023–1027. doi: 10.1016/j.fm.2010.06.012

Oro, L., Ciani, M., and Comitini, F. (2014). Antimicrobial activity of *Metschnikowia pulcherrima* on wine yeasts. *J. Appl. Microbiol.* 116, 1209–1217. doi: 10.1111/jam.12446

Pérez-Martín, F., Seseña, S., Fernández-González, M., Arévalo, M., and Palop, M. L. (2014). Microbial communities in air and wine of a winery at two consecutive vintages. *Int. J. Food Microbiol.* 190, 44–53. doi: 10.1016/j.ijfoodmicro.2014.08.020

Pérez-Nevado, F., Albergaria, H., Hogg, T., and Girio, F. (2006). Cellular death of two non-*Saccharomyces* wine-related yeasts during mixed fermentations with *Saccharomyces cerevisiae*. *Int. J. Food Microbiol.* 108, 336–345. doi: 10.1016/j.ijfoodmicro.2005.12.012

Pinto, C., Pinho, D., Cardoso, R., Custodio, V., Fernandes, J., Sousa, S., et al. (2015). Wine fermentation microbiome: a landscape from different Portuguese wine appellations. *Front. Microbiol.* 6:905. doi: 10.3389/fmicb.2015.00905

Sabate, J., Cano, J., Esteve-Zarzoso, B., and Guillamón, J. M. (2002). Isolation and identification of yeasts associated with vineyard and winery by RFLP analysis of ribosomal genes and mitochondrial DNA. *Microbiol. Res.* 157, 267–274. doi: 10.1078/0944-5013-00163

Sabate, J., Cano, J., Querol, A., and Guillamón, J. M. (1998). Diversity of *Saccharomyces* strains in wine fermentations: analysis for two consecutive years. *Lett. Appl. Microbiol.* 26, 452–455. doi: 10.1046/j.1472-765X.1998.00369.x

Sangorrín, M. P., Lopes, C. A., Belloch, C., Querol, A., and Caballero, A. C. (2007). *Candida patagonica* sp. nov., a new species of yeast from cellar surfaces. *Antonie Van Leeuwenhoek* 92, 77–81. doi: 10.1007/s10482-006-9137-z

Sannino, C., Francesca, N., Corona, O., Settanni, L., Cruciata, M., and Moschetti, G. (2013). Effect of the natural winemaking process applied at industrial level on the microbiological and chemical characteristics of wine. *J. Biosci. Bioeng.* 116, 347–356. doi: 10.1016/j.jbiosc.2013.03.005

Santamaría, P., Garijo, P., López, R., Tenorio, C., and Rosa Gutiérrez, A. (2005). Analysis of yeast population during spontaneous alcoholic fermentation: effect of the age of the cellar and the practice of inoculation. *Int. J. Food Microbiol.* 103, 49–56. doi: 10.1016/j.ijfoodmicro.2004.11.024

Santos, A., San Mauro, M., Bravo, E., and Marquina, D. (2009). PMKT2, a new killer toxin from *Pichia membranifaciens*, and its promising biotechnological properties for control of the spoilage yeast *Brettanomyces bruxellensis*. *Microbiology* 155, 624–634. doi: 10.1099/mic.0.023663-0

Tello, J., Cordero-Bueso, G., Aporta, I., Cabellos, J. M., and Arroyo, T. (2012). Genetic diversity in commercial wineries: effects of the farming system and vinification management on wine yeasts. *J. Appl. Microbiol.* 112, 302–315. doi: 10.1111/j.1365-2672.2011.05202.x

Tristezza, M., Vetrano, C., Bleve, G., Spano, G., Capozzi, V., Logrieco, A., et al. (2013). Biodiversity and safety aspects of yeast strains characterized from vineyards and spontaneous fermentations in the Apulia Region, Italy. *Food Microbiol.* 36, 335–342. doi: 10.1016/j.fm.2013.07.001

Vezinhet, F., and Hallet, J. (1992). Ecological survey of wine yeast strains by molecular methods of identification. *Am. J. Enol. Vitic.* 43, 83–86.

Wang, C., Garcia-Fernandez, D., Mas, A., and Esteve-Zarzoso, B. (2015). Fungal diversity in grape must and wine fermentation assessed by massive sequencing, quantitative PCR and DGGE. *Front. Microbiol.* 6:1156. doi: 10.3389/fmicb.2015.01156

Xufre, A., Albergaria, H., Inácio, J., Spencer-Martins, I., and Gírio, F. (2006). Application of fluorescence in situ hybridisation (FISH) to the analysis of yeast population dynamics in winery and laboratory grape must fermentations. *Int. J. Food Microbiol.* 108, 376–384. doi: 10.1016/j.ijfoodmicro.2006.01.025

Zott, K., Miot-Sertier, C., Claisse, O., Lonvaud-Funel, A., and Masneuf-Pomarede, I. (2008). Dynamics and diversity of non-*Saccharomyces* yeasts during the early stages in winemaking. *Int. J. Food Microbiol.* 125, 197–203. doi: 10.1016/j.ijfoodmicro.2008.04.001

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The Oenological Potential of *Hanseniaspora uvarum* in Simultaneous and Sequential Co-fermentation with *Saccharomyces cerevisiae* for Industrial Wine Production

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In oenology, the utilization of mixed starter cultures composed by *Saccharomyces* and non-*Saccharomyces* yeasts is an approach of growing importance for winemakers in order to enhance sensory quality and complexity of the final product without compromising the general quality and safety of the oenological products. In fact, several non-*Saccharomyces* yeasts are already commercialized as oenological starter cultures to be used in combination with *Saccharomyces cerevisiae*, while several others are the subject of various studies to evaluate their application. Our aim, in this study was to assess, for the first time, the oenological potential of *H. uvarum* in mixed cultures (co-inoculation) and sequential inoculation with *S. cerevisiae* for industrial wine production. Three previously characterized *H. uvarum* strains were separately used as multi-starter together with an autochthonous *S. cerevisiae* starter culture in lab-scale micro-vinification trials. On the basis of microbial development, fermentation kinetics and secondary compounds formation, the strain *H. uvarum* ITEM8795 was further selected and it was co- and sequentially inoculated, jointly with the *S. cerevisiae* starter, in a pilot scale wine production. The fermentation course and the quality of final product indicated that the co-inoculation was the better performing modality of inoculum. The above results were finally validated by performing an industrial scale vinification The mixed starter was able to successfully dominate the different stages of the fermentation process and the *H. uvarum* strain ITEM8795 contributed to increasing the wine organoleptic quality and to simultaneously reduce the volatile acidity. At the best of our knowledge, the present report is the first study regarding the utilization of a selected *H. uvarum* strain in multi-starter inoculation with *S. cerevisiae* for the industrial production of a wine. In addition, we demonstrated, at an industrial scale, the importance of non-*Saccharomyces* in the design of tailored starter cultures for typical wines.

Keywords: oenological non-*Saccharomyces*, wine alcoholic fermentation, *Hanseniaspora uvarum*, *Saccharomyces cerevisiae*, mixed fermentations, starter multi-strains, co-inoculation, sequential inoculation

INTRODUCTION

Fermentation associated with wine production represents complex biological processes denoted by several biochemical interactions between grape must and different micro-organisms such as fungi, yeasts and bacteria (Fleet, 2003). In particular, yeasts play a fundamental role, since they carry out the alcoholic fermentation (AF), i.e., the conversion of sugars to ethanol and CO_2, but they also determine the wine organoleptic properties by producing and secreting into the fermenting must several secondary metabolites (Lambrechts and Pretorius, 2000; Fleet, 2003; Romano et al., 2003; Jolly et al., 2006). The AF is initially promoted by the action of a heterogeneous consortium of yeasts belonging to different non-*Saccharomyces* species usually characterized by a low fermentative power (Heard and Fleet, 1985), while its final step is under the control of alcohol-tolerant *Saccharomyces cerevisiae* strains (Fleet and Heard, 1993). The function of non-*Saccharomyces* species throughout the AF is very significant, since they strongly contribute in determining the wine chemical composition. Autochthonous yeasts provide distinctive regional features to wines (Romano et al., 2003; Fleet, 2008; Ciani et al., 2010; Medina et al., 2013; Garofalo et al., 2015) thus advising their use as commercial starter cultures in order to differentiate wine productions. Some non-*Saccharomyces* yeasts are already commercialized as oenological starter cultures (e.g., *Torulaspora delbrueckii*, *Metschnikowia pulcherrima*, *Pichia kluyveri, Lachancea thermotolerans*) to be used in combination with *Saccharomyces cerevisiae* (Lu et al., 2015), while several others are the subject of various studies (e.g., *Hanseniaspora uvarum*, *Starmerella bacillaris*) (Masneuf-Pomarede et al., 2016).

The apiculate yeast *Hanseniaspora uvarum* (anamorph *Kloeckera apiculata*) is one of the yeast species more represented onto grape berries and they prevail in the first steps of spontaneous AF (Fleet and Heard, 1993). This yeast species is important in the production of volatile compounds in wine and the general chemical composition of wines made by *Hanseniaspora* spp./*S. cerevisiae* combinations may differ from reference wines produced with pure culture of *S. cerevisiae* (Zironi et al., 1993; Erten, 2002; Ciani et al., 2006; Gil et al., 2006). Previous reports indicated that several *H. uvarum* physiological properties of oenological interest are strain-dependent characters, such as ethanol production (Caridi and Ramondino, 1999), the volatile acidity associated with fermentation (Romano et al., 1992; Ciani and Maccarelli, 1998) and, most of all, the production of primary metabolites (i.e., glycerol, acetaldehyde) and secondary metabolites, such as ethyl acetate and hydrogen sulfide (Romano et al., 1997).

During a recent investigation, we have studied the oenological properties of 9 different *H. uvarum* strains isolated during the first 24 h of the spontaneous fermentation of Negroamaro grape must (De Benedictis et al., 2011). The chemical analysis of fermented must showed that all the strains produced low amounts of hydrogen sulfide and acetic acid, showing fructophilic character and relevant glycerol production. Analysis of volatile compounds indicated that in particular one strain, *H. uvarum* ITEM8795, could potentially enhance taste and flavor of wines, thus indicating its possible utilization for the formulation of mixed and/or sequential starters together with *S. cerevisiae* strains.

Indeed, for several non-*Saccharomyces* yeasts species has been demonstrated that they contribute to the analytical composition and the sensorial characteristics of wine, increasing the interest in the industrial application of apiculate yeasts (Pérez-Coello et al., 1999; Domizio et al., 2007; Fleet, 2008; Viana et al., 2008; Capozzi et al., 2015). In fact, the addition of non-*Saccharomyces* yeast species as part of mixed starter formulations, together with *S. cerevisiae* (and of malolactic bacteria), has been recently indicated as a way of mimic the spontaneous fermentations (Mendoza and Farías, 2010; Suzzi et al., 2012), conferring a particular aroma and characteristics to wines (Ciani et al., 2010; Comitini et al., 2011; Suárez-Lepe and Morata, 2012) without increasing/reducing the risks for wine quality and safety often associated with uncontrolled vinifications (Spano et al., 2010; Capozzi and Spano, 2011; Tristezza et al., 2013).

On the above basis, the aim of the present study was to assess the fermentation performances and interactions of mixed cultures and sequential inoculation of *H. uvarum* and *S. cerevisiae*. Data about microbial development, fermentation kinetics and secondary compound formation in lab-scale micro-vinification trials were further confirmed by utilization of the above mixed starter in pilot- and industrial-scale production of Negroamaro wine. At the best of our knowledge, the present investigation is the first report about the utilization of selected strain of *H. uvarum* in simultaneous and sequential co-fermentation with *S. cerevisiae* from micro-vinification up to the industrial scale in the production of a typical red wine.

MATERIALS AND METHODS

Yeast Strains

Yeast strains used in the present study are deposited in Agro-Food Microbial Culture Collection of ISPA (http://www.ispacnr.it/collezioni-microbiche/). The *Saccharomyces cerevisiae* strain ITEM6920 (S) and the *Hanseniaspora uvarum* strains ITEM8795 (H1), ITEM8797 (H2), ITEM8799 (H3) have been previously isolated from spontaneous fermentation of Negroamaro grapes (De Benedictis et al., 2011; Tristezza et al., 2012). All the strains had been previously identified and characterized for their oenological properties, and in particular, the *S. cerevisiae* strain ITEM6920 has been already used as starter culture for the industrial production of Negroamaro wine (De Benedictis et al., 2011; Tristezza et al., 2012). The yeast strains were sub-cultured on YEPD (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose, 20 g/L agar) and maintained at −80°C in glycerol 50% (Bleve et al., 2011). Screening of Killer-Sensitive pattern (killer, sensitive and neutral phenotypes) was carried out as described by Jacobs et al. (1988).

Microfermentations

Fermentation tests were carried out at 25°C in 500 mL flask containing 450 mL of Negroamaro grape must (205 g/L sugars, pH 3.44, assimilable nitrogen concentration 142.14 g/L) added with 20 mg/L of potassium metabisulphite. The must was clarified by centrifugation (10 min at 8000 g) and then

sterilized by membrane filtration through Millipore system (0.45 μm membrane). Each flask was inoculated with the required concentration of a yeast pre-culture in the same must (48 h at 25°C), as previously described (Grieco et al., 2011).

The *H. uvarum* strains were inoculated at 10^7 CFU/mL, while the *S. cerevisiae* strain ITEM6920 was inoculated, in a preliminary test, at three different concentrations: 10^7, 10^5, and 10^3 CFU/mL, in order to reach, respectively, the inoculation ratios *H. uvarum*:*S. cerevisiae* of 1:1, 100:1, and 10,000:1. Each *H. uvarum* strain was inoculated in combination with the *S. cerevisiae* strain in two different timings: simultaneous inoculum (SM) and sequential inoculum (SQ). In the case of SQ, *S. cerevisiae* was inoculated after *H. uvarum*, when alcohol content reach 5% v/v. Fermentation kinetics were monitored daily by gravimetric determinations until constant weight and then the samples were stored at −20°C until analysis. Each fermentation experiment was carried out in duplicate. A pure culture of the *S. cerevisiae* strain was also inoculated as positive control, as well as a non-inoculated must was used as negative control.

Pilot-Scale Vinification

The selected strains were tested in pilot-scale fermentation trials. The vinification was carried out in an experimental cellar using sterile stainless steel 100-L vessels (Grieco et al., 2011) by inoculating 90 L of Negroamaro must (240 g/L of total sugars, 232 mg/L of yeast assimilable nitrogen, pH 3.52, added with 20 g/hL of potassium metabisulphite) with 10^7 cell/mL of *H. uvarum* and 10^5 cell/mL of *S. cerevisiae*, both in simultaneous and sequential approach. *S. cerevisiae* inoculated alone was used as control. The kinetics of the alcoholic fermentation process was monitored daily measuring the density. Samples of must and wines were collected as single replicate and stored at −20°C for further analyses.

Industrial Vinification

Industrial fermentation was carried out in a 100,000 L stainless steel vessel. To start must fermentation on large scale, the initial inocula were prepared, transported to the winery and used as starters (Tristezza et al., 2012). The mixed starters cultures of *Hanseniaspora uvarum* strains ITEM8795 and *S. cerevisiae* ITEM6920, respectively corresponding to 7×10^{12} CFU/hL and 7×10^{10} CFU/hL, were mixed with 300 kg of and let for 6 h at room temperature. After this period, the yeast-must mixture was added to 7 tons of Negroamaro must (212.8 g/L of total sugars, pH 3.33, yeast assimilable nitrogen 158.8 g/L, added with 20 g/hL of potassium metabisulphite). The alcoholic fermentation process was carried out at 25°C and its kinetics was daily monitored by measuring the reducing sugars concentration and density. Samples of must and wines were collected as single replicate for further chemical and microbiological analysis.

Differential Enumeration of Yeast Populations

In order to determine microbial growth, must and wine samples were collected over the fermentation processes. Serial dilutions of each sample were spread on WL Nutrient Agar (WLN medium, Oxoid, UK) and Lysine Agar (LA medium, Oxoid, UK). LA medium was used for the enumeration of non-*Saccharomyces* yeast population while WLN was used for differential enumeration of total yeast population (Pallmann et al., 2001). The identification of *H. uvarum* and *S. cerevisiae* was carried out by performing a molecular assay. Yeast colonies, showing a typical phenotype, were selected from WLN plates, and their genomic DNA was extracted according to Tristezza et al. (2009). RAPD pattern of *H. uvarum* were performed according to De Benedictis et al. (2011), while interdelta profiles of *S. cerevisiae* were analyzed as described by Tristezza et al. (2012).

Analytical Determinations

The main chemical parameters of wines and musts were analyzed by Fourier Transform Infrared Spectroscopy (FTIR), employing the WineScan Flex (FOSS Analytical, DK). Samples were centrifuged at 8000 rpm for 10 min and then analyzed following the supplier's instructions. Acetaldehyde, ethyl acetate and acetoin were determined by gas-chromatography according to Mallouchos et al. (2003). The internal standard solution used was 4-methyl-1-pentanol. Free volatile compounds were extracted by solid phase extraction method (SPE) and analyzed by gas chromatography–mass spectrometry (GC–MS) as previously described (Tufariello et al., 2012). The Odor Activity Values (OAVs) were calculated according to Capone et al. (2013). To evaluate the contribution of a volatile compound to the aroma, the Odor Activity Value (OAV) was calculated as the ratio between the concentration of each compound and the perception threshold in a specified matrix reported in literature (Swiegers et al., 2005). An aromatic series was defined as a group of volatile compounds with similar aroma descriptors (i.e., floral, sweet, fruity, spicy, green, fatty). The value of each aromatic series was calculated as the sum of the OAVs of the compounds that integrate it. Fermentation rate (FR), fermentation purity (FP), and alcohol yield coefficient (AYC) were calculated according to Tristezza et al. (2012).

Statistical Treatment of Data

Statistical data processing was performed using the free software package PAST (Hammer et al., 2001).

RESULTS

Microfermentations

In a preliminary experiment we have studied the growth kinetics of the *H. uvarum/S. cerevisiae* mixed cultures (data not shown). The growth dynamics of the *H. uvarum* strains were comparable in the tests when the inoculum ratio were equivalent to 100:1 and 10.000:1, i.e., the *S. cerevisiae* starter inoculated at 10^5 and 10^3 CFU/mL, which reached a concentration of 10^7 CFU/mL respectively after 7 and 15 days. However, when the *S. cerevisiae* strain was inoculated at 10^7 CFU/mL (inoculum ratio of 1:1) the non-*Saccharomyces* cell concentration declined after 5 days (data not shown). For these reasons, the inoculum amount chosen for further experiments were 10^7 CFU/mL for *H. uvarum* strains and 10^5 CFU /mL for the *S. cerevisiae* starter (ratio 100:1). The fermentation kinetics of mixed cultures in micro-vinification

trials are reported as Supplementary Data (Supplementary Figure 1). Two different temporal approaches were tested: a simultaneous inoculation of the two species and a sequential inoculation with a delay of 2 days in the addition of *S. cerevisiae* after *H. uvarum*. The time courses of simultaneous trials were comparable to those of the *S. cerevisiae* pure culture. The sequential trials presented a decrement of the initial fermentation rate that was higher for ITEM8797 (H2) and ITEM8799 (H3). Nonetheless, all trials gave complete fermentations in 14 days.

The microbial population dynamics of the mixed fermentations are shown in **Figure 1**. In all simultaneous trials (**Figures 1A–C**), both strains reached a maximum population (around 10^8 CFU/mL) after 72 h. Viable counts of *S. cerevisiae* kept stable at 10^8 CFU/mL until the 10th day of fermentation. Then, in mixed fermentation with ITEM8795 (H1) (**Figure 1A**) and H3 (**Figure 1C**), cells counts slightly decrease at 10^7 CFU/mL. From the 3rd to the 5th day, all the three *H. uvarum* strains decreased in viable counts at 10^6 CFU/mL and kept stable until the 10th day. At the end of the fermentations, the number of viable cells of H2 was 10^5 CFU/mL, whereas for H1 and H3 it was up to 10^4 CFU/mL. In the three sequential trials (**Figures 1D–F**), *H. uvarum* reached a maximum (10^{10} CFU/mL) in 5 days and then decreased at 10^9 CFU/mL. By the end of the fermentations, viable counts were 10^5 CFU/mL for H1, 10^4 CFU/mL for H2 and 10^6 CFU/mL for H3. The strain of *S. cerevisiae* showed a similar trend in the three trials: reached a maximum (10^9 CFU/mL) 3 days after inoculation and kept constant until the end of the fermentations. The pure culture of *S. cerevisiae* ITEM6920 (S) used as control reached a maximum population (10^8 CFU/mL) in 3 days and kept constant until the 10th day post-inoculation. By the 14th day, viable counts were 10^7 CFU/mL. Moreover, the tests carried out to assess the killer toxin activity excluded any cross inhibition between the *H. uvarum* and *S. cerevisiae* strains under study (data not show).

The oenological parameters of the mixed fermentations and the pure culture are shown in **Table 1**. As expected considering the fermentation kinetics, all the trials finished the fermentation leaving in the must less than 3 g/L of residual sugars. The highest ethanol concentration was determined in the pure culture of *S. cerevisiae* while all the mixed fermentations reached a lower ethanol concentration ranging from 11.92 to 12.19 mL/100 mL. On the other hand, the production of glycerol was greater (6.15–6.33 g/L) in mixed fermentations than in the control (5.24 g/L). The activity of *H. uvarum* did not increase volatile acidity;

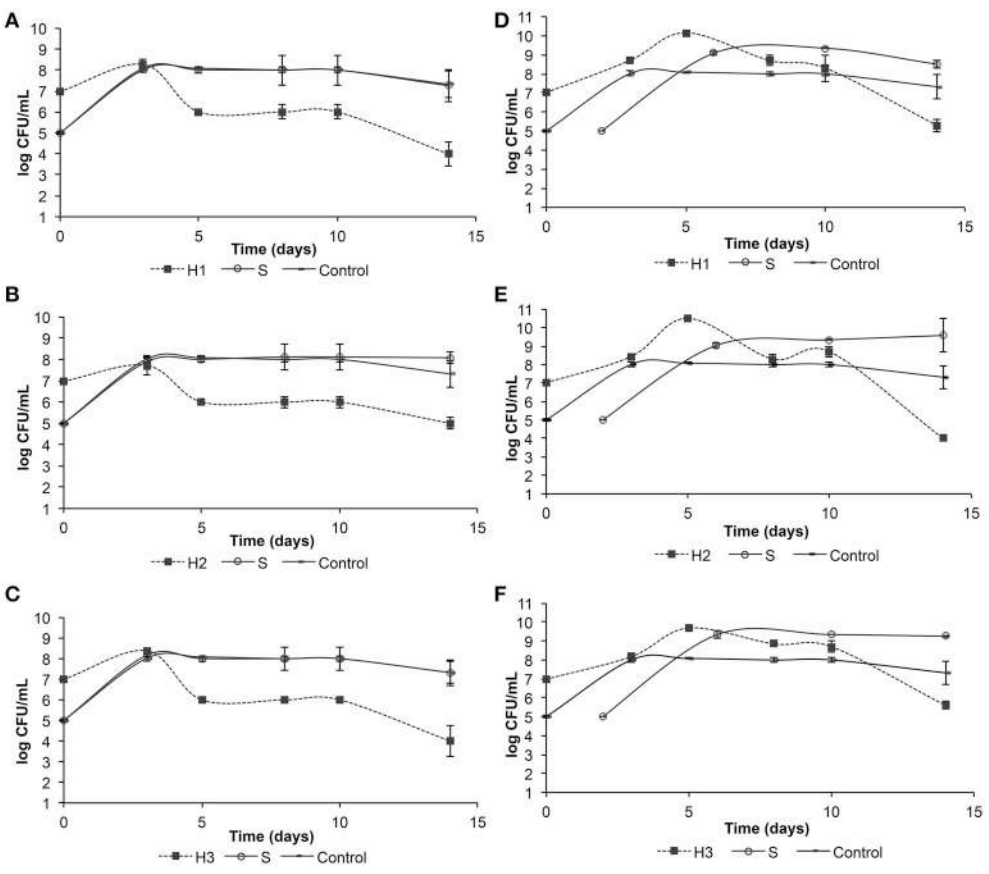

FIGURE 1 | Evolution of yeast populations in micro-vinification conditions with simultaneous inoculation (A, *H. uvarum* ITEM 8795 + *S. cerevisiae* ITEM 6920; B, *H. uvarum* ITEM 8797 + *S. cerevisiae* ITEM 6920; C, *H. uvarum* ITEM 8799 + *S. cerevisiae* ITEM 6920) and sequential inoculation (D, *H. uvarum* ITEM 8795 + *S. cerevisiae* ITEM 6920; E, *H. uvarum* ITEM 8797 + *S. cerevisiae* ITEM 6920; F, *H. uvarum* ITEM 8799 + *S. cerevisiae* ITEM 6920). Values are mean of two independent duplicates.

TABLE 1 | Concentration of major chemical compounds in fermented musts obtained with mixed cultures of *H. uvarum* /*S. cerevisiae* strains and with the pure culture of *S. cerevisiae* as control.

	Simultaneous			Sequential			Control
	H1+S	H2+S	H3+S	H1+S	H2+S	H3+S	S
Alcohol (mL/100 mL)	12.14 ± 0.114	12.05 ± 0.038	12.19 ± 0.104	11.98 ± 0.021	11.92 ± 0.028	11.96 ± 0.007	12.33 ± 0.007
Residual sugars (g/L)	2.09 ± 0.047	2.15 ± 0.153	2.19 ± 0.113	2.13 ± 0.092	2.25 ± 0.212	2.17 ± 0.099	2.18 ± 0.120
Total acidity (g/L)	6.30 ± 0.029	6.43 ± 0.087	6.46 ± 0.083	6.35 ± 0.064	6.52 ± 0.014	6.49 ± 0.028	6.34 ± 0.035
Volatile acidity (g/L)	0.34 ± 0.000	0.37 ± 0.008	0.34 ± 0.015	0.41 ± 0.014	0.40 ± 0.007	0.42 ± 0.014	0.41 ± 0.000
pH	3.34 ± 0.005	3.33 ± 0.014	3.33 ± 0.008	3.32 ± 0.007	3.31 ± 0.007	3.31 ± 0.000	3.29 ± 0.000
Tartaric acid (g/L)	1.87 ± 0.029	1.80 ± 0.068	1.92 ± 0.143	1.63 ± 0.028	1.66 ± 0.042	2.11 ± 0.092	1.73 ± 0.078
Glycerol (g/L)	6.32 ± 0.151	6.21 ± 0.266	6.32 ± 0.243	6.15 ± 0.042	6.33 ± 0.085	6.22 ± 0.007	5.24 ± 0.078
Acetaldehyde (mg/L)	20.05 ± 0.451	19.96 ± 0.382	20.32 ± 0.297	21.4 ± 0.600	21.95 ± 0.190	22.41 ± 0.216	24.22 ± 0.164
Ethyl acetate (mg/L)	84.78 ± 0.753	96.57 ± 0.822	98.33 ± 1.254	104.22 ± 2.660	107.53 ± 3.918	106.88 ± 2.674	44.53 ± 0.980
Acetoin (mg/L)	11.24 ± 1.045	12.33 ± 1.562	12.89 ± 1.664	12.77 ± 1.331	13.05 ± 1.258	12.87 ± 1.744	4.25 ± 0.563

Values are the mean of two injections of each replicate; the standard deviation values (±) are indicated; n.d. not detectable.

in fact the co-inoculated trials had a volatile acidity concentration statistically lower than the control. Fermentation purity (ratio between volatile acidity and ethanol produced) were also very low (0.03) for all samples, highlighting the good oenological performance of these mixed starters.

The capacity to produce a number of volatile compounds susceptible to be involved in the wine flavor formation (acetaldehyde, ethyl acetate and acetoin) was also assessed in mixed fermentations (**Table 1**). The acetaldehyde, one of the most important carbonyl compounds synthetized all through the alcoholic fermentation, was detected, within the range between 11.24 mg/L (H1+S) and 12.89 mg/L (H3+S) for simultaneous inoculation and within the range between 12.77 mg/L (H1+S) and 13.05 mg/L (H2+S) for sequential inoculation. The free acetaldehyde has a dual role in flavor formation; at moderate concentrations it contributes to fruity flavors, while high levels (>200 mg/L) suppress the aroma in wines. The ethyl acetate was identified in concentrations ranging from 84.78 mg/L (simultaneous inoculum with H1+S) to 107.53 mg/L (sequential inoculum with H2+S). Ethyl acetate may add pleasurable, fruity aroma to the general wine bouquet at low concentrations, whereas it appreciably affect the final aroma at a content higher than 150 mg/L (Lambrechts and Pretorius, 2000). The acetoin (3-hydroxy-2-butanone) odor threshold is relatively high (150 mg/L) and, consequently, its sensory meaning for the global aroma is nearly irrelevant. All the *H. uvarum* strains under study produced a low amount of the above compound, either in the simultaneous and in the sequential inoculum, within the range of 11.24 mg/L (simultaneous inoculum with H1+S) to 13.05 mg/L (sequential inoculum with H2+S).

To determine the effect of co-inoculums and sequential yeasts on the final composition of wine, experimental wines were analyzed by gas chromatography. The comparison of the results obtained is shown in **Figure 2**. Generally, simultaneous trials produced a higher amount of volatile compounds, esters, alcohols and terpenes. The co-inoculated couple H1+S presented the highest formation of esters (15.7 mg/L), alcohols (83 mg/L), and organic acids (20.4 mg/L). Also the co-inoculated couple H2+S presented high concentrations of alcohols (81.2 mg/L) and organic acids (19.4 mg/L) but lower amounts of esters (10.3 mg/L).

Analysis of these compounds provides a simply way of measuring the ability of different strains to produce wines with different profiles, since the main difference among wines inoculated with different yeast strains lies in the concentration of aromatic compounds rather than in the type of metabolite produced (Romano et al., 1997).

PCA was used to identify the specific volatile compounds best discriminating among the wines produced by co-inoculum (i.e., H1+S sm, H2+S sm, H3+S sm) and sequential (i.e., H1+S sq, H2+S sq, H3+S sq) techniques studied (**Figure 3**). PCA was initially applied to the concentrations of the volatile compounds in concentrations higher than their odor threshold are mainly considered as aroma-contributing substances (Gómez-Mínguez et al., 2007). The two principal components, PC1 and PC2, accounted for 68.16% of the total variance (43.53 and 24.63%, respectively). The second dimension (24.63% of explained variance) discriminates these two techniques studied, simultaneous (sm) and sequential (sq) inoculum.

However, samples H2+S sm, was associated to negative PC1(34% of explained variance), that discriminates H2+S sm and H1+S sm from the two other samples, Control S and H3+S sm for the high content, besides other variables, of ethyl octanoate, ethyl butanoate, terpens responsible of floral and fruity notes. The sample H1+S sm clustered at negative PC1 and PC2 scores thus showed relatively high correlations mainly with hexanoic and octanoic acids, phenylethylalcohol and isoamyl acetate. Samples H1+S sq and H2+S sq that cluster at positive PC1 scores scored high relative correlations with diethyl succinate, 2-methyl-propanoic acid, 2-methyl-1-propanol and 3-hydroxy-ethyl butanoate. Finally, H3+S sq that clusters at positive PC2 associated to 2,6-dimethyl-7-octen-2,6 diol. In conclusion, it was found that cultures in co-inoculum positively influenced the production of different classes of volatiles, terpenes, esters, acids and alcohols. In particular H1+S sm was characterized by a higher yield of most volatile

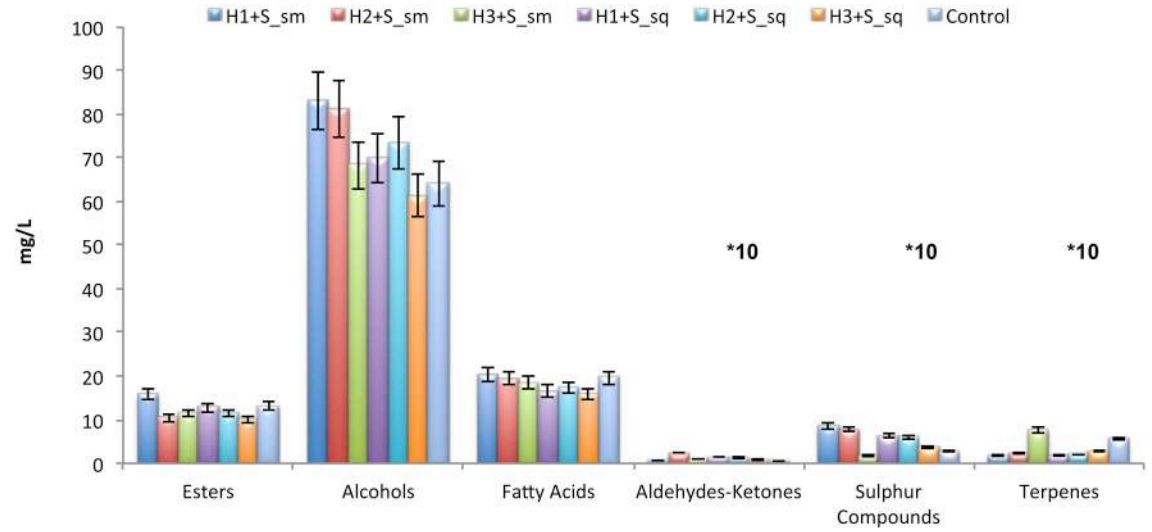

FIGURE 2 | Volatile composition of wines obtained in micro-vinification conditions with simultaneous (sm) and sequential (sq) inoculation. H1, *H. uvarum* ITEM 8795; H2, *H. uvarum* ITEM 8797; H3, *H. uvarum* ITEM 8799; S, *S. cerevisiae* ITEM 6920. The pure culture of *S. cerevisiae* ITEM 6920 (S) was used as control. The concentrations of the aldeydes-ketones, sulfur compounds and terpenes have been multiplied by a factor of 10. Error bars indicate standard deviation.

components that influence positively aroma bouquet, such as isoamyl acetate, ethyl octanoate, phenylethyl acetate (fruity notes), phenylethylalcohol (floral notes), hexanoic and octanoic acids.

Pilot-Scale Vinification

In reason of the performances in the micro-vinification trials, the *Hanseniaspora uvarum* strain ITEM8795 (H1) was selected to be tested in pilot-scale fermentations, both in simultaneous and sequential approaches, with *S. cerevisiae* ITEM6920. An identical amount (90 L) of the same Negroamaro must was inoculated with *S. cerevisiae* alone as control. Fermentation rate was higher for the two mixed starter fermentations than for that inoculated with the *S. cerevisiae* pure culture. Co-inoculation of *H. uvarum* and *S. cerevisiae* lead to a complete fermentation after 6 days (not shown). The three fermentations resulted in a different profile of sugars consumption (Supplementary Figure 2 in the Supplementary Data section). As can be observed, the simultaneous inoculation showed a good fermentation performance which led to a complete consumption of glucose in 4 days and fructose in 8 days. In addition, the sequential inoculation showed good fermentation properties with a complete consumption of glucose in 6 days and 5.7 g/L of residual fructose by the 12th day. On the contrary, the pure culture of *S. cerevisiae* showed a less efficient profile of sugar consumption with a complete consumption of glucose in the 8th day of fermentation and a residual fructose of 7.2 g/L at day 12. The development of yeast populations during the three fermentations is shown in Supplementary Figure 3 (Supplementary Data). The *H. uvarum* strain reached its maximum (10^8 CFU/mL) at the 2nd day, both in simultaneous and sequential trials; then, viable cells counts decreased at 10^3 CFU/mL (day 4th), subsequently at 10^2 CFU/mL, by the 6th day, and kept stable until the 11th day.

Viable cells counts of *S. cerevisiae* in simultaneous fermentation reached their maximum (10^8 CFU/mL) at the 2nd day and then slightly decreased at 10^7 CFU/mL until the end of fermentation (11th day). In sequential inoculation, *S. cerevisiae* reached a maximum population (10^9 CFU/mL) in 4 days and then gradually decreased. By the 11th day, viable counts were 10^7 CFU/mL. The pure culture of *S. cerevisiae* used as control showed a similar trend: reached a maximum (10^9 CFU/mL) 4 days after inoculation and constantly decreased to 10^7 CFU/mL until the end of the fermentations.

The analytical SPE/GC–MS method, used in this work for the analysis of wine samples, allowed the correct identification and quantification of 45 volatile compounds (**Table 2**). All the volatile compounds were grouped according to the belonging class (esters; aldehydes/ketons; alcohols; phenols; lactones; terpenes; acids). For each compound, the odor threshold (OTH) and the sensory odor descriptor were also reported. With respect to esters, it is important to highlight that wine produced by co-inoculation contained high concentrations of ethyl butyrate, isoamyl acetate, ethyl hexanoate responsible of fruity notes. On the contrary, concentrations of diethyl succinate, ethyl 9-decenoate, 2-phenylethyl acetate and diethyl malate were significantly lower in wines from co-inoculation assays. Ethyl esters are mainly synthesized by yeast starting from grape precursors and by ethanolysis of acylCoA that is formed during fatty acid synthesis or degradation.

Because alcohols are also important compounds influencing wine aroma, it is important to highlight that wine produced by co-inoculation contained higher 1-propanol, 1-butanol and isoamyl alcohols concentrations. Among identified alcohols, 2-phenylethanol was the second most abundant alcohol at concentrations higher than its threshold in all wines, contributing with fine rose's notes to wine aroma. In wines analyzed,

TABLE 2 | Concentration of major volatile compounds in fermented musts obtained with the mixed starter *H. uvarum/S. cerevisiae* used in sequential or simultaneous inoculum.

Volatile compounds	Odor threshold (μg/L)[a]	Odor descriptor	Odorant series[b]	H1+S Simultaneous	H1+S Sequential	S
				μg/L	μg/L	μg/L
ESTERS						
Ethyl butyrate	20 (a)	Fruity	1	425 ± 77	319 ± 84	386 ± 5
Isoamyl acetate	30(c)	Banana	1	2535 ± 1	2239 ± 140	2235 ± 49
Ethyl hexanoate	14 (b)		1	645 ± 118	561 ± 26	510 ± 17
Ethyl lactate	154,636 (c)	Acid. medicine	6	1028 ± 459	720 ± 104	1006 ± 32
Ethyl caprilate (octanoate)	5 (b)	Sweet. fruity	1.4	548 ± 111	573 ± 74	406 ± 42
3-hydroxy. ethyl butyrate	20,000 (b)	Caramel. Toasted	4	52 ± 34	65 ± 10	52 ± 2
Ethyl (decanoate) caprate	200 (c)	Sweet. fruity	1.4	219 ± 56	252 ± 71	188 ± 27
Diethyl succinate	200,000 (b)	Vinous	7	3735 ± 1820	4216 ± 1820	3851 ± 212
Ethyl 9 decenoate	14,100			200 ± 46	234 ± 88	102 ± 6
2-phenyl ethyl acetate	250 (a)	Floral	2	598 ± 93	696 ± 125	517 ± 65
Diethyl malate	760,000 (b)	Over-ripe. peach. cut grass	1	340 ± 164	525 ± 310	291 ± 31
4 hydroxy-3 methoxy benzoic acid ethyl ester (ethyl vanillate)	990 (b)	Sweet. vanillin	4.5	nd	4855 ± 21	Nd
Ethyl monosuccinate	1,000,000 (c)	Caramel. coffee	4	5648 ± 318	6052 ± 552	8476 ± 311
TOTAL				15,975 ± 3296	21,306 ± 3405	18,021 ± 799
CARBONYL COMPOUNDS						
Acetaldehyde	500 (a)	Pungent. ripe apple	1.6	269 ± 21	155 ± 65	125 ± 7
Acetoin	150,000			538 ± 192	nd	544 ± 26
Furfural	14,100 (c)			nd	nd	nd
Benzaldehyde	350 (c)	Sweet. fruity	1.4	94 ± 35	70 ± 6	58 ± 6
TOTAL				901 ± 248	224 ± 71	728 ± 38
ALCOHOLS						
1-propanol	830 (b)		1.6	312 ± 33	nd	211 ±17
Isobutanol	40,000 (b)		3.6	966 ± 566	701 ± 362	1427 ± 13
1-butanol	150,000 (b)	Medicinal. phenolic	6	109 ± 9	nd	178 ± 7
Isoamyl alcohol	30,000 (a)	Burnt. alcohol	4.6	14,785 ± 3772	13,968 ± 3525	15,754 ± 201
3-methyl-1-pentanol	50,000 (c)	Vinous. herbaceous. cacao	1.3.7	124 ± 43	118 ± 21	142 ± 7
1-hexanol	8000 (a)	Flower. green. cut grass	2.3	492 ± 196	491 ± 220	776 ± 22
(E)-3-hexen-1-ol				55 ± 31	79 ± 14	81 ± 5
(Z)-3-hexen-1-ol	400 (a)		3	66 ± 21	80 ± 2	56 ± 14
2.3-butanediol *(levo)*	15,0000 (b)	Fruity	1	2712 ± 1238	nd	1063 ± 48
2.3-butanediol *(meso)*		fruity		820 ± 79	nd	296 ± 30
Methionol	1000 (a)	Cooked vegetable	7	196 ± 82	203 ± 0	261 ± 8
Benzylalcohol	200,000 (b)	Sweet. fruity	1.4	190 ± 20	184 ± 30	179 ± 16
Phenylethylalcohol	10,000 (a)	Floral. roses	2	11,577 ± 2399	12,962 ± 3194	13,760 ± 1186
TOTAL				31,939 ± 8488	28,786 ± 7367	34,184 ± 1574
PHENOLS						
Guaiacol	10 (c)	Sweet. smoke	4.6	108 ± 22	nd	nd
Eugenol	6 (c)	Spices. clove. honey	4.5	nd	142 ± 62	42 ± 13
Ethyl phenol				nd	nd	nd
4 vinyl guaiacol	40 (a)	Spices. curry	5	363 ± 151	248 ± 54	218 ± 24
4 Hydroxy methyl acetophenone				nd	163 ± 42	nd
Siringol				299 ± 80		148 ± 0
TOTAL				770 ± 231	553 ± 158	408 ± 37
LACTONES						
Y-butyrolactone	35 (c)	Sweat. toasted	4	175 ± 116	96 ± 37	174 ± 10
Cis methyl 4 octanolide	67		4	nd	nd	89 ± 3
TOTAL				175 ± 116	96 ± 37	262 ± 13

(Continued)

TABLE 2 | Continued

Volatile compounds	Odor threshold (μg/L)[a]	Odor descriptor	Odorant series[b]	H1+S Simultaneous	H1+S Sequential	S
				μg/L	μg/L	μg/L
TERPENS						
Terpineol	110		2	73 ± 1	50 ± 0	30 ± 12
TOTAL						
ACIDS						
Isobutyric acid	2300 (b)	Rancid. butter. cheese	6	166 ± 138	93 ±31	212 ± 24
Butyric acid	173 (b)	Rancid. cheese. sweat	6	115 ± 50	83 ± 14	85 ± 3
(3 methyl butanoic) isovaleric acid	33 (c)	Sweet. acid	4.6	244 ± 58	269 ± 105	434 ± 10
Hexanoic acid	420 (b)	Sweet	6	2366 ± 96	2161 ± 67	2159 ± 115
Octanoic acid	500 (c)	Sweet. cheese	6	4716 ± 372	4372 ± 1098	3922 ± 149
Decanoic acid	1000 (b)	Rancid. fat	6	1178 ± 10	1344 ± 13	1278 ± 121
TOTAL				8785 ± 725	8322 ± 1328	8090 ± 422

The pure culture of S. cerevisiae was used as control.
Values expressed in μg/L are the mean of two injections. The standard deviation values (±) are indicated. n.d. not detectable.
[a]*(a) Guth (1997); (b) Etievant (1991); (c) Ferreira et al. (2000).*
[b]*Odorant series: 1 = Fruity; 2 = Floral; 3 = Green; 4 = Sweet; 5 = Spicy; 6 = Fatty; 7 = Others.*

we observed differences in α-terpineol concentration; in fact, this compound was identified and quantified in a major concentration in co-inoculated wine. Within the family of fatty acids, isobutyric, isovaleric, hexanoic, hexanoic, octanoic and decanoic acids were notable for their high concentrations in all wines and have been described with fruity, cheese, fatty, and rancid notes (Rocha et al., 2004).

The two mixed fermentations show an overall more complex aromatic profile than the pure culture of *S. cerevisiae*. Its sweet, spicy, floral odorant notes characterized the sequential mixed fermentation. Simultaneous fermentation of *H. uvarum* and *S. cerevisiae* was characterized by fruity and sweet aroma descriptors (**Table 2**).

Industrial Vinification

These large-scale experiments were conducted in a winery cellar of Salento by simultaneous inoculation, with the selected mixed starter *H. uvarum* ITEM8795/*S. cerevisiae* ITEM6920, of 7 tons of Negroamaro must. The data corresponding to the fermentation performance of the two isolates used and their ability to dominate the fermentation indicated that these two autochthonous yeast strains possess the fundamental properties required for starter cultures, in fact, the fermentations progressed regularly and sugar depletion was accomplished in 10 days.

Viable cells counts of the two yeast species throughout the fermentation are shown in **Figure 4**. *H. uvarum* dominated the early stages of fermentation and its population reached the maximum (10^9 CFU/mL) at the 2nd day; then gradually decreased to 10^4 CFU/mL and keep stable until the end of the fermentation period. *S. cerevisiae* dominate the fermentation from day 4th, when it reached a concentration of 10^9 CFU/mL; then slightly decreased to 10^6 CFU/mL and ultimate the fermentation by day 8.

The dominance of the inoculated strains was confirmed by molecular analysis. The electrophoresis patterns of green colonies isolated on WLN agar at middle fermentation stage are shown in **Figure 5A**. It can be observed that 9 out of 13 isolates have the same profile than that of the inoculated starter *H. uvarum* ITEM 8795 (H1), thus indicating that this strain got the upper hand of indigenous non-*Saccharomyces* strains. Likewise, the electrophoresis patterns of pale cream colonies isolated on WLN agar at the end of the fermentation are shown in **Figure 5B**. In this case, the 83% of isolates exhibit an identical profile to the one of the inoculated starter *S. cerevisiae* ITEM 6920, it being the evidence that the above starter was able to dominate the final steps of the AF. The results of chemical analysis of the wine obtained by co-fermentation *H. uvarum*/*S. cerevisiae* are shown in **Table 3**, in comparison to the same must fermented with the commercial starter in use in the winery. The total acidity was higher in must fermented by mixed starter (5.84 g/L), while volatile acidity was lower (0.43 g/L) than in must fermented with the commercial *S. cerevisiae* (5.49 and 0.45 g/L, respectively). Both starters were able to metabolize completely sugars. Furthermore, the mixed starter showed a lower alcohol content (13.99 mL/100 mL).

Comparation of Selected Volatile Compounds Concentration in Wine Produced in Lab-, Pilot-, and Industrial Scale

The influence of the mixed starter *H. uvarum*/*S. cerevisiae*, used to produce Negroamaro wine in laboratory-, pilot-, and industrial scale, on the organoleptic quality of wines was assessed by comparing the concentrations of specific volatile compounds, produced by yeast metabolism (**Table 4**). Each single analyzed compound, chosen between different esters, acids, alcohols,

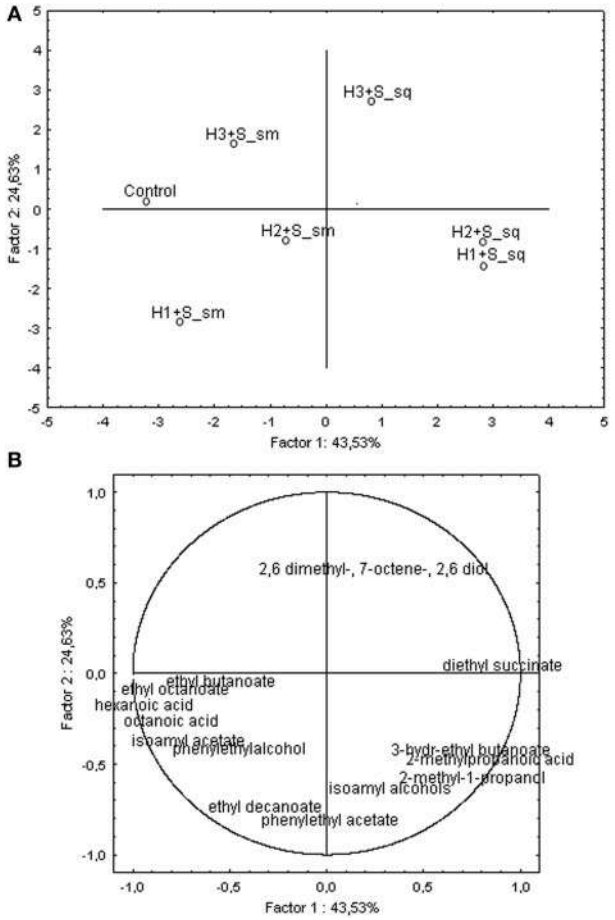

FIGURE 3 | Two-dimensional principal component analysis (PCA). Scores plot **(A)** for wines obtained in micro-vinification conditions and loading plot **(B)** for volatiles higher than odor threesold. Simultaneous (sm) and sequential (sq) inoculation. H1, *H. uvarum* ITEM 8795; H2, *H. uvarum* ITEM 8797; H3, *H. uvarum* ITEM 8799; S, *S. cerevisiae* ITEM 6920.

TABLE 3 | Analysis of final wine obtained by cofermentation of *H. uvarum* and *S. cerevisiae* in comparison to the same must fermented with the commercial starter in use in the industrial vinification.

Compound	Cofermentation *H. uvarum/S. cerevisiae*	Commercial starter
Alcohol (mL/100 mL)	13.99 ± 0.003	14.03 ± 0.01
Residual sugars (g/L)	n.d.	n.d.
Total acidity (g/L)	5.84 ± 0.067	5.49 ± 0.028
Volatile acidity (g/L)	0.43 ± 0.005	0.45 ± 0.003
pH	3.48 ± 0.009	3.44 ± 0.003
Malic acid (g/L)	1.1 ± 0.008	0.96 ± 0.005
Lactic acid (g/L)	0.18 ± 0.034	0.17 ± 0.023
Tartaric acid (g/L)	2.34 ± 0.105	1.89 ± 0.021
Citric acid (g/L)	0.45 ± 0.011	0.43 ± 0.02
Density (g/mL)	0.99093 ± 0.00003	0.99025 ± 0.000043
Dry matter (g/L)	22.79 ± 0.112	21.11 ± 0.111
Glycerol (g/L)	7.07 ± 0.014	7.01 ± 0.038
Methanol (mL/100 mL)	n.d.	n.d
Total polyphenols (mg/L)	547 ± 92	671 ± 25
Anthocyanins (mg/L)	410 ± 71	180 ± 22
Absorbance at 420	0.88 ± 0.001	0.81 ± 0.028
Absorbance at 520	0.97 ± 0.001	1.11 ± 0.031
Absorbance at 620	0.41 ± 0.001	0.23 ± 0.032

Values are the mean of three injections; the standard deviation values (±) are indicated; n.d. not detectable.

terpenes, and aldehydes, showed comparable concentration in the wines produced by the three vinifications.

When compared to the wines produced by using the *S. cerevisiae* as starter, the three wines produced by inoculation of the mixed starter showed an increment of acetate esters (ethyl acetate, isoamyl acetate, and phenylacetate) and fatty acids esters (ethyl hexanoate, ethyl octanoate, and ethyl decanoate). Esters is one of the large groups of volatiles found in wines. These compounds are important in young wine aroma and are among key compounds in the fruity flavors of wines (Rapp and Mandery, 1986). Ethyl acetate, in particular, adds complexity to the aroma of wine, with fruity notes at concentrations lower than 150 mg/L, while at higher concentrations it can donate a sour, vinegary off-odor. Its higher concentration was found in H1+S industrial scale (87.04 mg/L).

Regarding alcohols, in particular isoamylalcohols and 2-phenylethanol were determined in the analyzed wines and they resulted to be quantitatively the most representative compounds in this group, showing a higher concentrations of these molecules when compared to the wines produced by the *S. cerevisiae* starter. Isoamylalcohols can have both positive and negative impacts on wine aroma. In fact alcohols concentrations exceeding 400 mg/L can have a detrimental effect (Rapp and Versini, 1991; Romano et al., 1997), whereas lower concentrations impart positive fruity characters (Lambrechts and Pretorius, 2000; Saurina, 2010). In our sample the concentrations detected were below this threshold. However, 2-phenylethanol was the second most abundant alcohol at concentrations higher than its threshold (10 mg/L), contributing with fine rose's notes to wine aroma.

DISCUSSION

The utilization of non-*Saccharomyces* starters together with *Saccharomyces cerevisiae* in grape must fermentations has been investigated by Zironi and coworkers since 1993. The addition of yeasts belonging to non-*Saccharomyces* species as part of formulations of mixed starters, together with *S. cerevisiae*, has recently been indicated as a way to mimic the biotechnological potential associated with spontaneous fermentations to improve the quality of the wine (Rojas et al., 2001; Romano et al., 2003; Ciani et al., 2010).

Several non-*Saccharomyces* species, such as *H. uvarum*, *Zygosaccharomyces bailii, Lachancea thermotolerans*, *Candida cantarelli,* and *C. zemplinina* have been studied thus far in mixed fermentations with the scope of adding peculiar features to the wine (Toro and Vazquez, 2002; Ciani et al., 2006; Comitini et al., 2011; Suzzi et al., 2012; Gobbi et al., 2013; Garavaglia et al., 2015). In fact, a current trend in the wine market is to develop unique

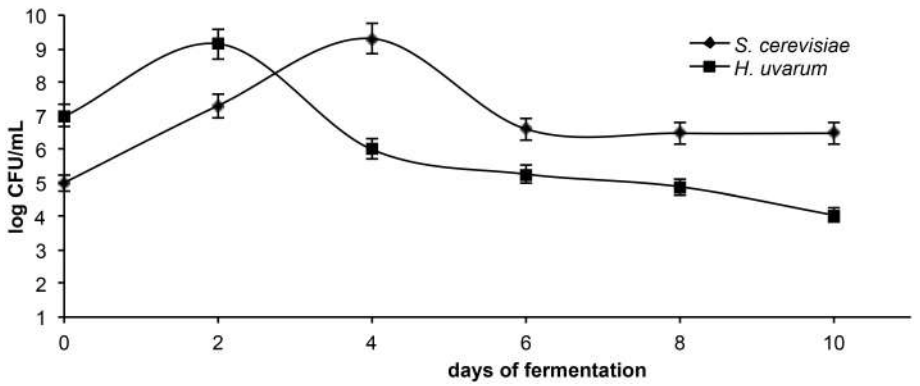

FIGURE 4 | Viable cell counts of the two inoculated yeast species throughout the industrial vinification.

TABLE 4 | Concentration of selected volatile compounds in wines obtained with the mixed starter *H. uvarum/S. cerevisiae* used in simultaneous inoculum.

	Lab-scale		Pilot-scale		Industrial-scale	
	Control	**H1+S**	**Control**	**H1+S**	**Control**	**H1+S**
ESTERS						
Isoamyl acetate	369.35	2330.00	2234.67	2239.18	312.05	2596.76
Ethyl hexanoate	434.51	510.00	510.49	560.85	433.73	547.57
Ethyl octanoate	371.69	604.91	406.42	573.12	476.00	661.15
3-Hydroxy-ethyl butanoate	53.79	69.78	52.23	65.35	51.41	67.74
Ethyl decanoate	183.24	230.00	188.19	252.18	234.87	229.38
Phenylethyl acetate	413.57	620.00	516.66	695.84	493.73	649.92
Ethyl acetate (mg/L)	42.11	84.78	22.07	92.04	25.05	87.04
ALCOHOLS						
Isoamyl alcohols	547.85	750.00	554.01	767.61	680.33	801.87
Phenylethylalcohol	11,480.03	11,994.45	13,760.43	12,962.32	10,555.51	11,716.07
ACIDS						
Hexanoic acid	2088.15	2246.68	2159.34	2366.45	2200.32	2246.68
Octanoic acid	3869.21	4574.65	3921.89	4716.22	3722.84	4574.65
TERPENS						
Terpineol	54.13	66.50	50.40	72.80	57.15	68.80
KETONS/ALDEHYDES						
Acetoin (mg/L)	4.11	11.24	7.65	11.85	6.05	11.34
Acetaldehyde (mg/L)	5.04	25.05	6.05	28.00	5.11	24.11

The pure culture of S. cerevisiae was used as control.

products, thus the mixed starter could be a good approach to give a special flavor and improve the quality of wines from both the organoleptic and microbiological point of view (Zironi et al., 1993; Mingorance-Cazorla et al., 2003; Capozzi et al., 2015; Lu et al., 2015; Masneuf-Pomarede et al., 2016). Moreover, in the contexst of the oenological production of Southern Italy (and other similar climates) denoted by high alcohol content and high total acidity, the preliminary utilization of a non-*Saccharomyces* starter (fructophylic and able to produce low amounts of acetic acid), might be an interesting approach in order to consume sugars in the early stage of fermentation, thus reducing the impact of osmotic stress for the *S. cerevisiae* starter (Rantsiou et al., 2012; Tofalo et al., 2012).

In the present investigation, we evaluated the fermentation performance of a culture of non-*Saccharomyces* yeasts belonging to the oenological species *H. uvarum* in micro-fermentation and, thereafter, in fermentations on pilot and industrial scale, conducted in mixed fermentations with yeasts belonging the species *S. cerevisiae*. These two different cultures were inoculated simultaneously or sequentially and the fermentation dynamics were studied in both fermentations. From the results of this series of tests, we obtained useful information on the kinetics of growth and fermentation activity, supported by analytical data of fermented musts and final wines.

In micro-fermentation trials, the presence of *S. cerevisiae* stimulated the persistence of the non-*Saccharomyces* strains

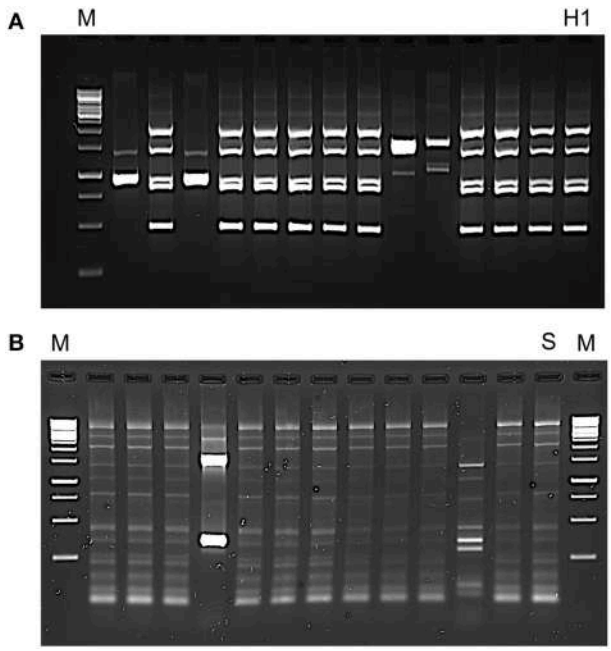

FIGURE 5 | Electrophoretic profiles patterns of (A) RAPD analysis with primer RM13 of *Hanseniaspora uvarum* randomly isolated from the large scale fermentation. The strain-specific profile of the 8795 strain is reported (H1); **(B)** interdelta region patterns obtained from *Saccharomyces cerevisiae* randomly isolated at the end of the large scale fermentation. The strain-specific profile for the 6920 strain is reported (S). Molecular marker (M): Thermo Scientific GeneRuler 1 Kb DNA Ladder.

during the fermentation process, in accordance with previous studies (Ciani et al., 2006; Mendoza et al., 2007; Mendoza and Farías, 2010), and this effect was more relevant in the sequential fermentations. Indeed, the three *H. uvarum* strains stayed viable, at significant high concentration levels of about 10^4–10^6 CFU/mL until the end of the fermentation even with an alcohol content of about 12% (v/v). On the other hand, in the simultaneous inoculation, the presence of the non-*Saccharomyces* strains since the early stages of fermentation seems to affect the cell growth and biomass production of *S. cerevisiae* probably due to the competition for nutrients (Mendoza et al., 2007; Domizio et al., 2011; Suzzi et al., 2012). However, the interactions between the two species during grape must/wine fermentation should be further studied and deepened. In fact, the knowledge about the metabolic interactions between *S. cerevisiae* and non-*Saccharomyces* strains in winemaking is still limited (Wang et al., 2015). Nevertheless, the fermentation rates of the mixed fermentation were comparable to that of the *S. cerevisiae* pure culture. Regardless the biomass production or fermentation rates, all the mixed cultures reached the completion but produced lower concentrations of ethanol than the pure culture of *S. cerevisiae* in accordance with previous studies (Mendoza and Farías, 2010; Mendoza et al., 2011).

The fermentations on a laboratory scale carried on regularly and the analysis of the corresponding fermented musts have not revealed the presence of compounds with possible negative impact to a level that will exceed the threshold of sensory perception. On the contrary, wines obtained with the association *H. uvarum*/*S. cerevisiae* showed some interesting characters. In fact, the evidence obtained during this investigation confirm previous data indicating that the combination and the interaction between the starter cultures of *S. cerevisiae* and non-*Saccharomyces* species has led to a reduction of acetic acid, even at concentrations lower than those produced by the pure culture of *S. cerevisiae* (Ciani et al., 2006; Mendoza and Farías, 2010; Domizio et al., 2011).

Several studies on the use of associated *S. cerevisiae* and non-*Saccharomyces* yeasts have highlighted many of the positive effects produced in these mixed fermentations such as the increasing in isoamyl acetate and 2-phenyl acetate (Moreira et al., 2008; Andorrà et al., 2010) or glycerol (Ciani and Ferraro, 1996) content in wine. Indeed, in the trial H1+S_sm, it was possible to note an increase of glycerol as well as of some volatile compounds, such as esters and aliphatic higher alcohols, as previously reported (Garde-Cerdán and Ancín-Azpilicueta, 2006). However, the impact of glycerol on the wine quality is still under discussion (Marchal et al., 2011).

These results were further confirmed in a pilot-scale vinification using a *H. uvarum* strain (ITEM 8795) in combination with *S. cerevisiae* ITEM 6920. The wines produced using two different strategies of inoculation (simultaneous and sequential) of the *H. uvarum*/*S. cerevisiae* starter were compared with that obtained after inoculation of a pure culture of *S. cerevisiae*, mainly focusing on their aromatic profile. It was also observed a different use of sugars in the tests in co-inoculation with *H. uvarum*. In fact, this fructophilic yeast interacts positively with the strain of *S. cerevisiae*, which is glucophilic, with the result of a more rapid utilization of the sugars (Ciani and Fatichenti, 1999). *H. uvarum* ITEM 8795, in simultaneous and sequential cultures, showed the maximal cell concentration after 2 days and then they die but remained in countable numbers until the end of the fermentation. This behavior of the apiculate yeast is in agreement with data reported in literature, which indicate that non-*Saccharomyces* yeasts dominate during the first 3–4 days of fermentations up to an ethanol concentration of about 4–7% (v/v) and then they start the phase of death (Fleet and Heard, 1993; Fleet, 2003). Moreover, it has been demonstrated that non-*Saccharomyces* yeasts kept their viability for longer period in composite cultures with *S. cerevisiae* (Ciani et al., 2006; Mendoza et al., 2007). The estimation of some of the principal volatile compounds confirmed that the *H. uvarum* ITEM 8975 did not form high amounts of ethyl acetate in mixed cultures (De Benedictis et al., 2011). However, in mixed cultures, the concentration of ethyl acetate produced are likely to contribute to the fruity notes and add to the general complexity to the produced wine (Ciani et al., 2006). The *H. uvarum* ITEM 8975 confirmed to be an acetoin low-producer even in multi-starter fermentations, it being this compound probably also consumed by the vigorously fermenting *S. cerevisiae* starter strain (Romano et al., 2003).

The amounts of acetaldehyde, a relevant secondary product of fermentation (Romano et al., 1997), did not appear to be negatively influenced by mixed cultures of *H. uvarum*, with a

behavior similar to that described by Ciani et al. (2006) during the studies of lab-scale *H. uvarum* multi-started fermentations.

Ethyl esters concentrations are influenced by yeast strain, fermentation temperature, aeration degree and sugar content. Both ethyl esters and acetate esters have a key importance in the whole wine aroma impressing a positive contribution by distinct sensory notes: sweet-fruity, grape-like odor, sweet-balsamic (Rapp, 1990; Swiegers and Pretorius, 2005). Indeed, wine yeasts such as *Hanseniaspora* spp. in mixed fermentations with *S. cerevisiae*, have improved the formation of esters with a positive sensorial impact, as well as the reduction of volatile acidity production (Rojas et al., 2003; Moreira et al., 2005; Medina et al., 2013). Chemical analysis of the wines produced using the mixed cultures *H. uvarum*/*S. cerevisiae* clearly differ from wine produced with the solo *S. cerevisiae*. Both mixed fermentations led to a higher content of esters such as 2-phenylethyl acetate, which is in agreement with previous studies conducted with *H. vineae* (Viana et al., 2011; Medina et al., 2013) and *H. guilliermondii* (Rojas et al., 2003; Moreira et al., 2011). This compound contributes to the rose, honey, fruity and flower aromas of wines (Swiegers et al., 2005). Likewise, 2-phenylethanol contributes with a floral (rose) aroma in the final wine (Swiegers et al., 2005) though, an excess in higher alcohols concentrations in wine would bring a strong, pungent smell and taste (Moreira et al., 2011). In our study, the use of the apiculate yeast *H. uvarum* in mixed starter culture with *S. cerevisiae* decreased the total higher alcohol content and resulted in a concentration of 2-phenylethyl alcohol just above its sensory threshold (Moreira et al., 2008; Medina et al., 2013). Mixed fermentations also resulted in decreases in isovaleric acid and increases in hexanoic, octanoic acid and ethyl octanoate. Moreover, the presence of higher levels of decanoic acid and ethyl decanoate was correlated with greater rates of cell lysis, which could contribute to the tropical fruit aroma, texture and body of wines (Medina et al., 2013). On the basis of the above findings, we can say that co-inoculation represents an alternative approach in commercial winemaking and its success strongly depends on the selection of suitable yeast strains. In this study carried out at industrial level, the use of selected yeasts provides good results in terms of lack of wine alterations. The scale-up of mixed fermentation, for the first time, to an industrial level was the key step to validate the results obtained in the laboratory and in pilot-scale. The winemaking process has largely confirmed both the evolution of the cultures inoculated and the analytical characteristics of wines given by the strains of *H. uvarum* and *S. cerevisiae* used for the fermentation. The results obtained were supported by the fact that both inoculated strains were dominant on indigenous microflora and, thus, they have certainly conducted the fermentative process. The data achieved during the present investigation confirmed the concept that oenological non-*Saccharomyces* yeasts represent a resource of great value for the winemaking industry. Indeed, the obtained results indicated the *H. uvarum* strain ITEM 8795 can be used in association with *S. cerevisiae* starter cultures in the in the winemaking conditions typical of Southern Italy (Puglia) wine production. The here-described multi-starter fermentation was able to enhance the quality, improve the aromatic profile and reduce the effect of the undesired characters of the final Negroamaro wine.

AUTHOR CONTRIBUTIONS

All authors significantly contributed to this paper. FG and GS conceived and designed the experiments; MTr, MTu, and VC performed the experiments; FG, GS, MTr, MTu, GM, and VC analyzed the data; FG was responsible for manuscript preparation and submission; FG, GS, MTr, MTu, GM, and VC reviewed the paper.

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SUPPLEMENTARY MATERIAL

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REFERENCES

Andorrà, I., Berradre, M., Rozès, N., Mas, A., Guillamón, J. M., and Esteve-Zarzoso, B. (2010). Effect of pure and mixed cultures of the main wine yeast species on grape must fermentations. *Eur. Food Res. Technol.* 231, 215–224. doi: 10.1007/s00217-010-1272-0

Bleve, G., Lezzi, C., Chiriatti, M. A., D'Ostuni, I., Tristezza, M., Di Venere, D., et al. (2011). Selection of non-conventional yeasts and their use in immobilized form for the bioremediation of olive oil mill wastewaters. *Bioresour. Technol.* 102, 982–989. doi: 10.1016/j.biortech.2010.09.059

Capone, S., Tufariello, M., and Siciliano, P. (2013). Analytical characterization of Negroamaro red wines by "Aroma Wheels." *Food Chem.* 141, 2906–2915. doi: 10.1016/j.foodchem.2013.05.105

Capozzi, V., Garofalo, C., Chiriatti, M. A., Grieco, F., and Spano, G. (2015). Microbial terroir and food innovation: the case of yeast biodiversity in wine. *Microbiol. Res.* 181, 75–83. doi: 10.1016/j.micres.2015.10.005

Capozzi, V., and Spano, G. (2011). Food microbial biodiversity and "Microbes of Protected Origin." *Front. Microbiol.* 2:237. doi: 10.3389/fmicb.2011.00237

Caridi, A., and Ramondino, D. (1999). Biodiversità fenotipica in ceppi di *Hanseniaspora* di origine enologica. *Enotecnico* 45, 71–74.

Ciani, M., Beco, L., and Comitini, F. (2006). Fermentation behavior and metabolic interactions of multistarter wine yeast fermentations. *Int. J. Food Microbiol.* 108, 239–245. doi: 10.1016/j.ijfoodmicro.2005.11.012

Ciani, M., Comitini, F., Mannazzu, I., and Domizio, P. (2010). Controlled mixed culture fermentation: a new perspective on the use of non-*Saccharomyces*

yeasts in winemaking. *FEMS Yeast Res.* 10, 123–133. doi: 10.1111/j.1567-1364.2009.00579.x

Ciani, M., and Fatichenti, F. (1999). Selective sugar consumption by apiculate yeasts. *Lett. Appl. Microbiol.* 28, 203–206. doi: 10.1046/j.1365-2672.1999.00505.x

Ciani, M., and Ferraro, L. (1996). Enhanced glycerol content in wines made with immobilized *Candida stellata* cells. *Appl. Environ. Microbiol.* 62, 128–132.

Ciani, M., and Maccarelli, F. (1998), Enological properties of non-*Saccharomyces* yeasts associated with wine-making. *World J. Microbiol. Biotechnol.* 14, 199–203. doi: 10.1023/A:1008825928354

Comitini, F., Gobbi, M., Domizio, P., Romani, C., Lencioni, L., Mannazzu, I., et al. (2011). Selected non-*Saccharomyces* wine yeasts in controlled multistarter fermentations with *Saccharomyces cerevisiae*. *Food Microbiol.* 28, 873–882. doi: 10.1016/j.fm.2010.12.001

De Benedictis, M., Bleve, G., Grieco, F., Tristezza, M., Tufariello, M., and Grieco, F. (2011), An optimized procedure for the enological selection of non-*Saccharomyces* starter cultures. *Anton. Leeuw. Int. J. G.* 99, 189–200. doi: 10.1007/s10482-010-9475-8

Domizio, P., Lencioni, L., Ciani, M., Di Blasi, S., Pontremolesi, C., and Sabatelli, M. P. (2007). Spontaneous and inoculated yeast population dynamics and their effect on organoleptic characters of Vinsanto wine under different process conditions. *Int. J. Food Microbiol.* 115, 281–289. doi: 10.1016/j.ijfoodmicro.2006.10.052

Domizio, P., Romani, C., Lencioni, L., Comitini, F., Gobbi, M., Mannazzu, I., et al. (2011). Outlining a future for non-*Saccharomyces* yeasts: selection of putative spoilage wine strains to be used in association with *Saccharomyces cerevisiae* for grape juice fermentation. *Int. J. Food Microbiol.* 147, 170–180. doi: 10.1016/j.ijfoodmicro.2011.03.020

Erten, H. (2002). Relations between elevated temperatures and fermentation behaviour of *Kloeckera apiculata* and *Saccharomyces cerevisiae* associated with winemaking in mixed cultures. *World J. Microbiol. Biotechnol.* 18, 373–378. doi: 10.1023/A:1015221406411

Etievant, P. X. (1991). "Wine," in *Volatile Compounds of Food and Beverages*, ed H. Maarse (New York, NY: Dekker), 483–546.

Ferreira, V., Lopez, R., and Cacho, J. F. (2000). Quantitative determination of the odorants of young red wines from different grape varieties. *J. Sci. Food Agric.* 80, 1659–1667. doi: 10.1002/1097-0010(20000901)80:11<1659::AID-JSFA693>3.0.CO;2-6

Fleet, G. H. (2003). Yeast interactions and wine flavor. *Int. J. Food Microbiol.* 86, 11–22. doi: 10.1016/S0168-1605(03)00245-9

Fleet, G. H. (2008). Wine yeast for the future. *FEMS Yeast Res.* 8, 979–995. doi: 10.1111/j.1567-1364.2008.00427.x

Fleet, G. H., and Heard, G. M. (1993). "Yeasts: growth during fermentation" in *Wine Microbiology and Biotechnology*, ed G. H. Fleet (Philadelphia, PA: Harwood), 27–55.

Garavaglia, J., de Souza Schneider, R. D. C., Mendes, S. D. C., Welke, J. E., Zini, C. A., Caramão, E. B., et al. (2015). Evaluation of *Zygosaccharomyces bailii* BCV 08 as a co-starter in wine fermentation for the improvement of ethyl esters production. *Microbiol. Res.* 173, 59–65. doi: 10.1016/j.micres.2015.02.002

Garde-Cerdán, T., and Ancín-Azpilicueta, C. (2006). Contribution of wild yeasts to the formation of volatile compounds in inoculated wine fermentations. *Eur. Food Res. Technol.* 222, 15–25. doi: 10.1007/s00217-005-0029-7

Garofalo, C., El Khoury, M., Lucas, P., Bely, M., Russo, P., Spano, G., et al. (2015). Autochthonous starter cultures and indigenous grape variety for regional wine production. *J. Appl. Microbiol.* 118, 1395–1408. doi: 10.1111/jam.12789

Gil, M., Cabellos, J. M., Arroyo, T., and Prodanov, M. (2006). Characterization of the volatile fraction of young wines from the Denomination of Origin "Vinos de Madrid" (Spain). *Anal. Chim. Acta* 563, 145–153. doi: 10.1016/j.aca.2005.11.060

Gobbi, M., Comitini, F., Domizio, P., Romani, C., Lencioni, L., Mannazzu, I., et al. (2013). *Lachancea thermotolerans* and *Saccharomyces cerevisiae* in simultaneous and sequential co-fermentation: a strategy to enhance acidity and improve the overall quality of wine. *Food Microbiol.* 33, 271–281. doi: 10.1016/j.fm.2012.10.004

Gómez-Mínguez, M. J., Cacho, J. F., Ferreira, V., Vicario, I. M., and Heredia, F. J. (2007). Volatile components of Zalema white wines. *Food Chem.* 100, 1464–1473. doi: 10.1016/j.foodchem.2005.11.045

Grieco, F., Tristezza, M., Vetrano, C., Bleve, G., Panico, E., Grieco, F., et al. (2011). Exploitation of autochthonous micro-organism potential to enhance the quality of Apulian wines. *Ann. Microbiol.* 61, 67–73. doi: 10.1007/s13213-010-0091-7

Guth, H. (1997). Identification of character impact odorants of different white wine varieties. *J. Agric. Food Chem.* 45, 3022–3026. doi: 10.1021/jf9608433

Hammer, Ø., Harper, D. A. T., and Ryan, P. D. (2001). PAST: Paleontological Statistics Software Package for education and data analysis. *Palaeontol. Electron.* 4, 9.

Heard, G. M., and Fleet, G. H. (1985). Growth of natural yeast flora during the fermentation of inoculated wines. *Appl. Environ. Microbiol.* 50, 727–728.

Jacobs, C. J., Fourie, I., and van Vuuren, H. J. J. (1988). Occurrence and detection of killer yeasts on Chenin Blanc grapes and grape skins. *S. Afr. J. Enol. Vitic.* 9, 28–31.

Jolly, N. P., Augustyn, O. P. H., and Pretorius, I. S. (2006). The role and use of non-*Saccharomyces* yeasts in wine production. *S. Afr. J. Enol. Vitic.* 27, 15–39.

Lambrechts, M. G., and Pretorius, I. S. (2000). Yeast and its importance to wine aroma. *S. Afr. J. Enol. Vitic.* 21, 97–129.

Lu, Y., Huang, D., Lee, P. R., and Liu, S.-Q. (2015). Assessment of volatile and non-volatile compounds in durian wines fermented with four commercial non-Saccharomyces yeasts. *J. Sci. Food Agric.* 96, 1511–1521. doi: 10.1002/jsfa.7253

Mallouchos, A., Skandamis, P., Loukatos, P., Komaitis, M., Koutinas, A., and Kanellaki, M. (2003). Volatile compounds of wines produced by cells immobilized on grape skins. *J. Agric. Food Chem.* 51, 3060–3066. doi: 10.1021/jf026177p

Marchal, A., Marullo, P., Moine, V., and Dubourdieu, D. (2011). Influence of yeast macromolecules on sweetness in dry wines: role of the *Saccharomyces cerevisiae* protein Hsp12. *J. Agric. Food Chem.* 59, 2004–2010. doi: 10.1021/jf103710x

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

Medina, K., Boido, E., Fariña, L., Gioia, O., Gomez, M. E., Barquet, M., et al. (2013). Increased flavour diversity of Chardonnay wines by spontaneous fermentation and co-fermentation with *Hanseniaspora vineae*. *Food Chem.* 141, 2513–2521. doi: 10.1016/j.foodchem.2013.04.056

Mendoza, L., and Farías, M. E. (2010). Improvement of wine organoleptic characteristics by non-*Saccharomyces* yeasts. *Curr. Res. Technol. Ed. Top. Appl. Microbiol. Microb. Biotechnol.* 2, 908–919.

Mendoza, L. M., Manca de Nadra, M. C., and Farias, M. F. (2007). Kinetics and metabolic behavior of a composite culture of *Kloeckera apiculata* and *Saccharomyces cerevisiae* wine related strains. *Biotechnol. Lett.* 29, 1057–1063. doi: 10.1007/s10529-007-9355-0

Mendoza, L. M., Merín, M. G., Morata, V. I., and Farías, M. E. (2011). Characterization of wines produced by mixed culture of autochthonous yeasts and *Oenococcus oeni* from the northwest region of Argentina. *J. Ind. Microbiol. Biotechnol.* 38, 1777–1785. doi: 10.1007/s10295-011-0964-1

Mingorance-Cazorla, L., Clemente-Jiménez, J. M., Martınez-Rodrıguez, S., Las Heras-Vazquez, F. J., and Rodrıguez-Vico, F. (2003). Contribution of different natural yeasts to the aroma of two alcoholic beverages. *World J. Microbiol. Biotechnol.* 19, 297–304. doi: 10.1023/A:1023662409828

Moreira, N., Mendes, F., de Pinho, P. G., Hogg, T., and Vasconcelos, I. (2008). Heavy sulphur compounds, higher alcohols and esters production profile of *Hanseniaspora uvarum* and *Hanseniaspora guilliermondii* grown as pure and mixed cultures in grape must. *Int. J. Food Microbiol.* 124, 231–238. doi: 10.1016/j.ijfoodmicro.2008.03.025

Moreira, N., Mendes, F., Hogg, T., and Vasconcelos, I. (2005). Alcohols, esters and heavy sulphur compounds production by pure and mixed cultures of apiculate wine yeasts. *Int. J. Food Microbiol.* 103, 285–294. doi: 10.1016/j.ijfoodmicro.2004.12.029

Moreira, N., Pina, C., Mendes, F., Couto, J. A., Hogg, T., and Vasconcelos, I. (2011). Volatile compounds contribution of *Hanseniaspora guilliermondii* and *Hanseniaspora uvarum* during red wine vinifications. *Food Control.* 22, 662–667. doi: 10.1016/j.foodcont.2010.07.025

Pallmann, C., Brown, J., Olineka, T., Cocolin, L., Mills, D., and Bisson, L. (2001). Use of WL medium to profile native flora fermentations. *Am. J. Enol. Vit.* 52, 198–203.

Pérez-Coello, M. S., Briones Pérez, A. I., Ubeda Iranzo, J. F., and Martin Alvarez, P. J. (1999). Characteristics of wines fermented with different *Saccharomyces*

cerevisiae strains isolated from the La Mancha region. *Food Microbiol.* 16, 563–573. doi: 10.1006/fmic.1999.0272

Rantsiou, K., Dolci, P., Giacosa, S., Torchio, F., Tofalo, R., Torriani, S., et al. (2012). *Candida zemplinina* can reduce acetic acid produced by *Saccharomyces cerevisiae* in sweet wine fermentations. *Appl. Environ. Microbiol.* 78, 1987–1994. doi: 10.1128/AEM.06768-11

Rapp, A. (1990). Natural flavours of wine: correlation between instrumental analysis and sensory perception. *J. Anal. Chem.* 337, 777–785. doi: 10.1007/BF00322252

Rapp, A., and Mandery, H. (1986). Wine aroma. *Experientia* 42, 873–883. doi: 10.1007/BF01941764

Rapp, A., and Versini, G. (1991). "Influence of nitrogen compounds in grapes on aroma compounds in wine," in *Proceedings of the International Symposium on Nitrogen in Grapes and Wine, Seattle, USA* (Davis, CA: American Society of Enology and Viticulture), 156–164.

Rocha, S. M., Rodrigues, F., Coutinho, P., Delgadillo, I., and Coimbra, M. A. (2004). Volatile composition of Baga red wine assessment of the identification of the would-be impact odourants. *Anal. Chim. Acta* 513, 257–262. doi: 10.1016/j.aca.2003.10.009

Rojas, V., Gil, J. V., Pinaga, F., and Manzanares, P. (2001). Studies on acetate ester production by non- *Saccharomyces* wine yeasts. *Int. J. Food Microbiol.* 70, 283–289. doi: 10.1016/S0168-1605(01)00552-9

Rojas, V., Gil, J. V., Piñaga, F., and Manzanares, P. (2003). Acetate ester formation in wine by mixed cultures in laboratories fermentations. *Int. J. Food Microbiol.* 86, 181–188. doi: 10.1016/S0168-1605(03)00255-1

Romano, P., Fiore, C., Paraggio, M., Caruso, M., and Capece, A. (2003). Function of yeast species and strains in wine flavour. *Int. J. Food Microbiol.* 86, 169–180. doi: 10.1016/S0168-1605(03)00290-3

Romano, P., Suzzi, G., Comi, G., and Zironi, R. (1992). Higher alcohol and acetic acid production by apiculate wine yeasts. *J. Appl. Bacteriol.* 73, 126–130. doi: 10.1111/j.1365-2672.1992.tb01698.x

Romano, P., Suzzi, G., Comi, G., Zironi, R., and Maifreni, M. (1997). Glycerol and other fermentation products of apiculate wine yeasts. *J. Appl. Microbiol.* 82, 615–618. doi: 10.1111/j.1365-2672.1997.tb02870.x

Saurina, J. (2010). Characterization of wines using compositional profiles and chemometric. *Trends Anal. Chem.* 29, 234–245. doi: 10.1016/j.trac.2009.11.008

Spano, G., Russo, P., Lonvaud-Funel, A., Lucas, P., Alexandre, H., Grandvalet, C., et al. (2010). Biogenic amines in fermented foods. *Eur. J. Clin. Nutr.* 64(suppl. 3), S95–S100. doi: 10.1038/ejcn.2010.218

Suárez-Lepe, J. A., and Morata, A. (2012). New trends in yeast selection for winemeking. *Trends Food Sci. Technol.* 23, 39–50. doi: 10.1016/j.tifs.2011.08.005

Suzzi, G., Schirone, M., Sergi, M., Marianella, R. M., and Fasoli, G., Aguzzi, et al. (2012). Multistarter from organic viticulture for red wine Montepulciano d'Abruzzo production. *Front. Microbiol* 3:135. doi: 10.3389/fmicb.2012.00135

Swiegers, J. H., Bartowsky, E. J., Henschke, P. A., and Pretorius, I. S. (2005). Yeast and bacterial modulation of wine aroma and flavour. *Aust. J. Grape Wine Res.* 11, 139–173. doi: 10.1111/j.1755-0238.2005.tb00285.x

Swiegers, J. H., and Pretorius, I. S. (2005). Yeast modulation of wine flavor. *Adv. Appl. Microbiol.* 57, 131–175. doi: 10.1016/S0065-2164(05)57005-9

Tofalo, R., Schirone, M., Torriani, S., Rantsiou, K., Cocolin, L., Perpetuini, G., et al. (2012). Diversity of *Candida zemplinina* strains from grapes and Italian wines. *Food Microbiol.* 29, 18 –26. doi: 10.1016/j.fm.2011.08.014

Toro, M. E., and Vazquez, F. (2002). Fermentation behaviour of controlled mixed and sequential cultures of *Candida cantarellii* and *Saccharomyces cerevisiae* wine yeasts. *World J. Microbiol. Biotechnol.* 18, 347–354. doi: 10.1023/A:1015242818473

Tristezza, M., Gerardi, C., Logrieco, A., and Grieco, F. (2009). An optimized protocol for the production of interdelta markers in *Saccharomyces cerevisiae* by using capillary electrophoresis. *J. Microbiol. Meth.* 78, 286–291. doi: 10.1016/j.mimet.2009.06.012

Tristezza, M., Vetrano, C., Bleve, G., Grieco, F., Tufariello, M., Quarta, A., et al. (2012). Autochthonous fermentation starters for the industrial production of Negroamaro wines. *J. Ind. Microbiol. Biotechnol.* 39, 81–92. doi: 10.1007/s10295-011-1002-z

Tristezza, M., Vetrano, C., Bleve, G., Spano, G., Capozzi, V., Logrieco, A., et al. (2013). Biodiversity and safety aspects of yeast strains characterized from vineyards and spontaneous fermentations in the Apulia Region, Italy. *Food Microbiol.* 36, 335–342. doi: 10.1016/j.fm.2013.07.001

Tufariello, M., Capone, S., and Siciliano, P. (2012). Volatile components of Negroamaro red wines produced in Apulian Salento area. *Food Chem.* 132, 2155–2164. doi: 10.1016/j.foodchem.2011.11.122

Viana, F., Belloch, C., Vallés, S., and Manzanares, P. (2011). Monitoring a mixed starter of *Hanseniaspora vineae–Saccharomyces cerevisiae* in natural must: impact on 2-phenylethyl acetate production. *Int. J. Food Microbiol.* 151, 235–240. doi: 10.1016/j.ijfoodmicro.2011.09.005

Viana, F., Gil, J. V., Genovés, S., Vallés, S., and Manzanares, P. (2008). Rational selection of non-*Saccharomyces* wine yeast for mixed starters based on ester formation and enological traits. *Food Microbiol.* 25, 778–785. doi: 10.1016/j.fm.2008.04.015

Wang, C., Mas, A., and Esteve-Zarzoso, B. (2015). Interaction between *Hanseniaspora uvarum* and *Saccharomyces cerevisiae* during alcoholic fermentation. *Int. J. Food Microbiol.* 206, 67–74. doi: 10.1016/j.ijfoodmicro.2015.04.022

Zironi, R., Romano, P., Suzzi, G., Battistuta, F., and Comi, G. (1993). Volatile metabolites produced in wine by mixed and sequential cultures of *Hanseniaspora guilliermondii* or *Kloeckera apiculata* and *Saccharomyces cerevisiae*. *Biotechnol. Lett.* 15, 235–238. doi: 10.1007/BF00128311

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frontiers in Microbiology

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Aroma Profile of Montepulciano d'Abruzzo Wine Fermented by Single and Co-culture Starters of Autochthonous *Saccharomyces* and Non-*saccharomyces* Yeasts

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Montepulciano d'Abruzzo is a native grape variety of *Vitis vinifera* L., grown in central Italy and used for production of high quality red wines. Limited studies have been carried out to improve its enological characteristics through the use of indigenous strains of *Saccharomyces cerevisiae*. The main objective of the present work was to test two indigenous strains of *S. cerevisiae* (SRS1, RT73), a strain of *Starmerella bacillaris* (STS12), one of *Hanseniaspora uvarum* (STS45) and a co-culture of *S. cerevisiae* (SRS1) and *S. bacillaris* (STS12), in an experimental cellar to evaluate their role in the sensory characteristic of Montepulciano d'Abruzzo wine. A *S. cerevisiae* commercial strain was used. Fermentations were conducted under routine Montepulciano d'Abruzzo wine production, in which the main variables were the yeast strains used for fermentation. Basic winemaking parameters, some key chemical analysis and aroma compounds were considered. *S. cerevisiae* strain dynamics during fermentation were determined by molecular methods. The musts inoculated with the co-culture were characterized by a faster fermentation start and a higher content of glycerol after 3 days of fermentation, as well as the musts added with strains *S. bacillaris* (STS12) and *H. uvarum* (STS45). At the end of fermentation the parameters studied were quite similar in all the wines. Total biogenic amines (BA) content of all the wines was low. Ethanolamine was the predominant BA, with a concentration ranging from 21 to 24 mg/l. Wines were characterized by esters and alcohols. In particular, 2-phenylethanol, 3-methylbut-1-yl methanoate, and ethyl ethanoate were the major aroma volatile compounds in all wines. Statistical analysis highlighted the different role played by aroma compounds in the differentiation of wines, even if it was impossible to select a single class of compounds as the most important for a specific yeast. The present study represents a further step toward the use of tailored autochthonous strains to impart the specific characteristics of a given wine which are an expression of a specific terroir.

Keywords: aroma compounds, autochthonous yeast strains, *Saccharomyces cerevisiae*, non-*Saccharomyces*, Montepulciano d'Abruzzo wine

INTRODUCTION

Wine fermentations constitute complex microbial ecosystems consisting of highly dynamic yeast communities which play a key role in shaping wine quality (Fleet, 2003). This complex array of relations influences the nutritional, hygienic, and aromatic features of the product through the consecutive growth and death of different species and strains within each species, during the fermentation process (Fleet, 2003; Liu et al., 2015). Many studies have been focused on the nature of these relations improving the knowledge about ecology, physiology, biochemistry, and molecular biology of the microrganisms involved in wine fermentation process underlying the ecological complexity and variability of these fermentations that extend beyond the species level (for a review see Liu et al., 2015).

Yeasts mainly impact on the wine flavor producing a large array of volatile substances (Howell et al., 2006). In this context the existing commercial yeast strains present some limits, especially because they reduce the uniqueness of wine bouquet (Alves et al., 2015). In fact, different yeast species and even different genotypes of *Saccharomyces cerevisiae* produce different wine aroma profiles (Alves et al., 2015; Barbosa et al., 2015; Vernocchi et al., 2015). This awareness opened new issues to meet wine-maker demand for "special yeasts for special traits" (Schuller and Casal, 2005; Sadoudi et al., 2012). Recently, the role of indigenous yeast strains has gained importance, as a tool to impart regional characters to wines. Indeed, the use of a "microarea-specific" starter culture highlighted the association between the volatile profile of wine and the geographical origin of the yeast used for the fermentation process (Tufariello et al., 2014).

The role of non-*Saccharomyces* (NS) yeasts in winemaking has been re-evaluated, leading to a more complex "flavor phenotype" producing more than 1300 volatile compounds e.g., esters, higher alcohols, acids, and monoterpenes (Swiegers et al., 2005; for a review see Jolly et al., 2014). Moreira et al. (2005) and Medina et al. (2013) demonstrated that *Hanseniaspora uvarum* increased the quantity of some desirable compounds, such as higher alcohols and esters, while Rantsiou et al. (2012) showed that inoculation with selected couples of *S. cerevisiae* and *Starmerella bacillaris* resulted in a decrease of about 0.3 g/l of acetic acid, maintaining high ethanol and glycerol levels.

Montepulciano d'Abruzzo is a red wine grape variety of *Vitis vinifera* L., grown widely in central Italy, most notably in Abruzzo, Marche, and Molise regions. However, it is mainly identified with Abruzzo, the region in which it is also the most common and cultivated red variety for over two centuries. The first report of the Montepulciano grape in Abruzzo is found in "*Saggio Itinerario Nazionale nel Paese dei Peligni*," written by Torcia (1972). It currently accounts for around 50% of the regional vineyard, that is, about 18.500 hectares (Regione Abruzzo, http://www.regione.abruzzo.it/). Montepulciano d'Abruzzo is used for production of high quality red wines characterized by fruity notes (apple, pear, cherry, etc.). The most famous example is Montepulciano d'Abruzzo "Colline Teramane" DOCG wine (recognition in 2003) produced in the Teramo province.

Despite the economic importance of Montepulciano d'Abruzzo "Colline Teramane" few studies have been performed to identify its enological characteristics. In a previous study Tofalo et al. (2011) highlighted that the major NS yeasts present during must fermentation of Montepulciano cultivar were *H. uvarum*, *Metschnikowia fructicola*, and *S. bacillaris*, representing 43, 31, and 11%, respectively, of the total NS population isolated. Selected strains of *H. uvarum* (STS45), *S. bacillaris (*STS12), and *S. cerevisiae* (SRS1 and RT73) were then studied to evaluate their fermentation performance and interactions in microvinifications (Suzzi et al., 2012a,b).

The aim of this study was to establish the role and the inter-strains variability of two indigenous strains of *S. cerevisiae* (SRS1, RT73), a strain of *S. bacillaris* (STS12), one of *H. uvarum* (STS45) and a co-culture of *S. cerevisiae* (SRS1), and *S. bacillaris* (STS12) in shaping Montepulciano d'Abruzzo wine aroma profile in an experimental cellar. A *S. cerevisiae* commercial strain was used. Vinifications were conducted under routine Montepulciano d'Abruzzo wine production. Basic winemaking parameters (residual sugar, glycerol, organic acids, etc.), biogenic amines (BA) and volatile metabolites were determined. *S. cerevisiae* strain dynamics were also determined by microsatellite analysis.

MATERIALS AND METHODS

Yeast Strains and Media

Non-*Saccharomyces* (*H. uvarum*, STS45 and *S. bacillaris,* STS12) and *S. cerevisiae* autochthonous strains (RT73 and SRS1) have been previously characterized for their oenological performances in Montepulciano d'Abruzzo microvinification trials (Suzzi et al., 2012a,b). A commercial strain (CS) of *S. cerevisiae* (Flower Fresh, Tecnofood, Pavia, Italy) was also used. All strains belong to the Culture Collection of the Faculty of BioScience and Technology for Food, Agriculture, and Environment (University of Teramo, Italy). Non-*Saccharomyces* and *S. cerevisiae* strains were routinely grown in YPD medium (1% w/v yeast extract, 2% w/v peptone, and 2% w/v glucose) for 48 h under aerobic conditions. All strains were stored at −80°C in YPD broth supplemented with glycerol (20% v/v final concentration; Sigma-Aldrich, Milan, Italy).

Cellar Vinifications

Vinifications were carried out in a cellar of Consorzio per la Ricerca Viticola ed Enologica in Abruzzo (CRIVEA), during the vintage 2011. Montepulciano d'Abruzzo must (235 g/l fermentable sugars, 8.17 titratable acidity (TTA) and pH 3.44) was separated in tanks of 50 l, after destemming and crushing and added with 100 mg/l potassium metabisulfite.The fermentations were performed in maceration with the skins. The tanks were inoculated with 10^6 cells/ml from 24 h pre-cultures grown in the same pasteurized must. Two *S. cerevisiae* strains (SRS1, RT73), a strain of *S. bacillaris* (STS12), one of *H. uvarum* (STS45), and a co-culture of SRS1+STS12 were used to conduct fermentations. All fermentations were carried out in triplicate at room temperature (maximum temperature variation from 9 to 19°C).When the fermentation ended, the yeast lees were allowed to settle for 7 days and then wines were racked in 40 l tanks and

stored at controlled temperature in the cellar for 3 months. Then the wines were placed into glass bottles (750 ml), crown-sealed, and stored at 15–20°C for up to 6 months until sensorial analyses were performed.

Enumeration and Yeast Isolation

Total viable yeast counts were performed after 3, 5, 7, 10, and 15 days, using Wallerstein Laboratory Nutrient Agar (WLN, Oxoid, Milan, Italy), according to Pallmann et al. (2001).

Analytical Determinations

The main wine analytical components (ethanol, reducing sugar, pH, volatile acidity, TTA, citric, lactic, malic, and tartaric acids, glycerol) were determined using a FOSS WineScan (FT-120) rapid scanning Fourier Transform Infrared Spectroscopy with FOSS WineScan software version 2.2.1. Samples were firstly centrifuged at 8000 g for 10 min and then analyzed following the manufacturer's instructions.

Microsatellite PCR Fingerprinting

Total DNA was extracted directly from musts and wines using the PowerSoil DNA Isolation Kit (MoBio Laboratories). Ten milliliter of each sample were centrifuged to collect cells. The DNA was then extracted according to manufacturer's protocol. Quantification of total DNA was achieved using a VersaFluor fluorimeter and a Fluorescent DNA Quantitation Kit (Bio-Rad, Milan Italy). DNA was used as a template for microsatellite PCR fingerprinting, as described by Vaudano and Garcia-Moruno (2008). PCR amplifications were performed in a thermocycler (MyCycler, Bio-Rad Laboatories, Milan, Italy) with the following PCR programme: 4 min of initial denaturation at 94°C, 28 cycles of 30 s at 94°C, 45 s at 56°C, 30 s at 72°C and, finally, 10 min at 72°C. The products were run on a 2.5% (w/v) agarose gel 1 × TAE buffer at 100 V for 80 min. Gels were stained with ethidium bromide. 1-kb plus DNA ladder (Life Technologies, Milan, Italy) was used as marker for the gel normalization.

Volatile Profiles

Volatile compounds were determined by solid phase microextraction coupled with gas chromatography (GC/MS-SPME) according to Suzzi et al. (2012a). Molecule identification was based on comparison of their retention times with those of pure compounds (Sigma-Aldrich, Milan, Italy) analyzed in the same conditions. The identification was further confirmed by comparing mass spectra of compounds with those contained in the available database (NIST version 2005). The data were expressed as the relative peak area (%) calculated from head space SPME (HS/SPME) gas chromatograms of the identified peaks. All determinations were performed in triplicate.

Biogenic Amines Determination

Biogenic amines (BA) were determined according to Manetta et al. (2016). BA were analyzed using an HPLC system consisting of an Alliance (Waters, Milford, MA, USA), equipped with a Waters 2695 separation module connected to a Waters 2996 photodiode array detector (PDA), set at 254 nm. A Supelcosil LC-18 column (5 μm particle size, 250 × 4.6 mm i.d.) from Sigma was used. The system was governed by Waters Empower personal computer software. All analyses were performed in triplicate.

Sensory Analysis

Sensory tests were performed at room temperature (20°C). Wine samples were coded with 3-digit numbers, were evaluated in triplicate and presented according to a completely randomized block design. Skilled judges ($n = 13$) were trained as stated in the ISO 8586-1: 1993 rules (ISO, 1993).

Descriptive analysis was carried out in only one session. Sensory profile was determined using nine descriptors (fruity, persistence, body, astringency, grassy, reduced, floral, tropical fruits, drupaceous fruits) as previously reported (Suzzi et al., 2012a). Samples were scored for selected descriptors on a 4 cm scale anchored with "low" and "high" intensity.

Statistical Analysis

All data were processed using Excel 2016 (Microsoft, USA) and MatLab 2009b (Mathworks, Natick, MA, USA) softwares. In particular, a Principal Component Analysis (PCA) was performed on SPME–GC data after auto-scaling. The volatile molecule data were used to build up a single matrix, which was submitted to a two-way hierarchical clustering analysis. A heat map, visualizing metabolite levels was then obtained in which values are represented by cell colored according to the *Z*-scores, where *Z* is the mean value of different vinifications with the same yeast strain (Ferrara et al., 2008; Serrazanetti et al., 2011). The significant differences of the main enological characteristics were determined by *F*-test.

RESULTS

Viable Counts and Strain Dynamics

In order to improve the quality of Montepulciano d'Abruzzo wine through the use of autochthonous wine yeasts, *Saccharomyces* and non-*Saccharomyces* strains isolated from the *terroir* "Colline Teramane" and characterized for their enological aptitudes (Suzzi et al., 2012a,b) were chosen for experimental cellar vinifications as reported in Materials and Methods. Six vinifications were carried out, two inoculated with single *S. cerevisiae* strains (SRS1 and RT73), two with single NS strains (*H. uvarum* STS45 and *S. bacillaris* STS12) and one with the simultaneous presence of SRS1 and STS12.

Fermentation trials inoculated with SRS1, RT73, and the co-culture (SRS1+STS12) started the fermentation quickly (**Figure 1**), reaching higher values of viable cells after 5 days. At the end of fermentation lower values were observed in must inoculated with *S. bacillaris* STS12 and *H. uvarum* STS45, even if a faster growth was observed during the first fermentation days.

To verify the dominance of inoculated strains on natural yeast population in the must, microsatellite analysis on total DNAs was performed. The *S. cerevisiae* SRS1, RT73, and CS were present during the whole fermentation process, confirming a clear dominance of these *S. cerevisiae* strains (**Figure 2**). As expected more complex profiles were detected in Montepulciano d'Abruzzo must inoculated with *S. bacillaris* STS12 and *H.*

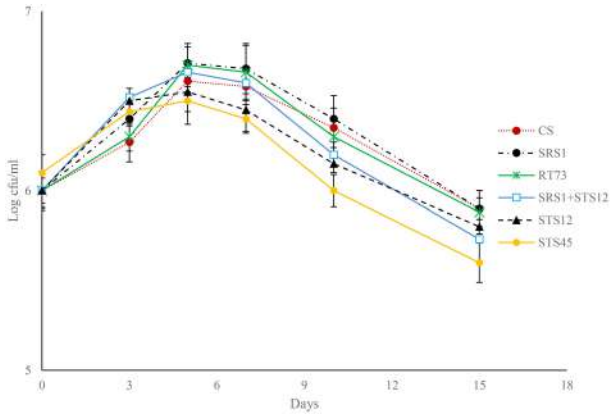

FIGURE 1 | Growth kinetic profiles of pure and mixed fermentation trials.

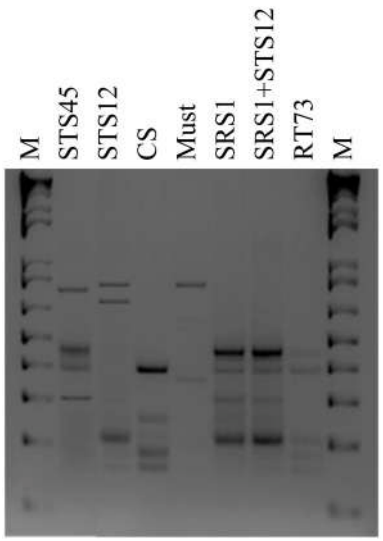

FIGURE 2 | Yeast strains electrophoretic patterns of microsatellite multiplex PCR (SC8132X, YOR267C and SCPTSY7) at the end of fermentation (15 days). Similar profiles were obtained after 3 days in inoculated fermentations. M: 1-kb plus DNA ladder (Life Technologies).

uvarum STS45 probably due to the presence of different indigenous *S. cerevisiae* strains.

Wine Characteristics

During the first days of fermentation a higher production of ethanol by NS and mixed cultures was observed (**Table 1**), whereas no differences were registered at the end of fermentation. In fact in all the six different conditions, must fermentations were completed according to reducing sugar concentration. The NS strains formed higher levels of glycerol up to 3.39 g/l after 3 days of fermentation, whereas the *S. cerevisiae* strains ranged from 1.38 to 2.20 g/l. The co-culture produced a wine with an intermediate glycerol content of 2.93 g/l. At the end of fermentation, the dominance of *S. cerevisiae* strains (**Figures 1**, **2**) made uniform all the wines with a glycerol content of about 10 g/l and an ethanol concentration of about of 14% (v/v). Similar behaviors were observed for other parameters such as volatile acidity, pH, TTA, and organic acids concentration. In all the samples the consumption of malic acid started before alcoholic fermentation was completed. This fact could be related to an high number of malolatic bacteria on grapes, as reported by Renouf et al. (2006), who found *Oenococcus oeni* and other lactic acid bacteria at the beginning of alcoholic fermentation. On the other hand, Nehme et al. (2010) reported simultaneous fermentations by inoculated yeasts and malolactic acid bacteria. Obtained wines were also analyzed for the presence of BA. In all wines cadaverine, tryptamine, β- phenylethylamine, tyramine, and histamine were below the limit of detection for the method used (Manetta et al., 2016). The levels of ethanolamine, ethylamine, isoamilamine, and putrescine had no significant changes. Their content was quite similar in all samples with ethanolamine which was the most abundant amine found ranging from 21 to 24 mg/l (data not shown).

Volatile Compounds

The volatile metabolites of the Montepulciano d'Abruzzo wines obtained with autochthonous strains of *S. cerevisiae* and NS and a mixed culture have been identified for a total of 101. Aroma compounds belonged to eight different families such as alcohols, aldehydes, ketones, esters, acids, terpenes, phenols, and aromatic compounds. The number of metabolites ranged from 63 for the wine produced by strain SRS1, 53 by STS45, 51 by CS, 49 by RT73, and mixed culture SRS1+STS12 and 47 by STS12 (**Table 2**). **Table 2** shows the main volatile molecules identified in relation to starter culture used. In the table only the main components of each aroma profile are reported. Nevertheless their presence represented at least the 95% of the total area in all the wine. Esters represented the major group for all the wines, followed by alcohols. The wine obtained with the co-culture showed the lowest relative percentage of alcohols in the heat space (about 14%), while those produced with *S. cerevisiae* CS and SRS1 were characterized by the highest ones, about 24.78 and 28.08%, respectively. In particular 2-phenylethanol (line 30, rose odor) had the highest relative percentage, ranging from 8.07% (SRS1+STS12) to 21.2% (CS). Differences were observed also for 2-methyl-1-propanol (line 15) and 1-hexanol (line 28, fruity and erbal odor) prevailing in wines fermented by NS and co-culture. Regarding esters the relative percentage in the heat space ranged from 57.42% (CS) to 77.38% (mixed culture), with more differences on the relative quantities of compounds among strains, as it can be easily evaluated from **Figure 3** built in order to better visualize the wine characterizing volatile molecules in relation to the starter used. The main esters present in the wines were 3-methylbut-1-yl methanoate (line 45, fruit aroma), ranging from 19.97% (CS) to 41.7% (RT73) followed by ethyl ethanoate (line 60), due to the large quantities of ethanol present. Isoamyl acetate was produced in relevant quantities only by the *S. cerevisiae* strains (line 44, banana aroma), whereas ethyl octanoate (line 65) and ethyl decanoate (line 59), generally associated to fruity aroma, were produced only by CS and SRS1. Ethyl hexanoate (line 63), related to red apple, fruity apple

TABLE 1 | Montepulciano d'Abruzzo wine characteristics fermented with autochthonous *S. cerevisiae* strains (SRS1 and RT73), commercial strain (CS), co-culture (SRS1+STS12) and non-*Saccharomyces* strains (STS12 and STS45) after 3 and 15 days (in parentheses) of fermentation.

Parameters	Strains					
	CS	SRS1	RT73	SRS1+STS12	STS12	STS45
Ethanol* (% v/v)	0.36 ± 0.18^{a}	0.41 ± 0.08^{a}	0.91 ± 0.68^{a}	2.44 ± 1.69^{b}	2.77 ± 0.15^{bc}	2.05 ± 0.09^{c}
	$(14.40 \pm 0.04)^{d}$	$(14.26 \pm 0.19)^{d}$	$(14.24 \pm 0.08)^{d}$	$(14.21 \pm 0.08)^{d}$	$(14.24 \pm 0.13)^{d}$	$(14.10 \pm 0.11)^{d}$
Reducing sugar (g/l)	215 ± 10^{a}	220 ± 14^{a}	197 ± 17^{a}	166 ± 34^{ab}	167 ± 12^{b}	174 ± 12^{b}
	$(2.07 \pm 0.13)^{c}$	$(2.18 \pm 0.28)^{c}$	$(2.06 \pm 0.22)^{c}$	$(1.81 \pm 0.10)^{d}$	$(1.96 \pm 0.14)^{d}$	$(1.95 \pm 0.12)^{d}$
pH	3.29 ± 0.09^{a}	2.94 ± 0.61^{a}	3.33 ± 0.05^{a}	3.36 ± 0.01^{a}	3.33 ± 0.10^{a}	3.35 ± 0.09^{a}
	$(3.30 \pm 0.02)^{a}$	$(3.34 \pm 0.04)^{a}$	$(3.31 \pm 0.04)^{a}$	$(3.33 \pm 0.06)^{a}$	$(3.33 \pm 0.07)^{a}$	$(3.31 \pm 0.10)^{a}$
Volatile acidity** (g/l)	0.07 ± 0.04^{a}	0.05 ± 0.02^{a}	0.09 ± 0.02^{ab}	0.14 ± 0.06^{bc}	0.14 ± 0.02^{c}	0.16 ± 0.05^{c}
	$(0.47 \pm 0.06)^{d}$	$(0.58 \pm 0.15)^{d}$	$(0.55 \pm 0.12)^{d}$	$(0.51 \pm 0.08)^{d}$	$(0.57 \pm 0.09)^{d}$	$(0.49 \pm 0.09)^{d}$
Titratable acidity*** (g/l)	6.03 ± 0.30^{a}	5.97 ± 0.33^{a}	5.89 ± 0.14^{a}	6.10 ± 0.19^{a}	6.20 ± 0.21^{a}	6.27 ± 0.22^{a}
	$(7.02 \pm 0.19)^{b}$	$(6.55 \pm 0.26)^{b}$	$(6.80 \pm 0.18)^{b}$	$(6.78 \pm 0.38)^{b}$	$(7.02 \pm 0.27)^{b}$	$(7.48 \pm 0.13)^{c}$
Citric acid (g/l)	0.15 ± 0.01^{a}	0.06 ± 0.01^{b}	0.14 ± 0.02^{a}	0.20 ± 0.03^{c}	0.19 ± 0.04^{bc}	0.27 ± 0.04^{c}
	$(0.59 \pm 0.09)^{de}$	$(0.55 \pm 0.02)^{d}$	$(0.57 \pm 0.07)^{d}$	$(0.59 \pm 0.03)^{d}$	$(0.63 \pm 0.04)^{e}$	$(0.62 \pm 0.05)^{de}$
Lactic acid	0.15 ± 0.08^{a}	0.30 ± 0.05^{b}	0.15 ± 0.02^{a}	0.03 ± 0.01^{c}	–	0.16 ± 0.02^{a}
	$(1.10 \pm 0.22)^{d}$	$(1.04 \pm 0.11)^{d}$	$(1.15 \pm 0.19)^{d}$	$(1.14 \pm 0.13)^{d}$	$(1.01 \pm 0.17)^{d}$	$(0.77 \pm 0.15)^{e}$
Malic acid (g/l)	1.00 ± 0.10^{a}	1.08 ± 0.21^{ab}	1.17 ± 0.06^{b}	1.18 ± 0.09^{b}	1.20 ± 0.08^{b}	1.17 ± 0.12^{ab}
	$(0.34 \pm 0.21)^{cde}$	$(0.10 \pm 0.04)^{c}$	$(0.21 \pm 0.09)^{cd}$	$(0.21 \pm 0.08)^{cd}$	$(0.39 \pm 0.11)^{d}$	$(0.55 \pm 0.12)^{de}$
Tartaric acid (g/l)	6.88 ± 0.21^{ab}	7.22 ± 0.38^{a}	6.78 ± 0.31^{ab}	6.59 ± 0.38^{b}	6.79 ± 0.21^{ab}	6.30 ± 0.19^{b}
	$(3.0 \pm 0.06)^{c}$	$(2.98 \pm 0.18)^{c}$	$(3.11 \pm 0.27)^{c}$	$(2.87 \pm 0.27)^{c}$	$(2.96 \pm 0.17)^{c}$	$(2.99 \pm 0.15)^{c}$
Glycerol (g/l)	2.2 ± 1.5^{ab}	1.38 ± 0.19^{a}	1.67 ± 0.65^{a}	2.9 ± 1.2^{b}	3.39 ± 0.27^{b}	3.02 ± 0.24^{b}
	$(10.96 \pm 0.28)^{c}$	$(10.12 \pm 0.08)^{d}$	$(10.0 \pm 0.17)^{d}$	$(10.16 \pm 0.41)^{d}$	$(10.22 \pm 0.19)^{d}$	$(10.71 \pm 0.54)^{d}$

*ml of alcohol/100 ml of wine, **expressed as acetic acid, ***expressed as tartaric acid.

Data are expressed as average ± SD. Same letters indicate samples in the same line with non-significant differences ($p < 0.05$).

or estery flavor was completely absent in the co-culture wine. Acids ranged from 2.8% (STS45) to 4.73% (RT73). The other compounds were present only in low amount or absent (**Table 2**).

In order to understand the variability among the strains, 101 aroma compounds data were submitted to PCA analysis (**Figures 4A–C**) to generate a visual representation of the wine discrimination on the basis of the specific aroma profiles generated by the strains used. The first three principal components were able to explain >50% of the total variances. Wines showed similar aroma profiles with differences for some compounds as reported above. The first 3 PCs score plot (**Figure 4A**) highlighted an overlapping of wines produced with SRS1+STS12, STS12, and RT73, while wines obtained with CS, SRS1, and STS45 were well differentiated. For a clearer comprehension of the loadings plot (**Figure 4C**), only the first 2 PCs of it were reported along with the first 2 PCs scores plot (**Figure 4B**). When collapsing the scores plot in two dimensions, separations between the different strains remained the same, except for an overlapping of CS and SRS1. Looking at the loading plot it was impossible to select a single class of compounds as the most important for a specific yeast (even observing the 3rd component, data not shown). However, *H. uvarum* STS45 was characterized by sulfur compounds (thiolane and 2-thiophene-acetic acid), some aromatic compounds (2-phenylacetaldeide, 5H-dibenzo[b,f]azepine, toluene and 1,3 dimethyl, 2-ethyl benzene) and hydocarbons such as 3-heptene and 2-heptamethyl nonene. Moreover, most of aldehydes such as heptanal, nonanal, and decanal can be found in the first quadrant of the loading plot correlated with CS and SRS1. Most of alcohols with even number of C atoms such as 1-butanol, 1-hexanol, and 3-mehyl 1 pentanol were present in the 3rd quadrant related to RT73 and to the co-culture SRS1+STS12.

Sensory Analysis

Sensory analysis revealed the influence of yeast strains on some of the considered descriptors. The wines fermented with SRS1 and the co-culture were characterized by a good floral and a highest persistence (**Figure 5**). Moreover negative attributes such as reduced and grassy were not very pronounced (significantly lower compared to STS12 and STS45 respectively). In particular, the co-culture had the lowest reduced aroma of all theses. Wines obtained with STS12 and STS45 were mainly characterized by grassy and reduced aroma. RT73 produced balanced wines with negative and positive attributes arranged in good proportions. Wines fermented with CS presented significantly low persistence, unwanted characteristic for Montepulciano d'Abruzzo wine. However these wines showed good aroma descriptors. In general, sensory analysis highlighted that the most interesting wines were those produced with SRS1 and the co-culture since they were characterized by a good floral, a highest persistence and, above all, have the reduced and grassy not too marked, as often it happens also in high quality Montepulciano d'Abruzzo wines.

TABLE 2 | Main volatile compounds identified (expressed as percentage of the peak area of each compound compared to the total area) ad in the wines produced by *S. cerevisiae* and non-*Saccharomyces* strains.

Line	Compounds	CS	SRS1	RT73	SRS1+STS12	STS12	STS45
ALCOHOL							
1*	(2E)-3,7-dimethylocta-2,6-dien-1-ol	–	0.1 ± 0.09	–	–	–	–
2	3,7,11-Trimethyl-1,6,10-dodecatrien-3-ol	–	0.07 ± 0.01	0.2 ± 0.09	–	–	–
3	1-butanol	0.3 ± 0.0	0.3 ± 0.1	0.7 ± 0.11	0.53 ± 0.05	0.6 ± 0.1	0.6 ± 0.02
4	1-dodecanol	–	0.07 ± 0.05	–	–	–	–
5	1-nonanol	–	–	0.03 ± 0.02	–	–	–
6	1-octanol	0.37 ± 0.15	0.4 ± 0.1	–	–	–	–
7	Oct-1-en-3-ol	–	0.03 ± 0.01	0.07 ± 0.03	0.1 ± 0.01	0.1 ± 0.02	0.1 ± 0.01
8	1-pentanol	–	–	0.07 ± 0.01	0.17 ± 0.02	0.2 ± 0.01	0.1 ± 0.01
9	1-undecanol	0.03 ± 0.01	–	–	–	–	–
10	2,2 ethoxyethoxy ethanol	–	–	–	–	0.1 ± 0.01	–
11	2,3-butandiol	0.35 ± 0.10	0.27 ± 0.11	0.7 ± 0.11	0.3 ± 0.1	0.5 ± 0.02	0.2 ± 0.02
12	2,3-dimethyl-2-hexanol	–	0.1 ± 0.03	–	–	–	–
13	2-decen-1-ol	–	–	–	–	0.2 ± 0.02	0.1 ± 0.01
14	2-ethyl-1-hexanol	0.2 ± 0.05	0.17 ± 0.03	–	–	–	–
15	2-methyl 1-propanol	0.97 ± 0.12	1.73 ± 0.60	1.93 ± 0.77	2.17 ± 0.15	2.5 ± 0.7	2.8 ± 0.8
16	2-octanol	–	–	–	0.13 ± 0.05	0.1 ± 0.01	0.1| ± 0.01
17	2-pentanol	–	–	0.03 ± 0.01	0.13 ± 0.01	0.2 ± 0.01	–
18	3,4-dimethyl-2-hexanol	0.23 ± 0.09	0.1 ± 0.04	0.03 ± 0.01	0.07 ± 0.02	–	–
19	3-hexen-1-ol	–	0.07 ± 0.01	0.13 ± 0.04	0.2 ± 0.08	0.2 ± 0.01	0.1 ± 0.01
20	3-methyl-1-pentanol	–	–	0.03 ± 0.01	0.07 ± 0.01	0.1 ± 0.01	–
21	3-(methylthio)-1-propanol	0.07 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	–	–	–
22	5-methyl-2-hexanol	–	–	0.03 ± 0.01	0.4 ± 0.12	0.7 ± 0.03	0.1 ± 0.01
23	5-methoxy-1-pentanol	0.17 ± 0.07	0.07 ± 0.02	–	–	–	–
24	6,10,13-trimethyl-1-tetradecanol	0.03 ± 0.01	0.03 ± 0.01	–	–	–	0.2 ± 0.01
25	3,7-dimethyloct-6-en-1-ol	–	0.1 ± 0.02	–	–	–	–
26	Phenylmethanol	0.23 ± 0.06	0.23 ± 0.04	0.13 ± 0.04	0.13 ± 0.05	0.1 ± 0.01	0.1 ± 0.01
27	2-ethoxyethanol	–	–	0.03 ± 0.01	–	–	–
28	1-hexanol	0.63 ± 0.05	0.83 ± 0.32	1.5 ± 0.7	2.27 ± 0.15	1.7 ± 0.7	1.3 ± 0.6
29	1-heptanol	–	0.07 ± 0.01	–	–	–	–
30	2-phenylethanol	21.2 ± 7.59	16.03 ± 5.72	12.77 ± 5.98	8.07 ± 1.59	11.7 ± 2.5	10.4 ± 2.68
	Total	24.78	20.80	18.41	14.74	19.0	16.2
ALDEHYDES							
31	3-furaldehyde	–	–	–	0.1 ± 0.01	0.3 ± 0.08	0.4 ± 0.2
32	Benzaldehyde	0.83 ± 0.05	0.87 ± 0.15	0.17 ± 0.05	0.63 ± 0.15	1.0 ± 0.3	1.0 ± 0.33
33	2-Phenylacetaldehyde	–	–	–	–	–	0.1 ± 0.03
34	Carbaldeide	–	–	–	–	0.1 ± 0.01	0.1 ± 0.02
35	Decanal	0.17 ± 0.02	0.17 ± 0.08	–	–	–	–
36	Furan-2-carbaldehyde	0.17 ± 0.05	0.23 ± 0.05	0.07 ± 0.01	0.07 ± 0.01	–	–
37	Heptanal	0.07 ± 0.01	–	–	–	–	–
38	Nonanal	0.27 ± 0.01	0.3 ± 0.16	–	–	–	–
	Total	1.51	1.57	0.24	0.80	1.4	1.6
KETONS							
39	2,3-butanedione	–	–	0.17 ± 0.0.01	0.13 ± 0.01	–	–
40	(E)-1-(2,6,6-Trimethyl-1-cyclohexa-1,3-dienyl)but-2-en-1-one	0.1 ± 0.01	0.07 ± 0.01	–	–	–	0.1 ± 0.01

(Continued)

TABLE 2 | Continued

Line	Compounds	CS	SRS1	RT73	SRS1+STS12	STS12	STS45
41	3-hexanone	–	–	0.03 ± 0.01	0.07 ± 0.01	0.1 ± 0.01	0.1 ± 0.03
42	3-hydroxy-2-butanone	–	0.07 ± 0.01	0.03 ± 0.01	0.07 ± 0.01	0.1 ± 0.01	0.1 ± 0.02
	Total	0.1	0.14	0.23	0.27	0.2	0.3
ESTERS							
43	2-methylbut-1-yl ethanoate	–	–	2.53 ± 0.38	5.4 ± 0.52	3.8 ± 0.7	4.1 ± 1.59
44	3-methylbut-1-yl ethanoate	3.37 ± 0.8	3.47 ± 1.02	2.13 ± 0.73	0.63 ± 0.23	0.6 ± 0.2	0.6 ± 0.23
45	3-methylbut-1-yl methanoate	19.97 ± 1.05	31.1 ± 11.19	41.7 ± 2.68	37.33 ± 3.13	36.4 ± 3.78	39.8 ± 3.89
46	Ethyl furan-2-carboxylate	0.07 ± 0.01	0.13 ± 0.01	–	–	0.1 ± 0.01	–
47	Pentan-2-yl methanoate	–	0.03 ± 0.01	–	–	–	–
48	3,7-dimethyloct-6-enyl methanoate	0.07 ± 0.01	–	–	–	–	–
49	2-methylpropyl acetate	–	–	0.23 ± 0.1	0.43 ± 0.05	0.2 ± 0.04	0.4 ± 0.2
50	2-phenylethyl ethanoate	1.0 ± 0.2	0.73 ± 0.16	0.13 ± 0.05	0.1 ± 0.01	0.1 ± 0.01	0.2 ± 0.1
51	Hexyl ethanoate	0.07 ± 0.01	0.67 ± 0.26	–	–	–	–
52	Ethyl phenylacetate	0.1 ± 0.01	0.1 ± 0.01	–	–	–	–
53	Ethyl 3-phenylpropanoate	–	0.07 ± 0.01	–	–	–	–
54	Benzyl 2-hydroxybenzoate	0.6 ± 0.1	0.67 ± 0.17	0.27 ± 0.15	0.17 ± 0.05	0.2 ± 0.03	0.2 ± 0.08
55	Butanedioic acid, diethyl ester	2.0 ± 0.26	1.8 ± 0.40	1.3 ± 0.78	1.5 ± 0.65	1.3 ± 0.5	0.9 ± 0.03
56	Ethyl butanoate	0.63 ± 0.05	1.23 ± 0.41	1.37 ± 0.11	1.3 ± 0.12	1.2 ± 0.37	1.2 ± 0.2
57	Ethyl 2-methylbutanoate	0.1 ± 0.01	0.1 ± 0.01	0.07 ± 0.01	0.1 ± 0.01	0.1 ± 0.02	0.1 ± 0.04
58	Ethyl 3-methylbutanoate	0.07 ± 0.01	0.1 ± 0.01	0.07 ± 0.01	0.1 ± 0.01	0.1 ± 0.01	0.1 ± 0.02
59	Ethyl decanoate	3.37 ± 0.51	1.03 ± 0.56	0.33 ± 0.2	0.13 ± 0.05	–	–
60	Ethyl ethanoate	9.07 ± 0.92	15.07 ± 2.44	17.13 ± 1.44	20.93 ± 4.66	17.6 ± 3.54	13.9 ± 2.73
61	Ethyl heptanoate	0.2 ± 0.01	0.3 ± 0.08	–	–	–	0.1 ± 0.02
62	3-Oxohexanedioic Acid Diethyl Ester	–	0.03 ± 0.01	–	4.3 ± 1.08	–	–
63	Ethyl hexanoate	3.73 ± 0.75	5.03 ± 2.49	2.73 ± 1.08	–	4.6 ± 1.79	2.8 ± 0.74
64	methyl 3-metoxy-aminopropanoate	–	–	0.03 ± 0.01	–	–	–
65	Ethyl octanoate	12.27 ± 0.97	7.0 ± 1.94	0.3 ± 0.1	–	–	–
66	Methyl octanoate	–	2.37 ± 0.98	–	–	–	–
67	Ethyl 2-hydroxypropanoate	0.73 ± 0.31	1.2 ± 0.85	2.13 ± 0.97	1.1 ± 0.9	–	0.8 ± 0.06
68	Ethyl 2-methylpropanoate	–	0.07 ± 0.01	–	1.23 ± 0.78	1.3 ± 0.75	–
69	Ethyl undecanoate	–	–	1.3 ± 0.86	2.63 ± 1.1	4.6 ± 1.23	4.0 ± 1.35
	Total	57.42	71.30	73.75	77.38	72.20	69.2
ACIDS							
70	3-methyl butanoic acid	–	–	0.23 ± 0.09	0.3 ± 0.02	0.4 ± 0.03	0.4 ± 0.11
71	Acetic acid	2.27 ± 0.47	3.03 ± 1.05	3.73 ± 1.71	3.77 ± 1.19	3.0 ± 1.25	1.7 ± 0.64
72	4-hydroxy-butanoic acid	–	0.07 ± 0.01	–	–	–	–
73	Hexanoic acid	0.53 ± 0.2	0.23 ± 0.05	0.2 ± 0.03	0.23 ± 0.05	0.6 ± 0.05	0.3 ± 0.17
74	Octanoic acid	1.2 ± 0.75	0.33 ± 0.1	0.57 ± 0.13	0.1 ± 0.01	0.5 ± 0.06	0.4 ± 0.09
75	Propanoic acid	0.07 ± 0.01	–	–	–	–	–
	Total	4.07	3.66	4.73	4.40	4.50	2.8
TERPENS							
76	3,7,7-trimethylbicyclo[4.1.0]hept-3-ene	0.07 ± 0.02	–	–	–	–	–
77	2-(4-Methyl-1-cyclohex-3-enyl)propan-2-ol	–	0.03 ± 0.01	–	–	–	–
	Total	0.07	0.03				

(Continued)

TABLE 2 | Continued

Line	Compounds	CS	SRS1	RT73	SRS1+STS12	STS12	STS45
AROMATICS							
78	Phenylethene	0.07 ± 0.03	0.13 ± 0.08	–	0.03 ± 0.02	0.1 ± 0.03	0.1 ± 0.02
79	1-methyl-4-(1-methylethyl)benzene	0.17 ± 0.08	0.1 ± 0.01	–	–	–	0.1 ± 0.01
80	1,3 dymethyl, 2-ethyl benzene	–	–	–	–	0.1 ± 0.02	0.3 ± 0.04
81	Toluene	–	–	–	–	–	0.1 ± 0.02
82	Dithiolane	–	–	–	–	0.1 ± 0.05	0.1 ± 0.04
	Total	0.24	0.23		0.03	0.3	0.7
PHENOLS							
83	4,4′-(propane-2,2-diyl)diphenol	–	0.1 ± 0.01	–	–	–	–
84	4-methyl phenol	–	0.1 ± 0.02	–	–	–	0.1 ± 0.01
	Total		0.2				0.1
OTHERS							
85	1-methoxy octane	–	–	–	0.07 ± 0.01	–	0.2 ± 0.01
86	1-chlorooctane	–	0.07 ± 0.01	0.17 ± 0.08	–	–	–
87	1-methoxy-2-methyl-propane	0.13 ± 0.05	–	–	0.17 ± 0.02	–	–
88	4-amino-1,2,4-triazole	0.4 ± 0.1	–	–	–	–	–
89	Pentamine	0.13 ± 0.01	0.03 ± 0.02	–	–	–	–
90	2-pentylfuran	0.03 ± 0.01	0.07 ± 0.03	–	–	–	–
91	3-heptene	–	–	–	–	–	0.1 ± 0.01
92	Ciclo-heptane	0.27 ± 0.05	0.2 ± 0.17	–	0.13 ± 0.01	–	–
93	Decamethyl cyclopentasiloxane	–	–	–	0.23 ± 0.1	0.6 ± 0.09	0.6 ± 0.13
94	2-heptamethyl nonene	–	–	–	0.17 ± 0.08	0.3 ± 0.1	0.9 ± 0.09
95	5H-dibenzo[b,f]azepine	–	–	–	–	–	0.1 ± 0.02
96	Silane	1.4 ± 0.31	0.1 ± 0.43	1.0 ± 0.73	–	–	–
97	Indole	–	–	0.03 ± 0.02	–	–	–
98	Thiolane	–	–	0.2 ± 0.01	0.4 ± 0.07	0.1 ± 0.07	1.9 ± 0.8
99	Dihydrofuran-2(3H)-one	–	–	0.17 ± 0.07	0.3 ± 0.01	0.3 ± 0.02	0.1 ± 0.09
100	2-thiophene acetic acid	–	–	0.27 ± 0.09	0.37 ± 0.12	0.1 ± 0.04	4.2 ± 1.33
101	3-thiopheneethanol	2.4 ± 0.6	0.63 ± 0.25	–	–	–	–
	Total	4.76	1.1	1.84	1.84	1.4	8.1

*, *metabolite number corresponding in the heatmap.*

DISCUSSION

Montepulciano d'Abruzzo is a native grape variety of *V. vinifera* L., grown in central Italy and used for production of high quality red wines. Limited studies have been carried out to improve its enological characteristics through the use of indigenous wine yeasts. The interest for autochthonous strains as single or mixed cultures in combination with *S. cerevisiae* is gaining more and more importance since they are potentially associated to a particular terroir and therefore adapted to a specific grape must reflecting the biodiversity of a particular area (Bokulich et al., 2014; Capozzi et al., 2015). For this reason, the application of indigenous mixed non-*Saccharomyces*/*Saccharomyces* starter, able to mimic wine biodiversity, could be a valid alternative to spontaneous fermentations, since the multi-starter ability to increase the organoleptic properties of wine and to minimize the microbial spoilage (Comitini et al., 2011; Ciani and Comitini, 2015).

In this study the organoleptic properties of Montepulciano d'Abruzzo wine and the fermentation of two indigenous strains of *S. cerevisiae* (SRS1, RT73), a strain of *S. bacillaris* (STS12), one of *H. uvarum* (STS45), and a co-culture of *S. cerevisiae* (SRS1) and *S. bacillaris* (STS12) were evaluated. The data highlighted that at 3 days faster fermentations were obtained in the musts inoculated with NS yeasts, in agreement with other authors (Mendoza et al., 2007; Fleet, 2008; Ciani et al., 2010; Suzzi et al., 2012b). Also the co-culture SRS1+STS12 showed a good fermentation kinetic in comparison with SRS1. The positive interaction between *S. cerevisiae* and *S. bacillaris* has been highlighted by other authors (Rantsiou et al., 2012; Suzzi et al., 2012b). The sugar consumption was faster in SRS1+STS12 co-culture than in *S. cerevisiae* pure cultures probably because of the osmotolerant and fructophilic character of this non-*Saccharomyces* yeast. In fact, it consumes sugars at the early stage of the fermentation, alleviating the *S. cerevisiae* from the osmotic stress, thereby improving also the fermentation kinetics (Rantsiou et al., 2012; Englezos et al., 2015).

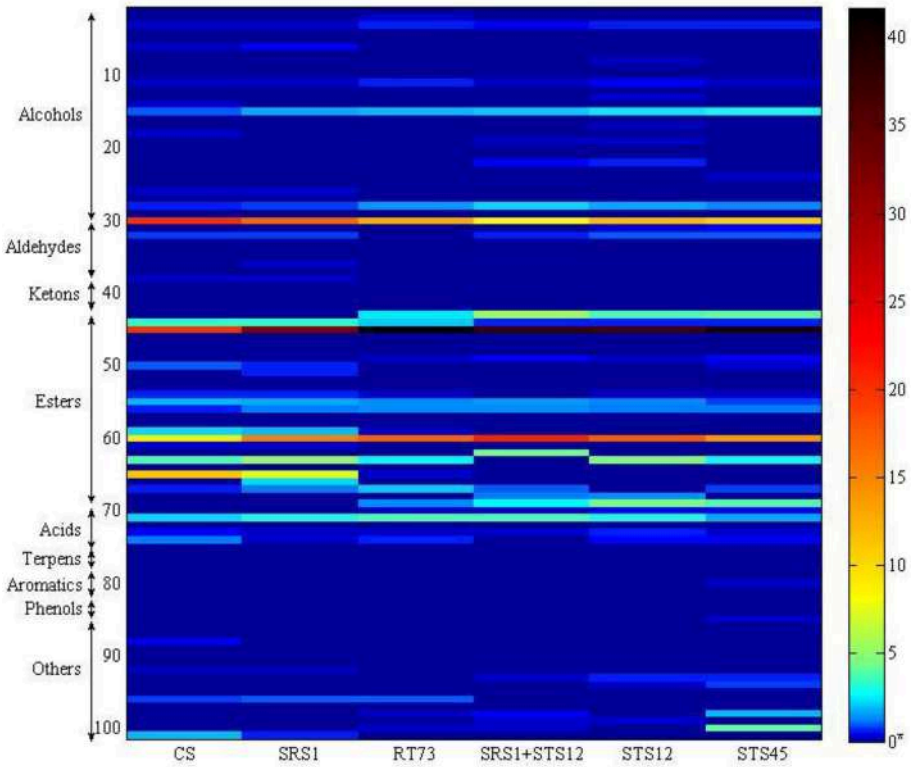

FIGURE 3 | Heatmap representing volatile profile of autochthonous *S. cerevisiae* strains (SRS1 and RT73), commercial strain (CS), co-culture (SRS1+STS12), and non-*Saccharomyces* strains (STS12 and STS45). Compounds were organized by chemical families, and with the indication of the number of compounds per family. Each line corresponds to one metabolite, and each column corresponds to each strain. For the correspondence between number and volatile compound see **Table 2**. *The quantitative analysis of wine aroma compounds was carried out on the basis of the relative peak area (Qi) calculated from head space SPME (HS/SPME) gas chromatograms after addition of known amounts of analyte standards.

H. uvarum STS45 showed a good fermentation kinetic at the beginning however at the end of fermentation it showed the lowest viable count values. The disappearance of *Hanseniaspora* yeasts can be associated to their low ethanol tolerance or to the production of other toxic compounds besides ethanol (Egli et al., 1998; Fleet, 2003). *S. bacillaris* STS12 showed better fermentation kinetic than STS45 and a higher number of viable cells at the end of fermentation. Some authors reported that *S. bacillaris* was able to complete Macabeo must fermentation even if with a slight delay compared to the *S. cerevisiae* fermentation (Andorrà et al., 2010).

The enological parameters during the first days of fermentation highlighted the metabolic cooperation between inoculated and indigenous strains, although at the end of fermentation all wines showed similar characteristics due to the dominance of *S. cerevisiae* strains. In fact, also wines inoculated with NS wine yeasts showed low values of residual sugar and an ethanol concentration of about 14%, probably due to the contribution of indigenous *Saccharomyces* population present in the must at the start of fermentation. The wine organoleptic properties are related to the presence of several compounds deriving from the yeast metabolism (Capozzi et al., 2015) and the dominance or competitiveness of a starter strain could have an influence on the sensorial quality of wine by imposing its aromatic profile or deleting the collaborative role of natural *S. cerevisiae* populations. In this study the microsatellites analysis performed directly on the must allowed to establish the dominance of all *S. cerevisiae* strains (SRS1, RT73, and CS) during all the fermentation process shaping wine aroma and the presence of other non-starter yeasts during fermentation with NS strains. In *S. cerevisiae*, microsatellites have been described as abundant and highly polymorphic in length (Richards et al., 2009), and for this reason, they are used as a reproducible and portable typing method (Hennequin et al., 2001; Schuller et al., 2004; Bradbury et al., 2005; Legras et al., 2005; Tofalo et al., 2013).

In all wines, the volatile acidity was below the legal limit of 1.2 g/l of acetic acid (Office Internationale de la Vigne et du Vin, 2009), since higher values can confer to wine a detrimental acidic flavor (Bely et al., 2003). In this context it is interesting to underline that despite acetic acid production is considered as a common pattern in apiculate yeasts (Romano et al., 2003), we found that wines inoculated with *H. uvarum* STS45 did not show an increased volatile acidity, in agreement with other authors (Andorrà et al., 2010; Suzzi et al., 2012b). In addition all wines showed low quantity of BA indicating the low decarboxylase activity of wine yeasts and indigenous malolactic bacteria (Marcobal et al., 2006; Smit et al., 2008; Suzzi et al., 2012b).

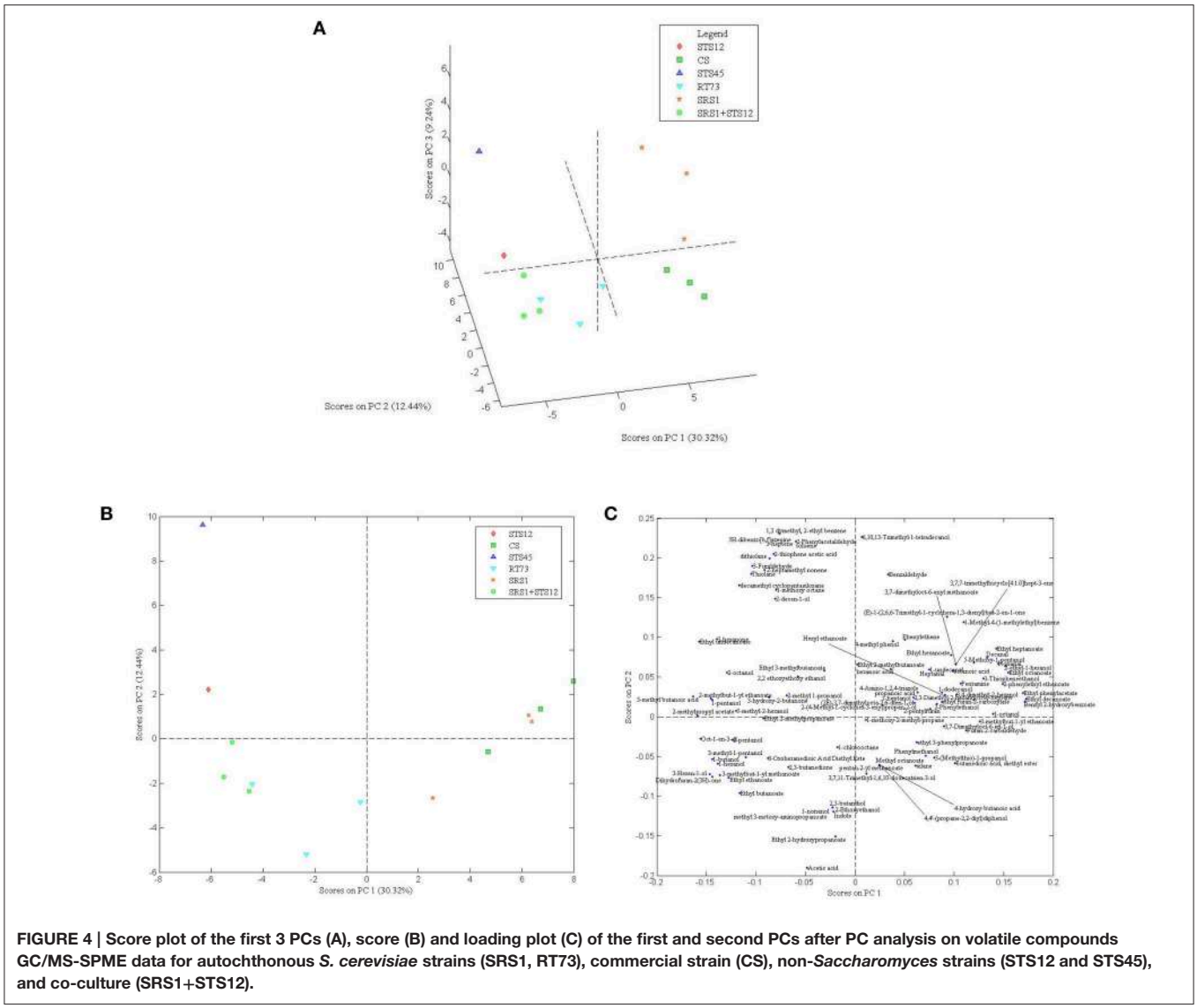

FIGURE 4 | Score plot of the first 3 PCs (A), score (B) and loading plot (C) of the first and second PCs after PC analysis on volatile compounds GC/MS-SPME data for autochthonous *S. cerevisiae* strains (SRS1, RT73), commercial strain (CS), non-*Saccharomyces* strains (STS12 and STS45), and co-culture (SRS1+STS12).

Esters were the most representative compounds in all wines according to Ferreira et al. (1995) and according to Suzzi et al. (2012a) the fruity character attributed to the aroma of Montepulciano wines is mainly related to apple, pear, and cherry notes. In fact, esters are a group of volatile compounds, arise from yeast metabolic activity, that impart a mostly pleasant smell (Capozzi et al., 2015). The wines produced with SRS1 and CS were well differentiated by other wines as shown by PCA and sensory analyses acquiring the aromatic fingerprinting of the strain.

Specific features were also shown by wines produced with STS45. These wines were characterized by the presence of sulfur compounds. Sulfur compounds have different sensory properties and, although most of them could negatively affect the wine aroma, they can also give a positive contribute by adding fruity notes (Swiegers and Pretorius, 2005).

The wines produced with RT73 and SRS1+STS12 clustered together in the PCA analysis, however sensory analysis revealed that wines obtained with the co-culture showed interesting olfactory and tasting properties such as fruity, good body, and persistence which are important characteristics for red wines. In addition the simultaneously malolactic and alcoholic fermentation suggested a possible impact of lactic acid bacteria on the final wines. In fact it is well known as the role of malolactic fermentation is more than a deacidification, affecting the quality of wine positively, such as volatile acids and negatively such BA production (Liu, 2002; Renouf et al., 2006). In all the wines the content of BA was lower than the detection limits, confirming that lactic acid bacteria vary on the production of these compounds (Lonvaud-Funel, 2001).

The data obtained in this study highlighted that the use of NS autochthonous yeasts positively influence wine aroma profile. In particular STS45 produced wines with a specific aroma fingerprinting. In conclusion the natural cultures applied in cellar vinification in this study can be considered as a useful tool that take the advantages of the spontaneous fermentation,

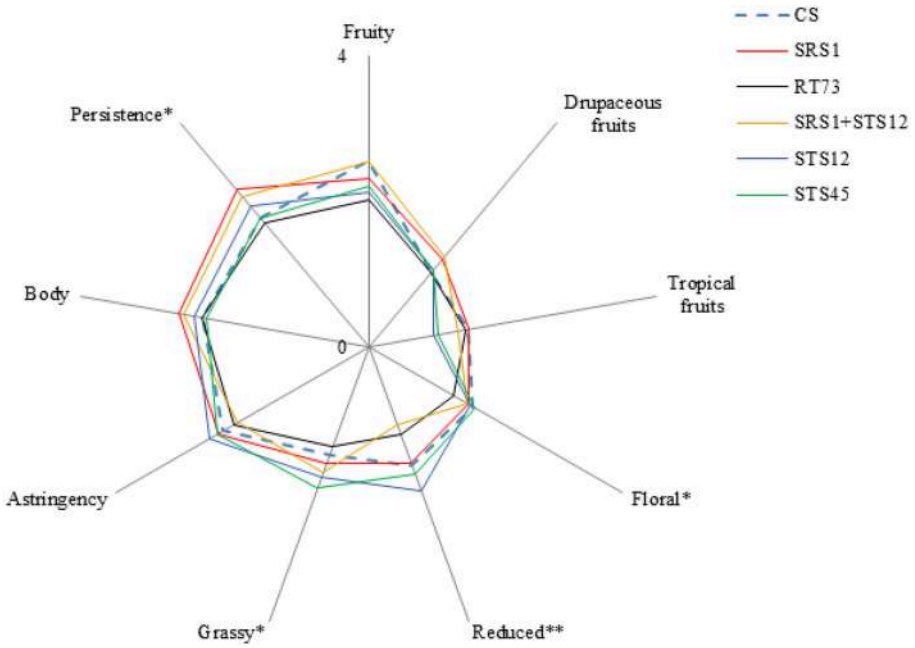

FIGURE 5 | Descriptive analysis of obtained wines. $^{*}p < 0.05$, $^{**}p < 0.01$.

enhancing the chemical and organoleptic characteristics of the wine and avoiding the risk of stuck fermentations and microbial contamination.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: GS, RT. Performed the experiments: GP, MS, PG, FP. Analyzed the data: MS, DP, GA, RL. Wrote the paper: GS, RT, MS.

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REFERENCES

Alves, Z., Melo, A., Figueiredo, A. R., Coimbra, M. A., Gomes, A. C., and Rocha, S. M. (2015). Exploring the *Saccharomyces cerevisiae* volatile metabolome: indigenous versus commercial strains. *PLoS ONE* 11:e0143641. doi: 10.1371/journal.pone.0143641

Andorrà, I., Berradre, M., Rozès, N., Mas, A., Guillamón, J. M., and Esteve-Zarzoso, B. (2010). Effect of pure and mixed cultures of the main wine yeast species on grape must fermentations. *Eur. Food Res. Technol.* 231, 215–224. doi: 10.1007/s00217-010-1272-0

Barbosa, C., García-Martínez, J., Pérez-Ortín, J. E., and Mendes-Ferreira, A. (2015). Comparative transcriptomic analysis reveals similarities and dissimilarities in *Saccharomyces cerevisiae* wine strains response to nitrogen availability. *PLoS ONE* 10:e0122709. doi: 10.1371/journal.pone.0122709

Bely, M., Rinaldi, A., and Dubourdieu, D. (2003). Influence of assimilable nitrogen on volatile acidity production by *Saccharomyces cerevisiae* during high sugar fermentation. *J. Biosci. Bioeng.* 96, 507–512. doi: 10.1016/S1389-1723(04)70141-3

Bokulich, N. A., Thorngate, J. H., Richardson, P. M., and Mills, D. A. (2014). Microbial biogeography of wine grapes is conditioned by cultivar, vintage, and climate. *PNAS* 111, E139–E148. doi: 10.1073/pnas.1317377110

Bradbury, J. E., Richards, K. D., Niederer, H. A., Lee, S. A., Dunbar, P. R., and Gardner, R. C. (2005). A homozygous diploid subset of commercial wine yeast strains. *Antonie Leeuwenhoek* 89, 27–37. doi: 10.1007/s10482-005-9006-1

Capozzi, V., Garofalo, C., Chiriatti, M. A., Grieco, F., and Spano, G. (2015). Microbial terroir and food innovation: the case of yeast biodiversity in wine. *Microbiol. Res.* 181, 75–83. doi: 10.1016/j.micres.2015.10.005

Ciani, M., and Comitini, F. (2015). Yeast interactions in multi-starter wine fermentation. *Curr. Opin. Food Sci.* 1, 1–6. doi: 10.1016/j.cofs.2014.07.001

Ciani, M., Comitini, F., Mannazzu, I., and Domizio, P. (2010). Controlled mixed culture fermentation: a new perspective on the use of non- *Saccharomyces* yeasts in winemaking. *FEMS Yeast Res.* 10, 123–133. doi: 10.1111/j.1567-1364.2009.00579.x

Comitini, F., Gobbi, M., Domizio, P., Romani, C., Lencioni, L., Mannazzu, I., et al. (2011). Selected non-*Saccharomyces* wine yeasts in controlled multistarter fermentations with *Saccharomyces cerevisiae*. *Food Microbiol.* 28, 873–882. doi: 10.1016/j.fm.2010.12.001

Egli, C. M., Edinger, W. D., Mitrakul, C. M., and Henick-Kling, T. (1998). Dynamics of indigenous and inoculated yeast populations and their effect on the sensory character of Riesling and Chardonnay wines. *J. Appl. Microbiol.* 85, 779–789. doi: 10.1046/j.1365-2672.1998.00521.x

Englezos, V., Rantsiou, K., Torchio, F., Rolle, L., Gerbi, V., and Cocolin, L. (2015). Exploitation of the non-*Saccharomyces* yeast *Starmerella bacillaris* (synonym *Candida zemplinina*) in wine fermentation: physiological and molecular characterizations. *Int. J. Food Microbiol.* 199, 33–40. doi: 10.1016/j.ijfoodmicro.2015.01.009

Ferrara, C. T., Wang, P., Neto, E. C., Stevens, R. D., Bain, J. R., Wenner, B. R., et al. (2008). Genetic networks of liver metabolism revealed by integration of metabolic and transcriptional profiling. *PLoS Genet.* 4:e1000034. doi: 10.1371/journal.pgen.1000034.

Ferreira, B., Hary, C., Bard, M. H., Táisant, C., Olsson, A., and Lefur, Y. (1995). Effect of skin-contact and setting on the level of the C18:2, C18:3 fatty acids and C6 compounds in Burgundy Chardonnay musts and wines. *Food Qual. Prefer.* 6, 35–41. doi: 10.1016/0950-3293(94)P4210-W

Fleet, G. H. (2003). Yeast interactions and wine flavour. *Int. J. Food Microbiol.* 86, 11–22. doi: 10.1016/S0168-1605(03)00245-9

Fleet, G. H. (2008).Wine yeast for the future. *FEMS Yeast Res.* 8, 979–995. doi: 10.1111/j.1567-1364.2008.00427.x

Hennequin, C., Thierry, A., Richard, G. F., Lecointre, G., Nguyen, H. V., Gaillardin, C., et al. (2001). Microsatellite typing as a new tool for identification of *Saccharomyces cerevisiae* strains. *J. Clin. Microbiol.* 39, 551–559. doi: 10.1128/JCM.39.2.551-559.2001

Howell, K. S., Cozzolino, D., Bartowsky, E. J., Fleet, G. H., and Henschke, P. A. (2006). Metabolic profiling as a tool for revealing *Saccharomyces* interactions during wine fermentation. *FEMS Yeast Res.* 6, 91–101. doi: 10.1111/j.1567-1364.2005.00010.x

ISO (1993). *Sensory Analysis—General Guidance for the Selection, Training, and Monitoring of Assessors. Part 1: Selected Assessors*. International Organization for Standardization, ISO 8586–1:1993.

Jolly, N. P., Varela, C., and Pretorius, I. S. (2014). Not your ordinary yeast: non-*Saccharomyces* yeasts in wine production uncovered. *FEMS Yeast Res.* 14, 215–237. doi: 10.1111/1567-1364.12111

Legras, J. L., Ruh, O., Merdinoglu, D., and Karst, F. (2005). Selection of hypervariable microsatellite loci for the characterization of *Saccharomyces cerevisiae*. *Int. J. Food Microbiol.* 102, 73–83. doi: 10.1016/j.ijfoodmicro.2004.12.007

Liu, S. Q. (2002). A review Malolactic fermentation in wine-beyond deacidification. *J. Appl. Microbiol.* 92, 589–601. doi: 10.1046/j.1365-2672.2002.01589.x

Liu, Y., Rousseaux, S., Tourdot-Maréchal, R., Sadoudi, M., Gougeon, R., Schmitt-Kopplin, P., et al. (2015). Wine microbiome, a dynamic world of microbial interactions. *Crit. Rev. Food Sci. Nutr.* doi: 10.1080/10408398.2014.983591. [Epub ahead of print].

Lonvaud-Funel, A. (2001). Biogenic amines in wines: role of lactic acid bacteria. *FEMS Microbiol. Lett.* 199, 9–13. doi: 10.1111/j.1574-6968.2001.tb10643.x

Manetta, A. C., Di Giuseppe, L., Tofalo, R., Martuscelli, M., Schirone, M., Giammarco, M., et al. (2016). Evaluation of biogenic amines in wine: determination by an improved HPLC-PDA method. *Food Control* 62, 351–356. doi: 10.1016/j.foodcont.2015.11.009

Marcobal, Á., Martín-Álvarez, P. J., Polo, M.,C., Muñoz, R., and Moreno-Arribas, M. V. (2006). Formation of biogenic amines throughout the industrial manufacture of red wine. *J. Food Prot.* 69, 397–404.

Medina, K., Boido, E., Fariña, L., Gioia, O., Gomez, M. E., Barquet, M., et al. (2013). Increased flavour diversity of Chardonnay wines by spontaneous fermentation and co-fermentation with *Hanseniaspora vineae*. *Food Chem.* 141, 2513–2521. doi: 10.1016/j.foodchem.2013.04.056

Mendoza, L. M., Mancade Nadra, M. C., and Farías, M. E. (2007). Kinetics and metabolic behavior of a composite culture of *Kloeckera apiculata* and *Saccharomyces cerevisiae* wine related strains. *Biotechnol. Lett.* 29, 1057–1063. doi: 10.1007/s10529-007-9355-0

Moreira, N., Mendes, F., Hogg, T., and Vasconcelos, I. (2005). Alcohols, esters and heavy sulphur compounds production by pure and mixed cultures of apiculate wine yeasts. *Int. J. Food Microbiol.* 103, 285–294. doi: 10.1016/j.ijfoodmicro.2004.12.029

Nehme, N., Mathieu, F., and Taillandier, P. (2010). Impact of the co-colture of *Saccharomyces cerevisiae- Oenococcus oeni* on malolactic fermentetion and partial characterization of a yeast –derived inihibitory peptidic fraction. *Food Microbiol.* 27, 150–157. doi: 10.1016/j.fm.2009.09.008

Office Internationale de la Vigne et du Vin (2009). *Compendium of International Methods of Wine and Must Analysis*. Paris: International Organisation of Vine and Wine (OIV).

Pallmann, C. L., Brown, J. A., Olineka, T. L., Cocolin, L. S., Mills, D. A., and Bisson, L. F. (2001). Use of WL medium to profile native flora fermentations. *Am. J. Enol. Vitic.* 52, 198–203.

Rantsiou, K., Dolci, P., Giacosa, S., Torchio, F., Tofalo, R., Torriani, S., et al. (2012). *Candida zemplinina* can reduce acetic acid production by *Saccharomyces cerevisiae* in sweet wine fermentations. *Appl. Environ. Microbiol.* 78, 1987–1994. doi: 10.1128/AEM.06768-11

Renouf, V., Claisse, O., Miot-Sertier, C., and Lonvaud-Funel, A. (2006). Lactic acid bacteria evolution during winemaking: use of *rpoB* gene as a target for PCR-DGGE analysis. *Food Microbiol.* 23, 136–145. doi: 10.1016/j.fm.2005.01.019

Richards, K. D., Goddard, M. R., and Gardner, R. C. (2009). A database of microsatellite genotypes for *Saccharomyces cerevisiae*. *Antonie Leeuwenhoek* 96, 355–359. doi: 10.1007/s10482-009-9346-3

Romano, P., Fiore, C., Paraggio, M., Caruso, M., and Capece, A. (2003). Function of yeast species and strains in wine flavour. *Int. J. Food Microbiol.* 86, 169–180. doi: 10.1016/S0168-1605(03)00290-3

Sadoudi, M., Tourdot-Maréchal, R., Rousseaux, S., Steyer, D., GallardoChacón, J. J., Ballester, J., et al. (2012). Yeast-yeast interactions revealed by aromatic profile analysis of Sauvignon Blanc wine fermented by single or co-culture of non-*Saccharomyces* and *Saccharomyces* yeasts. *Food Microbiol.* 32, 243–253. doi: 10.1016/j.fm.2012.06.006

Schuller, D., and Casal, M. (2005). The use of genetically modified *Saccharomyces cerevisiae* strains in the wine industry. *Appl. Microbiol. Biotechnol.* 68, 292–304. doi: 10.1007/s00253-005-1994-2

Schuller, D., Valero, E., Dequin, S., and Casal, M. (2004). Survey of molecular methods for the typing of wine yeast strains. *FEMS Microbiol. Lett.* 231, 19–26. doi: 10.1016/S0378-1097(03)00928-5

Serrazanetti, D. I., Ndagijimana, M., Sado, S. L., Corsetti, A., Vogel, R. F., Ehrmann, M., et al. (2011). Acid stress-mediated metabolic shift in *Lactobacillus sanfranciscensis* LSCE1. *Appl. Environ. Microbiol.* 77, 2656–2666. doi: 10.1128/AEM.01826-10

Smit, A. Y., du Toit, W. J., and du Toit, M. (2008). Biogenic amines in wine: understanding the headache. *South Afr. J. Enol. Vitic.* 29, 109–127.

Suzzi, G., Arfelli, G., Schirone, M., Corsetti, A., Perpetuini, G., and Tofalo, R. (2012a). Effect of grape indigenous *Saccharomyces cerevisiae* strains on Montepulciano d'Abruzzo red wine quality. *Food Res. Int.* 46, 22–29. doi: 10.1016/j.foodres.2011.10.046

Suzzi, G., Schirone, M., Sergi, M., Marianella, R. M., Fasoli, G., Aguzzi, I., et al. (2012b). Multistarter from organic viticulture for red wine Montepulciano d'Abruzzo production. *Front. Microbiol.* 3, 135. doi: 10.3389/fmicb.2012.00135

Swiegers, J. H., Bartowsky, E. J., Henschke, P. A., and Pretorius, I. S. (2005). Yeast and bacterial modulation of wine aroma and flavour. *Aust. J. Grape Wine Res.* 11, 139–173. doi: 10.1111/j.1755-0238.2005.tb00285.x

Swiegers, J. H., and Pretorius, I. S. (2005). Yeast modulation of wine flavor. *Adv. Appl. Microbiol.* 57, 131–175. doi: 10.1016/S0065-2164(05)57005-9

Tofalo, R., Perpetuini, G., Schirone, M., Fasoli, G., Aguzzi, I., Corsetti, A., et al. (2013). Biogeographical characterization of Saccharomyces cerevisiae wine yeast by molecular methods. *Front. Microbiol.* 4, 166. doi: 10.3389/fmicb.2013.00166

Tofalo, R., Schirone, M., Telera, G. C., Manetta, A. C., Corsetti, A., and Suzzi, G. (2011). Influence of organic viticulture on non-*Saccharomyces* wine yeast populations. *Ann. Microbiol.* 61, 57–66. doi: 10.1007/s13213-010-0102-8

Torcia, M. (1972). *Saggio Itinerario Nazionale pel Paese de' Peligni*. Abruzzo: Edizioni digitali del CISVA.

Tufariello, M., Chiriatti, M., Grieco, F., Perrotta, C., Capone, S., Rampino, P., et al. (2014). Influence of autochthonous *Saccharomyces cerevisiae* strains on volatile profile of Negroamaro wines. *LWT Food Sci. Technol.* 58, 35–48. doi: 10.1016/j.lwt.2014.03.016

Vaudano, E., and Garcia-Moruno, E. (2008). Discrimination of *Saccharomyces cerevisiae* wine strains using microsatellite multiplex PCR and band pattern analysis. *Food Microbiol.* 25, 56–64. doi: 10.1016/j.fm.2007.08.001

Vernocchi, P., Patrignani, F., Ndagijimana, M., Chaves Lopez, C., Suzzi, G., Gardini, F., et al. (2015). Trebbiano wine produced by using *Saccharomyces cerevisiae* strains endowed with β-glucosidase activity. *Ann. Microbiol.* 65, 1565–1571. doi: 10.1007/s13213-014-0995-8

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Sequential Fermentation with Selected Immobilized Non-*Saccharomyces* Yeast for Reduction of Ethanol Content in Wine

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The average ethanol content of wine has increased over the last two decades. This increase was due to consumer preference, and also to climate change that resulted in increased grape maturity at harvest. In the present study, to reduce ethanol content in wine, a microbiological approach was investigated, using immobilized selected strains of non-*Saccharomyces* yeasts namely *Starmerella bombicola*, *Metschnikowia pulcherrima*, *Hanseniaspora osmophila,* and *Hanseniaspora uvarum* to start fermentation, followed by inoculation of free *Saccharomyces cerevisiae* cells. The immobilization procedures, determining high reaction rates, led a feasible sequential inoculation management avoiding possible contamination under actual winemaking. Under these conditions, the immobilized cells metabolized almost 50% of the sugar in 3 days, while *S. cerevisiae* inoculation completed all of fermentation. The *S. bombicola* and *M. pulcherrima* initial fermentations showed the best reductions in the final ethanol content (1.6 and 1.4% v/v, respectively). Resulting wines did not have any negative fermentation products with the exception of *H. uvarum* sequential fermentation that showed significant amount of ethyl acetate. On the other hand, there were increases in desirable compounds such as glycerol and succinic acid for *S. bombicola*, geraniol for *M. pulcherrima* and isoamyl acetate and isoamyl alcohol for *H. osmophila* sequential fermentations. The overall results indicated that a promising ethanol reduction could be obtained using sequential fermentation of immobilized selected non-*Saccharomyces* strains. In this way, a suitable timing of second inoculation and an enhancement of analytical profile of wine were obtained.

Keywords: ethanol reduction, immobilized cells, non-*Saccharomyces* yeast, sequential fermentation, wine

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INTRODUCTION

Over the last few decades, there has been a progressive increase in the ethanol content in wine due to new wine styles arising from consumer preference, and to the global climate change that is often associated with increased grape maturity (Jones et al., 2005; Grant, 2010; MacAvoy, 2010; Alstona et al., 2011; Gonzalez et al., 2013). However, wine with high levels of ethanol can be perceived negatively due to health concerns, wine quality reduction and taxation rates according to ethanol content (Guth and Sies, 2001; Athès et al., 2004; Gawel et al., 2007).

In this context, several lines of research are aimed at reducing the ethanol content of wines, which have generally focused on vineyard management and winemaking practices, and particularly on the de-alcoholization of wine (Belisario-Sánchez et al., 2009; Kutyna et al., 2010; Stoll et al., 2010; Schmidtke et al., 2012; Bindon et al., 2013). Considering microbiological applications, several strategies that use genetically modified *Saccharomyces cerevisiae* strain have also been proposed for reduction of alcohol content in wine (Ehsani et al., 2009; Kutyna et al., 2010; Varela et al., 2012). More recently, Tilloy et al. (2014) used evolution-based strategies together with breeding strategies to show that evolved or hybrid strains can led to ethanol reductions of 0.6 to 1.3% (v/v) in comparison with the ancestral strains.

Another approach to reduce the production of ethanol might be the use of non-*Saccharomyces* wine yeast as part of the natural microbiota present on grapes and winemaking equipment during grape juice fermentation (Renouf et al., 2005, 2007). The use of non-*Saccharomyces* yeast in combination with *S. cerevisiae* has been proposed to improve the quality and enhance the complexity of wine (Jolly et al., 2014; Capozzi et al., 2015). Thus, the use of controlled multistarter fermentation using selected cultures of non-*Saccharomyces* and *S. cerevisiae* yeast strains has been encouraged (Ciani and Comitini, 2011; Comitini et al., 2011; Domizio et al., 2011; Magyar and Tóth, 2011; Di Maio et al., 2012; Ehsani et al., 2012; Morata et al., 2012; Jolly et al., 2014). In this context, non-*Saccharomyces* wine yeast and multistarter fermentation might have a role in the reduction of the ethanol content in wine. The wide variability amongst non-*Saccharomyces* yeast regarding ethanol yield, fermentation efficiency, biomass production, by-product formation, and respiro-fermentative metabolism might be used to reduce the ethanol concentration in wine. Among non-*Saccharomyces* wine yeasts some strains/species showed low ethanol yield and sugar consumption by respiration (Crabtree negative). Using these selected strains, 1–2% v/v of ethanol reduction was achieved but prolonged time of sequential inoculation or high level of acetic acid were shown (Contreras et al., 2014; Gobbi et al., 2014; Quirós et al., 2014).

Sequential fermentation adequately setup might be an attractive tool for the use of non-*Saccharomyces* yeast for the reduction of the ethanol content in wine. This fermentative approach, in which an initial inoculation of a non-*Saccharomyces* strain is followed by inoculation of the *S. cerevisiae* starter strain, would allow the metabolism of the first inoculated yeast to be exploited without too great an influence on the *S. cerevisiae* strain. To benefit from the metabolic particularities of some non-*Saccharomyces* yeast in sequential fermentation (i.e., low ethanol yield, low fermentation efficiency), the inoculation level and the duration of the interval between the first and second inoculations are fundamental. An enhancement of the inoculation level of non-*Saccharomyces* yeast will improve the competitiveness toward wild yeast and *S. cerevisiae* starter strain and, at the same time, this will increase the expression of their metabolic activity.

In the present study, we evaluated the initial use of immobilized non-*Saccharomyces* yeast in sequential fermentation trials in terms of reduction of the ethanol content in the wine. The immobilization procedures allowed high inoculation rates, with the consequent high reaction rates, to reduce the delay before the *S. cerevisiae* starter strain inoculation. This also avoids possible contamination under actual winemaking conditions, due to this late inoculation of *S. cerevisiae*.

MATERIALS AND METHODS

Yeast Strains

The non-*Saccharomyces* yeast strains used in this study were *Starmerella bombicola* (formerly named *Candida stellata*) DiSVA66, (DBVPG # 3827; Industrial Yeast Collection of the University of Perugia), *Metschnikowia pulcherrima* DiSVA269, *Hanseniaspora osmophila* DiSVA253, and *Hanseniaspora uvarum* DiSVA252. These were obtained from the Yeast Collection of the Department of Life and Environmental Sciences (DiSVA) of the Polytechnic University of Marche (Italy). All of the strains were previously selected and used in mixed fermentation trials to enhance the analytical and aromatic profile of the wine, as well as to improve the wine complexity (Ciani and Ferraro, 1998; Comitini et al., 2011; Domizio et al., 2011). These were used here as the initial fermentation trials for sequential fermentations with *S. cerevisiae* commercial strain Lalvin EC1118 (Lallemand Inc., Toulouse, France), which was also used in pure culture as the control.

All of the strains were maintained at −80°C for long-term storage, in cryovials supplemented with 80% (w/v) glycerol as the cryoprotective agent. Subsequently, the strains were cultured on Yeast Peptone Dextrose (YPD) agar medium at 25°C for 48–72 h, and stored at 4°C.

MEDIA

Synthetic Grape Juice

Synthetic grape juice (SGJ) for the micro-fermentation trials was prepared using three different solutions: solution A (500 mL), solution B (250 mL), and solution C (250 mL). The three solutions were sterilized at 121°C for 20 min separately and then combined aseptically (Ciani and Ferraro, 1996). Solution A contained 110 g D-glucose, 110 g D-fructose, 10 mg ergosterol, and 1 ml Tween 80. Four milliliters of ergosterol stock solution (Tween 80, 6.25 mL; ergosterol, 62.5 mg in ethanol to make 25 mL) was added to the glucose-fructose solution to complete solution A. Solution B contained 6 g L-(+)-tartaric acid, 3 g L-(−)-malic acid, and 0.5 g citric acid. Solution C was a mix of 1.7 g Yeast Nitrogen Base without amino acids and without ammonium sulfate (DIFCO), 0.2 g $CaCl_2$, 2 g casamino acids, 0.8 g arginine-HCl, 1 g L-(2)-proline, and 0.1 g L-(2)-tryptophan. Solutions B and C were buffered at pH 3.5 with NH_4OH and H_3PO_4, respectively.

Natural Grape Juice

Natural grape juice (NGJ) was obtained during 2014 vintage and came from Verdicchio, a white grape variety that is grown in the Marche region, in central Italy. The main characteristics of the grape juice were: pH 3.39; total acidity, 8.27 g/L; free SO_2,

12 mg/L; total SO_2, 48 mg/L; malic acid, 3.3 g/L; initial sugar content, 202 g/L; yeast assimilable nitrogen, 160 mg N/L.

Immobilization Procedures

Modified YPD medium (0.5% yeast extract, 0.1% peptone, 2% dextrose, all w/v) was used to produce biomass at 25°C for 72 h in a rotary shaker (150 rpm). This biomass, for the immobilization system, were harvested by centrifugation, washed three times with sterile distilled water and added to 2.5% Na-alginate (Carlo Erba, Milan, Italy), at a ratio of 5% (wet w/v). Using a peristaltic pump, this mixture was then dripped into $CaCl_2$ (0.1 M) to induce gelation. After 1 h, the beads formed were washed several times with sterile distilled water and then used immediately. The inoculum for the immobilized cells of the non-*Saccharomyces* was 10% (wet w/v), which corresponded to an inoculum of *ca*. 2×10^8 cells/mL in SGJ or NGJ.

Fermentation Conditions

To evaluate the influence of the sequential inoculations using immobilized non-*Saccharomyces* cells on the ethanol content, several fermentation trials were set up with SGJ and NGJ. In SGJ, the inoculated immobilized non-*Saccharomyces* cells were removed after 48 or 72 h, and the free *S. cerevisiae* cells (1×10^6 cell/mL) were inoculated into the partially fermented grape juice. In NGJ, the sequential fermentation trials were conducted by inoculation of the *S. cerevisiae* starter culture after only 72 h, with and without the removal of the immobilized cells.

Cultures of *S. cerevisiae* were pre-incubated in SGJ at 25°C in a rotary shaker (150 rpm) for 48 h, harvested by centrifugation, and washed with sterile distilled water, with the procedure standardized to provide an inoculation level of 1×10^6 cells/mL. Before the *S. cerevisiae* inoculation, 1 g of beads containing non-*Saccharomyces* yeast were collected and maintained under agitation in 50 mL 1% Na-citrate solution (w/v) for 1 h, to release the cells. Cell viability was then evaluated by standard plate counting techniques, in YPD medium. Parallel control trials were carried out using free *S. cerevisiae* cells only.

The fermentation trials were carried out in 1-L glass minifermenters that contained 400 mL SGJ or NGJ under static conditions at 25°C and in duplicate. The minifermenters had two ports, one for gas flow and the other for the inoculation of beads, and a septum of a glass frit, to maintain the beads in the medium and to allow carbon dioxide to come out.

The weight loss of the minifermenters due to CO_2 evolution was followed until the end of the fermentation trials (constant weight for three consecutive days). Samples of media and beads were taken after 48, 72 h, and at the end of the fermentation and underwent chemical and microbiological analysis respectively.

Analytical Determinations

Ethanol was measured by gas-liquid chromatography (GLC) analysis (AOAC, 1990). Acetaldehyde, ethyl acetate, and higher alcohols were determined by direct injection into the GLC system. Samples were injected into a column of 30 m × 0.32 mm, with 0.25 μm film thickness (Zebron ZB-WAXPlus; Phenomenex, Torrance, California, USA) with an internal standard of 1-pentanol (162 mg/L). Nitrogen was used as the carrier gas. A Shimadzu gas chromatograph (Japan) equipped with a flame ionization detector was used. The oven temperature ranged from 40°C to 200°C. The temperature of the injector and the detector was 220°C. The volatile compounds were extracted using an ether–hexane (1:1) extraction technique, and evaluated by capillary GLC. For quantification, and before their extraction, the samples were spiked with a known amount of 3-octanol, as the internal standard (1.6 mg/l). A glass capillary column was used: 0.25 μm Supelcowax 10 (length, 60 m; internal diameter, 0.32 mm). One microliter was injected in split–splitless mode, with 60 s splitless; temperature of injection, 220° C; temperature of detector, 250°C; carrier gas, helium; and flow rate, 2.5 mL/min. The temperature program was: 50°C for 5 min; 3°C /min to 220°C, and then 220°C for 20 min. The compounds were identified and quantified by comparisons with external calibration curves for each compound. The glucose, fructose, glycerol, and succinic acid concentrations were determined using specific enzyme kits (Megazyme International Ireland). Volatile acidity (expressed as grams acetic acid per liter) was quantified by steam distillation, according to the official analytical methods (EC, 2000).

Statistical Analysis

Analysis of variance (ANOVA) was applied to the experimental data for the main enological characteristics of the wines. The means were analyzed using the STATISTICA 7 software. The significant differences were determined using Duncan tests, and the data were considered significant if the associated *P*-values were <0.05.

RESULTS

Fermentation Kinetics and Main Fermentation Parameters of Synthetic Grape Juice Trials

In the SGJ fermentation trials, the inoculated immobilized non-*Saccharomyces* cells were removed after 48 and 72 h, and the partial fermented SGJ was then inoculated with free *S. cerevisiae* cells (**Figure 1**). The 48 h SGJ trials (**Figure 1A**) showed that the control *S. cerevisiae* improved the fermentation kinetics compared to the sequential fermentation trials with the initial non-*Saccharomyces* yeast. The sequential fermentation trials in SGJ with *M. pulcherrima* and *S. bombicola* showed overlapping fermentation kinetics that were quicker than for the *H. uvarum* and *H. osmophila*. Moreover, all of the sequential fermentation trials showed less final CO_2 evolved when compared to the control *S. cerevisiae*.

The data for the fermentation parameters of the sequential fermentations inoculated after 48 h in SGJ are summarized in **Table 1**. All of the sequential fermentation trials showed significantly lower ethanol content when compared with the control *S. cerevisiae*, with residual sugar less than 3 g/L. In particular, the sequential fermentation trials carried out with *M. pulcherrima*, *S. bombicola,* and *H. uvarum* showed comparable lower final ethanol production, while with *H. osmophila* there was higher ethanol content. Data for the ethanol yield basically

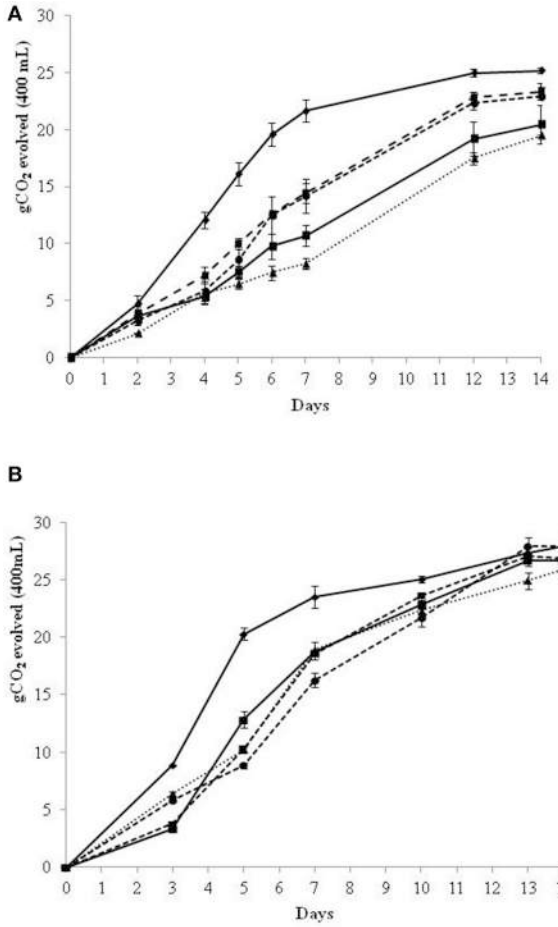

FIGURE 1 | Growth kinetics in sequential fermentation trials of immobilized non-*Saccharomyces* yeast and control *S. cerevisiae* on synthetic grape juice (SGJ). Beads were removed at 48 h **(A)** and 72 h **(B)** of fermentation. —◆—*S. cerevisiae* control culture; ····▲····*H. osmophila/S. cerevisiae*; - -●- -*S. bombicola/S. cerevisiae*; —■—*H. uvarum/S. cerevisiae*; - ■ - *M. pulcherrima/S. cerevisiae.*

confirmed those of ethanol production. At 48 h of fermentation, the control *S. cerevisiae* showed a sugar consumption of 25%, while the non-*Saccharomyces* sequential fermentation trials using the immobilized cells, showed significantly lower sugar consumption, from 11 to 21%; with the exception of *M. pulcherrima* (38%). Again, and with the exception of *M. pulcherrima*, the other sequential fermentation trials resulted in significant increases in glycerol, in comparison with the control *S. cerevisiae*. For the volatile acidity, there were no significant differences seen, while the succinic acid content significant increased in all fermentation trials (with the exception of *H. osmophila*) with the immobilized non-*Saccharomyces* yeast, particularly with *S. bombicola*.

Since, 48 h sequential fermentation showed a limited sugar consumption and with the aim to enhance the fermentation performance of immobilized cells, 72 h sequential fermentation was evaluated. In 72 h SGJ sequential fermentation trials (**Figure 1B**), the control *S. cerevisiae* showed essentially the same fermentation kinetics as described above for the 48 h trials. Over these first 3 days of fermentation with the immobilized non-*Saccharomyces* species, *H. osmophila,* and *S. bombicola* showed enhanced fermentation kinetics in comparison with *M. pulcherrima* and *H. uvarum*. After removal of the beads and inoculation of *S. cerevisiae*, all of the sequential fermentation trials showed lower fermentation kinetics when compared with the control *S. cerevisiae*, although all condition achieved comparable amounts of CO_2 evolution at the end of the fermentation trials.

In this regard, the inoculation delay for the addition of free *S. cerevisiae* cells from 48 to 72 h resulted in further reductions in ethanol content in comparison with the control *S. cerevisiae*, paired with a small increase in the residual sugars (**Table 2**). *H. uvarum* was the only sequential fermentation trial that did not show any difference in ethanol reduction from the 48 to 72 h inoculation of *S. cerevisiae*. Data for the ethanol yield confirmed this trend. Indeed, and with the exception of *H. uvarum*, all of these sequential fermentation trials showed a significant reduction in ethanol yield in comparison with the control *S. cerevisiae*. As expected, there was increased sugar consumption of the immobilized non-*Saccharomyces* yeast from 48 to 72 h, which varied from 37 to 52% of total sugars. For glycerol content, only the *S. bombicola* and *H. uvarum* sequential fermentation trials showed a significant increase when compared with the control *S. cerevisiae* (**Table 2**). All sequential fermentation trials showed significant, but limited, increases in volatile acidity and succinic acid (although the succinic acid content did not reach statistical significance for *H. uvarum*).

Fermentation Kinetics and Main Fermentation Parameters of Natural Grape Juice Trials

After identifying the delay time of the inoculum of *S. cerevisiae* starter (72 h) that allows the immobilized cells to consume around 50% of initial sugars in a synthetic medium, we carried out non-*Saccharomyces* immobilized cells sequential fermentations in NGJ (Verdicchio grape juice) to evaluate the overall fermentation parameters and the analytical profile of wines. The fermentation kinetics of the sequential fermentation trials in NGJ, conducted with the removal of the immobilized cells, showed similar behaviors to those in SGJ for 72 h (**Figure 2A**). Different fermentation kinetics were shown in NGJ without the removal of beads, and hence in the continued presence of the immobilized non-*Saccharomyces* species (**Figure 2B**). These data highlighted that in the presence of the immobilized cells during the whole fermentation process, this resulted in increased fermentation kinetics by day 7, with the same CO_2 evolved as for the control *S. cerevisiae*. After this time, all of the fermentation kinetics showed overlap (**Figure 2B**).

The data regarding to the main fermentation parameters in NGJ both with or without beads removal are reported in **Table 3**, and they confirm the significantly lower ethanol content in all of the sequential fermentation trials, in comparison with the control *S. cerevisiae*, which were all accompanied by little or no residual sugars (<2 g/L).

TABLE 1 | The main fermentation parameters for non-*Saccharomyces* yeast in sequential fermentation trials with synthetic grape juice, with *S. cerevisiae* EC1118 inoculated after 48 h of fermentation.

Fermentation trials	Sugar consumed at 48 h (%)	End of fermentation					
		Sugar consumed (g/L)	Ethanol (% v/v)	Ethanol yield (g/g)	Glycerol (g/L)	Volatile acidity (as acetic acid g/L)	Succinic acid (g/l)
S. cerevisiae control culture	24.56 ± 0.62^{a}	218.77 ± 0.10^{b}	12.54 ± 0.02^{a}	0.452 ± 0.001^{a}	5.32 ± 0.44^{b}	0.38 ± 0.02^{d}	0.37 ± 0.04^{c}
H. osmophila/S. cerevisiae	20.98 ± 0.01^{b}	217.83 ± 0.05^{c}	11.76 ± 0.12^{b}	0.426 ± 0.004^{b}	6.70 ± 0.02^{a}	0.54 ± 0.01^{c}	$0.44 \pm 0.06^{b,c}$
M. pulcherrima/S. cerevisiae	38.33 ± 0.01^{c}	219.41 ± 0.04^{a}	11.40 ± 0.07^{c}	0.410 ± 0.002^{c}	5.64 ± 0.29^{b}	0.63 ± 0.02^{b}	0.53 ± 0.04^{b}
S. bombicola/S. cerevisiae	17.42 ± 0.75^{d}	217.67 ± 0.14^{c}	11.36 ± 0.01^{c}	0.412 ± 0.000^{c}	7.54 ± 0.70^{a}	0.57 ± 0.00^{c}	1.03 ± 0.01^{a}
H. uvarum/S. cerevisiae	10.56 ± 1.71^{e}	218.83 ± 0.05^{b}	11.48 ± 0.00^{c}	0.414 ± 0.000^{c}	6.99 ± 0.07^{a}	0.68 ± 0.01^{a}	0.49 ± 0.01^{b}

The initial sugar concentration was 220 g/L. Data are means ± standard deviations from two independent experiments. Data with different superscript letters (^{a,b,c,d,e}) within each column are different according to Duncan tests (0.05%).

TABLE 2 | The main fermentation parameters for non-*Saccharomyces* yeast in sequential fermentation trials with synthetic grape juice, with *S. cerevisiae* EC1118 inoculated after 72 h of fermentation.

Fermentation trials	Sugar consumed at 72 h (%)	End of fermentation					
		Sugar consumed (g/L)	Ethanol (% v/v)	Ethanol yield (g/g)	Glycerol (g/L)	Volatile acidity (as acetic acid g/L)	Succinic acid (g/L)
S. cerevisiae control culture	43.82 ± 0.03^{b}	220.00 ± 0.11^{a}	12.36 ± 0.27^{a}	0.443 ± 0.009^{a}	5.18 ± 0.07^{c}	0.36 ± 0.00^{d}	0.39 ± 0.00^{c}
H. osmophila/S. cerevisiae	52.16 ± 2.30^{a}	216.90 ± 0.28^{c}	11.03 ± 0.16^{b}	$0.401 \pm 0.005^{b,c}$	5.25 ± 0.17^{c}	0.66 ± 0.01^{a}	0.71 ± 0.10^{b}
M. pulcherrima/S. cerevisiae	37.22 ± 2.12^{d}	217.79 ± 0.04^{b}	11.01 ± 0.22^{b}	0.400 ± 0.008^{c}	5.07 ± 0.02^{c}	0.51 ± 0.01^{b}	0.59 ± 0.03^{b}
S. bombicola/S. cerevisiae	42.20 ± 1.63^{b}	216.74 ± 0.14^{c}	11.08 ± 0.00^{b}	$0.403 \pm 0.000^{b,c}$	7.46 ± 0.01^{a}	$0.49 \pm 0.01^{b,c}$	1.12 ± 0.10^{a}
H. uvarum/S. cerevisiae	39.54 ± 071^{c}	215.86 ± 0.37^{d}	11.58 ± 0.43^{b}	$0.423 \pm 0.000^{a,b}$	6.18 ± 0.19^{b}	0.47 ± 0.01^{c}	$0.55 \pm 0.01^{b,c}$

The initial sugar concentration was 220 g/L. Data are means ± standard deviations of two independent experiments. Data with different superscript letters (^{a,b,c,d}) within each column are different according to Duncan tests (0.05%).

The same was also observed for the ethanol yield, which confirmed that the reduction of ethanol content was mainly due to the lower yield. In this context, the main by-products showed some variations: the *S. bombicola* sequential fermentation trial confirmed the highest production of glycerol and succinic acid as previously reported (Ciani and Ferraro, 1998), while *H. osmophila* showed the lowest glycerol content. Compared to the control *S. cerevisiae*, in the NGJ sequential trials *H. uvarum* showed a significant increase in volatile acidity, while the other sequential fermentation trials showed comparable or lower values.

Viability and Cell Release from the Beads

Two important features to monitor in the use of these immobilized cells are the loss of cell viability and the cells released from the beads, which is closely related to the conservation of the structure of the matrix. The data reported in **Table 4** show that in SGJ, the viability of all of the non-*Saccharomyces* yeast after 48 and 72 h was around 1×10^9 cell/g, without significant loss of cell viability after their use. The low levels of cells released after their use (*ca.* 1×10^2 cell/mL after 48 h, and 1×10^3 cell/mL after 72 h) confirmed the high cell viabilities, thus indicating the good integrity of the beads. In NGJ, there was comparable high cell viability, although there was also an increase in the cell release (about 1×10^4 cell/mL), which indicated some break-up of the matrix.

Ethanol Reduction

Figure 3 summarizes the ethanol reductions obtained across all of the sequential and control fermentation trials. The data for SGJ indicate that the delay in the *S. cerevisiae* inoculation from 48 to 72 h generally promoted further ethanol reductions. Indeed, from 48 to 72 h, the ethanol reductions were from 1.18 to 1.28% (v/v) for *S. bombicola*, from 1.14 to 1.35% (v/v) for *M. pulcherrima*, and from 0.78 to 1.33% (v/v) for *H. osmophila*. In contrast, this was opposite for *H. uvarum*, that ranged from

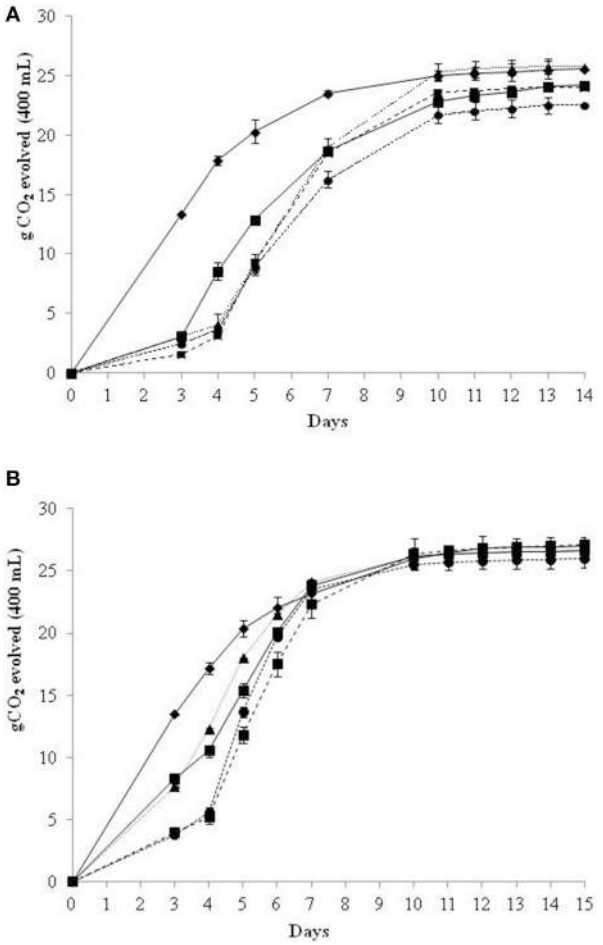

FIGURE 2 | Growth kinetics in sequential fermentation trials of immobilized non-*Saccharomyces* yeast and control *S. cerevisiae* on natural grape juice (NGJ). Beads were removed at 72 h **(A)** and without beads removal **(B)**: *S. cerevisiae* control culture; *H. osmophila/S. cerevisiae*; *S. bombicola/S. cerevisiae*; *H. uvarum/S. cerevisiae*; *M. pulcherrima/S. cerevisiae*.

1.06 to 0.78% (v/v). In NGJ, the immobilized non-*Saccharomyces* yeast showed a comparable or little bit lower ethanol reduction than that exhibited by sequential fermentation trials at 72 h in SGJ, without any significant differences among the non-*Saccharomyces* species. However, significantly greater reductions in ethanol were obtained in the trials without the beads removal, thus leaving the non-*Saccharomyces* yeast in the fermentation trials with the *S. cerevisiae*. In these fermentation trials, there were generally significant improvements in the ethanol reductions over the 72 h NGJ sequential fermentation trials: from 1.10 to 1.46% (v/v) for *M. pulcherrima*, from 1.17 to 1.64% (v/v) for *S. bombicola*, and from 1.04 to 1.21% (v/v) for *H. uvarum*. Only the *H. osmophila* sequential fermentation trial did not show any statistically significant variation here (from 1.04 to 1.00% [v/v]).

The Main Volatile Compounds in Sequential Fermentations on Natural Grape Juice

To determine the influence of the non-*Saccharomyces* sequential fermentation trials on the aromatic profile of the wine, the main volatile compounds on NGJ were assayed, and the results are summarized in **Table 5**.

An increase in ethyl acetate was showed in all sequential fermentations with the exception of *S. bombicola* trials, in both with or without beads removal. However, only *H. uvarum* sequential fermentation showed unacceptable level of this compound (around the threshold value for a negative impact on the aromatic profiles amounting to 175 mg/L).

Regarding to the other esters, all sequential fermentations showed comparable or lower amount of phenyl ethyl acetate, ethyl hexanoate, and ethyl octanoate when compared with the control *S. cerevisiae* while significant increases in ethyl butyrate content was showed in *S. bombicola* (both with and without beads removal trials) and *H. uvarum* (only without beads removal). Furthermore, *H. osmophila* sequential fermentation showed an increase in isoamyl acetate, which is responsible of the fruity note, in both trials while *M. pulcherrima* and *S. bombicola* sequential fermentations exhibited an enhancement of this ester only without beads removal trials.

Regarding to the higher alcohols, sequential fermentations showed variable production of n-propanol. Isobutanol, amylic alcohol, and hexanol did not show significant differences between sequential fermentations and control trials while an increase in isoamyl alcohol in *H. osmophila* and *M. pulcherrima* but only without beads removal trials was found. A generalized reduction in β-phenyl ethanol content was shown in all sequential fermentation trials when compared with the control *S. cerevisiae*. On the contrary, an enhancement in acetaldehyde content was found in all sequential fermentations.

Regarding the volatile terpenes, a significant increase in geraniol content was exhibited by *M. pulcherrima* sequential fermentation confirming the capability of this yeast strain to liberate volatile terpens by glycosidase activity (Comitini et al., 2011).

DISCUSSION

Different microbiological approaches have been proposed to reduce the ethanol content in wine, such as genetically modified *S. cerevisiae* yeast (Ehsani et al., 2009; Kutyna et al., 2010; Varela et al., 2012; Rossouw et al., 2013), evolution-based strategies, together with breeding strategies (Abalos et al., 2011; Tilloy et al., 2014) and the use of non-*Saccharomyces* wine yeast (Contreras et al., 2014; Gobbi et al., 2014; Quirós et al., 2014).

In this last approach, the strategies include the need to manage the fermentation on the basis of several enological traits of the non-*Saccharomyces* species used. Several wine yeast species could be selected for their low ethanol yield, alcoholic fermentation efficiency, biomass and by-product formation as a result of the diversion of carbon away from ethanol production (Ciani and Maccarelli, 1998; Gobbi et al., 2014). On the other hand, these non-*Saccharomyces* wine yeasts could be used to promote sugar consumption via respiration rather than fermentation, through partial aeration of the grape juice (Gonzalez et al., 2013).

Both these approaches have indicated the promising use of non-*Saccharomyces* wine yeast to limit ethanol production. The use of various amounts of oxygen added during the first stages

TABLE 3 | The main fermentation parameters for non-*Saccharomyces* yeast in sequential fermentation trials with natural grape juice, with *S. cerevisiae* EC1118 inoculated after 72 h of fermentation and without removing the beads.

	With removing beads					Without removing the beads				
Fermentation trials	***S. cerevisiae* control culture**	***H. osmophila*/ *S. cerevisiae***	***M. pulcherrima*/ *S. cerevisiae***	***S. bombicola*/ *S. cerevisiae***	***H. uvarum*/ *S. cerevisiae***	***S. cerevisiae* control culture**	***H. osmophila*/ *S. cerevisiae***	***M. pulcherrima*/ *S. cerevisiae***	***S. bombicola*/ *S. cerevisiae***	***H. uvarum*/ *S. cerevisiae***
Sugar consumed at 72 h (%)	47.72 ± 0.75^{b}	54.76 ± 1.94^{a}	39.80 ± 0.38^{g}	$41.34 \pm 0.77^{e,f}$	43.57 ± 2.17^{c}	48.46 ± 0.02^{b}	55.26 ± 0.26^{a}	$40.79 \pm 0.19^{f,g}$	$42.08 \pm 0.003^{d,e}$	$42.83 \pm 0.72^{c,d}$
Sugar consumed (g/L)	201.39 ± 0.14^{a}	199.74 ± 0.03^{c}	199.74 ± 0.03^{c}	199.67 ± 0.13^{c}	200.83 ± 0.0^{b}	200.58 ± 0.46^{b}	200.81 ± 0.31^{b}	200.62 ± 0.38^{b}	$201.04 \pm 0.25^{a,b}$	$200.90 \pm 0.2^{a,b}$
Ethanol (% v/v)	12.06 ± 0.04^{a}	11.12 ± 0.11^{c}	$11.06 \pm 0.09^{c,d}$	10.99 ± 0.06^{d}	11.12 ± 0.04^{c}	12.00 ± 0.03^{a}	11.00 ± 0.04^{d}	10.54 ± 0.03^{f}	10.36 ± 0.05^{g}	10.79 ± 0.05^{e}
Ethanol yield (g/g)	0.476 ± 0.001^{a}	0.439 ± 0.004^{c}	$0.437 \pm 0.003^{c,d}$	$0.434 \pm 0.002^{d,e}$	$0.436 \pm 0.00^{c,d}$	0.472 ± 0.001^{b}	0.432 ± 0.002^{e}	0.414 ± 0.002^{g}	0.406 ± 0.002^{h}	0.423 ± 0.002^{f}
Glycerol (g/L)	$5.87 \pm 0.19^{d,e}$	5.36 ± 0.08^{f}	$5.88 \pm 0.11d^{e}$	7.63 ± 0.04^{b}	6.02 ± 0.03^{d}	5.68 ± 0.27^{e}	4.80 ± 0.18^{g}	$5.80 \pm 0.30^{d,e}$	8.43 ± 0.20^{a}	6.74 ± 0.21^{c}
Volatile acidity (as acetic acid g/L)	$0.56 \pm 0.01^{c,d}$	0.49 ± 0.0^{d}	$0.58 \pm 0.02^{b,c}$	$0.55 \pm 0.1^{c,d}$	0.67 ± 0.00^{a}	$0.63 \pm 0.07^{b,c}$	$0.59 \pm 0.03^{a,b,c}$	$0.65 \pm 0.04^{a,b}$	$0.58 \pm 0.02^{b,c}$	0.67 ± 0.06^{a}
Succinic acid (g/L)	$0.52 \pm 0.02^{d,e}$	0.70 ± 0.10^{c}	0.60 ± 0.02^{c}	0.90 ± 0.04^{b}	0.47 ± 0.00^{e}	$0.53 \pm 0.01^{d,e}$	$0.65 \pm 0.02^{c,d}$	$0.66 \pm 0.11^{c,d}$	1.16 ± 0.03^{a}	$0.57 \pm 0.12^{c,d,e}$

The initial sugar concentration was 202 g/L. Data are means ± standard deviations. Data with different superscript letters (^{a,b,c,d,e,f,g}) within each column are different according to Duncan tests (0.05%).

TABLE 4 | Non-*Saccharomyces* viable cell counts and cells released from the beads in synthetic grape juice and natural grape juice at 48 and 72 h of fermentation trials.

Fermentation trials	Synthetic grape juice				Natural grape juice	
	48 h		72 h		72 h	
	Viable cells (Log cell/g)	Cells released (Log CFU/mL)	Viable cells (Log cell/g)	Cells released (Log CFU/mL)	Viable cells (Log cell/g)	Cells released (Log CFU/mL)
H. osmophila/S. cerevisiae	9.64 ± 0.01	2.28 ± 0.04	9.64 ± 0.02	3.30 ± 0.05	9.36 ± 0.21	4.19 ± 0. 20
M. pulcherrima/S. cerevisiae	9.58 ± 0.82	2.03 ± 0.01	9.40 ± 0.02	3.02 ± 0.03	8.60 ± 0.34	4.64 ± 0.11
S. bombicola/S. cerevisiae	9.40 ± 0.70	2.57 ± 0.04	9.58 ± 0.40	3.56 ± 0.03	8.60 ± 0.34	4.60 ± 0.06
H. uvarum/S. cerevisiae	9.36 ± 0.24	2.02 ± 0.01	9.36 ± 0.03	3.25 ± 0.10	8.82 ± 0.21	4.03 ± 0.01

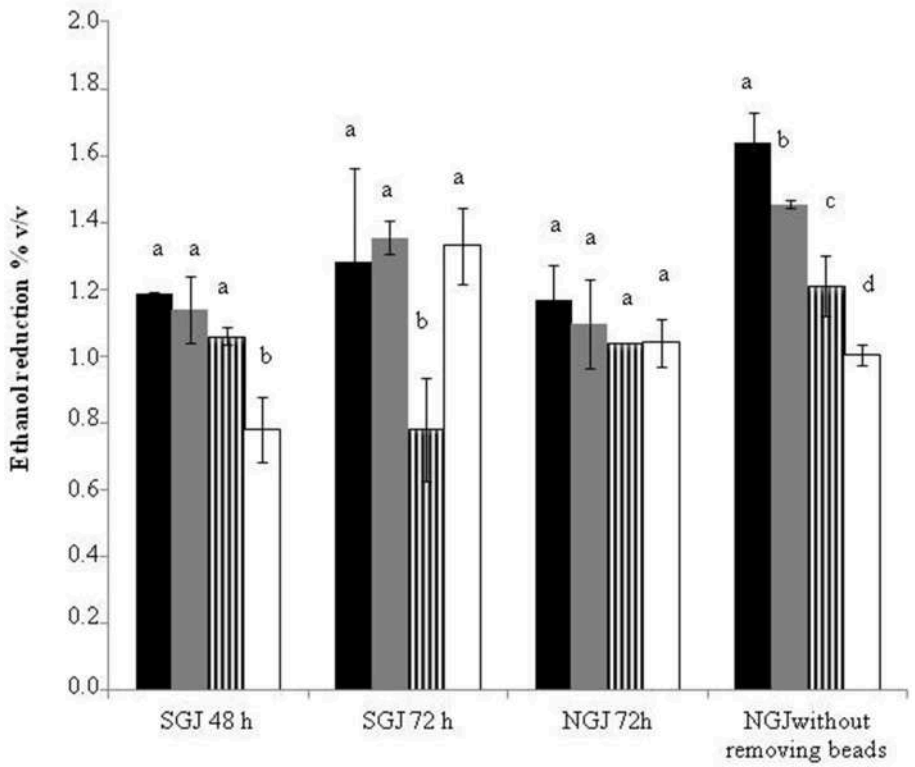

FIGURE 3 | Ethanol reduction with synthetic grape juice and natural grape juice. ■ *S. bombicola/S. cerevisiae*; ▒ *M. pulcherrima/S. cerevisiae*; ▥ *H. uvarum/S. cerevisiae*; □ *H. osmophila/S. cerevisiae*. Data with different superscript letters (^{a,b,c,d}) within each trials are different according to Duncan tests (0.05%).

of fermentation of mixed fermentations results in significant reduction in ethanol production (Contreras et al., 2015b; Morales et al., 2015). Indeed, under limited aerated conditions, *M. pulcherrima*, *Torulaspora delbrueckii,* and *Zygosaccharomyces bailii* mixed fermentations resulted in reduced ethanol content, from 1.5 to 2.2% (v/v), while under high agitation and aeration rates this resulted in unacceptable amounts of acetic acid by *S. cerevisiae* partner strain (Quirós et al., 2014; Contreras et al., 2015b). However, the effects of aerobic conditions on the analytical and sensorial profiles and oxygen modulation in mixed fermentations were not evaluated and for these reasons, the aeration of grape juice requires further investigations. Recently, under anaerobic conditions, a reduction in the alcohol level was achieved in fermentations performed using sequential inoculation with a strain of *M. pulcherrima* (Contreras et al., 2014, 2015a). Using a strain of *M. pulcherrima* in sequential fermentation trials, 50% of sugar consumed was achieved in white and red grape juice, with a delay of the second inoculation with *S. cerevisiae* strain of 9 and 17 days resulting in an ethanol reduction of 0.9 and 1.6% (v/v), respectively (Contreras et al., 2014). However, a long delay for timing of second inoculation is difficult to manage under winery conditions, because of wild microflora contamination, where the competitiveness of the non-*Saccharomyces* strain is low, and the wild *S. cerevisiae* strains can easily dominate the fermentation process.

TABLE 5 | The main volatile compounds (mg/L) of sequential fermentation trials in natural grape juice with *S. cerevisiae* EC1118 inoculated after 72 h of fermentation with and without removing beads.

	With removing beads					Without removing beads				
Fermentation trials	***S. cerevisiae* control culture**	***H. osmophila*/ *S. cerevisiae***	***M. pulcherrima*/ *S. cerevisiae***	***S. bombicola*/ *S. cerevisiae***	***H. uvarum*/ *S. cerevisiae***	***S. cerevisiae* control culture**	***H. osmophila*/ *S. cerevisiae***	***M. pulcherrima*/ *S. cerevisiae***	***S. bombicola*/ *S. cerevisiae***	***H. uvarum*/ *S. cerevisiae***
ESTERS										
Ethyl butyrate	0.17 ± 0.01^{e}	0.20 ± 0.01^{c}	0.21 ± 0.01^{c}	0.25 ± 0.04^{b}	0.20 ± 0.01^{c}	0.19 ± 0.01^{e}	0.20 ± 0.06^{c}	0.21 ± 0.00^{d}	0.26 ± 0.01^{b}	0.53 ± 0.02^{a}
Ethyl acetate	36.94 ± 0.69^{f}	51.52 ± 0.03^{e}	83.10 ± 2.12^{c}	36.68 ± 1.64^{f}	195.79 ± 2.9^{a}	36.94 ± 0.40^{f}	68.04 ± 3.35^{d}	87.42 ± 0.51^{c}	45.44 ± 0.31^{e}	165.68 ± 0.9^{b}
Phenyl ethylacetate	0.043 ± 0.007^{a}	0.036 ± 0.004^{a}	0.037 ± 0.004^{a}	0.027 ± 0.001^{a}	0.035 ± 0.01^{a}	0.031 ± 0.002^{a}	0.029 ± 0.014^{a}	0.032 ± 0.007^{a}	0.027 ± 0.007^{a}	0.034 ± 0.00^{a}
Ethyl hexanoate	0.11 ± 0.01^{a}	$0.09 \pm 0.00^{b,c}$	$0.10 \pm 0.00^{a,b}$	0.01 ± 0.00^{f}	0.08 ± 0.01^{c}	0.11 ± 0.001^{a}	0.05 ± 0.03^{d}	0.03 ± 0.00^{e}	0.01 ± 0.00^{f}	0.11 ± 0.00^{a}
Ethyl octanoate	$0.089 \pm 0.003^{a,b}$	0.020 ± 0.00^{e}	$0.084 \pm 0.001^{a,b}$	0.096 ± 0.00^{a}	$0.032 \pm 0.01^{d,e}$	0.076 ± 0.002^{b}	$0.028 \pm 0.037^{d,e}$	0.074 ± 0.001^{b}	0.059 ± 0.00^{c}	0.037 ± 0.00^{d}
Isoamyl acetate	0.20 ± 0.01^{d}	0.27 ± 0.10^{c}	0.22 ± 0.01^{d}	0.22 ± 0.01^{d}	0.22 ± 0.01^{d}	0.22 ± 0.06^{d}	0.41 ± 0.02^{a}	0.41 ± 0.01^{a}	0.33 ± 0.02^{b}	0.22 ± 0.01^{d}
ALCOHOLS										
n-propanol	64.2 ± 0.95^{d}	35.7 ± 0.4^{e}	36.3 ± 0.3^{e}	86.5 ± 0.4^{b}	34.5 ± 2.7^{e}	65.1 ± 0.5^{d}	75.1 ± 1.5^{c}	106.0 ± 2.3^{a}	57.7 ± 1.8^{d}	88.1 ± 2.4^{b}
Isobutanol	8.6 ± 1.1^{b}	7.0 ± 1.1^{b}	$4.4 \pm 0.1^{b,c}$	$4.5 \pm 0.7b^{c}$	$4.2 \pm 1.7^{b,c}$	7.9 ± 2.8^{b}	$5.6 \pm 2.2^{b,c}$	$4.0 \pm 0.8b^{c}$	$5.2 \pm 0.2^{b,c}$	11.6 ± 3.4^{a}
Amylc alcohol	$10.2 \pm 0.3^{b,c}$	$8.6 \pm 0.5^{b,c}$	15.7 ± 1.2^{a}	$8.6 \pm 1.4^{b,c}$	10.6 ± 2.4^{b}	$9.3 \pm 0.02^{b,c}$	16.1 ± 0.1^{a}	$10.1 \pm 2.7^{b,c}$	11.1 ± 0.4^{b}	$10.2 \pm 2.7^{b,c}$
Isoamylic alcohol	99.8 ± 0.1^{c}	106.8 ± 0.4^{b}	$94.9 \pm 0.7^{c,d}$	81.9 ± 0.8^{e}	84.7 ± 2.4^{e}	97.9 ± 1.2^{c}	129.4 ± 0.2^{a}	106.6 ± 1.9^{b}	$92.1 \pm 0.1^{c,d}$	83.7 ± 0.1^{e}
β-Phenyl ethanol	$30.8 \pm 1.2^{a,b}$	$23.3 \pm 0.5^{c,d}$	21.0 ± 1.4^{d}	$26.9 \pm 0.7^{b,c}$	$23.3 \pm 0.5^{c,d}$	$31.9 \pm 2.9^{a,b}$	19.8 ± 2.1^{d}	32.6 ± 1.7^{a}	$30.2 \pm 1.6^{a,b}$	20.3 ± 3.1^{d}
Hexanol	$0.047 \pm 0.000^{a,b}$	0.049 ± 0.001^{a}	0.026 ± 0.00^{e}	0.040 ± 0.003^{c}	0.27 ± 0.00^{e}	$0.048 \pm 0.001^{a,b}$	0.048 ± 0.02^{a}	0.035 ± 0.004^{d}	$0.047 \pm 0.00^{a,b}$	$0.043 \pm 0.004^{b,c}$
CARBONYL COMPOUNDS										
Acetaldehyde	53.3 ± 0.7^{f}	113.3 ± 1.4^{b}	116.9 ± 1.4^{b}	87.14 ± 3.4^{d}	112.3 ± 3.1^{b}	51.3 ± 0.4^{f}	95.2 ± 0.8^{c}	73.8 ± 2.6^{e}	70.1 ± 2.7^{e}	126.2 ± 2.9^{a}
Acetoin	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
MONOTERPENS										
Linalool	0.006 ± 0.001^{a}	$0.005 \pm 0.002^{a,b}$	$0.005 \pm 0.00^{a,b}$	0.003 ± 0.00^{b}	0.003 ± 0.00^{b}	0.006 ± 0.00^{a}	0.006 ± 0.00^{a}	$0.004 \pm 0.001^{a,b}$	$0.005 \pm 0.00^{a,b}$	0.003 ± 0.00^{b}
Nerol	0.217 ± 0.002^{a}	0.081 ± 0.003^{c}	0.065 ± 0.001^{d}	0.048 ± 0.003^{e}	0.030 ± 0.003^{f}	0.216 ± 0.002^{a}	$0.074 \pm 0.035^{c,d}$	0.077 ± 0.004^{c}	0.096 ± 0.007^{b}	0.063 ± 0.00^{d}
Geraniol	0.188 ± 0.012^{b}	0.193 ± 0.010^{b}	0.266 ± 0.002^{a}	0.153 ± 0.001^{c}	0.111 ± 0.01^{e}	0.193 ± 0.010^{b}	0.131 ± 0.097^{d}	0.255 ± 0.015^{a}	$0.138 \pm 0.006^{c,d}$	0.149 ± 0.00^{c}

Data are means ± standard deviations of two independent experiment. Data with different superscript letters (^{a,b,c,d,e,f}) within each column are different according to Duncan tests (0.05%). ND = not detected

In this context, the management of non-*Saccharomyces* yeast in mixed fermentation trials with the aim to reduce the ethanol content is a crucial step. Indeed, to achieve sugar consumption of about 50% using non-*Saccharomyces* yeast as the starter culture, a long delay of the *S. cerevisiae* starter inoculation would be needed. In the present study, we evaluated the use of four selected non-*Saccharomyces* strains in immobilized forms in both SGJ and NGJ, to obtain high inoculation levels and the consequent high metabolic activity, to reduce the time of the second inoculation. Under these conditions, with a delay of 3 days, we obtained a sugar consumption that ranged from 40 to 54% with an ethanol reduction from 1.0 to 1.17% (v/v). Without removing beads, the alcohol reduction was further enhanced. Under these conditions, *M. pulcherrima* and *S. bombicola* confirmed the benefits for ethanol reduction in mixed fermentation trials than for those with *H. uvarum* and *H. osmophila*, which showed alcohol reductions of 1.4 and 1.6% v(/v), respectively. As previously shown, *M. pulcherrima* and *S. bombicola* in mixed fermentations, can reduce the ethanol content (Ciani and Ferraro, 1998; Contreras et al., 2014, 2015a,b; Quirós et al., 2014). The significant enhancement of by-products such as glycerol or succinic acid do not justify the ethanol reduction obtained. Other fermentation by-products that were not evaluated in this study and coming from glycerol-pyruvic fermentation or other metabolic pathways could explain this result. Moreover, pyruvate-metabolism is strictly linked to amino-acids, organic acids, and lipids biosynthesis and, consequently, sugar carbon could follow these pathways (Gancedo and Serrano, 1989).

Another important feature that should be highlighted is the analytical profile of final wines. In this context, the wine obtained showed in general comparable or better analytical profiles then for the control *S. cerevisiae*. Indeed, all fermentation trials showed a limited increase of acetaldehyde content and only *H. uvarum* sequential fermentation exhibited significant amount of ethyl acetate that negatively affect the aroma profile of final wine. On the other hand, an enhancement in sequential fermentations of some desired compounds was shown. In particular, *S. bombicola* showed an enhancement in of glycerol and succinic acid, *M. pulcherrima* exhibited an increase in geraniol while *H. osmophila* displayed a significant increase in isoamyl acetate and isoamyl alcohol.

In conclusion, the non-*Saccharomyces M. pulcherrima* and *S. bombicola* are both promising wine yeast species for use in immobilized forms in sequential fermentation trials to reduce the ethanol content in wine. The use of high inoculation levels and immobilization procedures, however, results in substantial increases in the management costs of the fermentation process. For these reasons, further investigations are necessary to explore reductions in the bead concentrations, modulation of grape juice aeration, and evaluation of the sensorial profile of the resulting wine.

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AUTHOR CONTRIBUTIONS

LC, LO, FC, and MC contributed equally to this manuscript. All authors participated in the design and discussion of the research. LC and LO carried out the experimental part of the work.LC, LO, MC, and FC carried out the analysis of the data and wrote the manuscript. All authors have read and approved the final manuscript.

REFERENCES

Abalos, D., Vejarano, R., Morata, A., González, C., and Suárez-Lepe, J. A. (2011). The use of furfural as a metabolic inhibitor for reducing the alcohol content of model wines. *Eur. Food Res. Technol.* 232, 663–669. doi: 10.1007/s00217-011-1433-9

Alstona, J. M., Fuller, K. B., James, T., Lapsley, J. T., and Soleas, G. (2011). Too much of a good thing? Causes and consequences of increases in sugar content of California wine grape. *J. Wine Econom.* 6, 135–159. doi: 10.1017/S1931436100001565

AOAC (1990). *Official Methods of Analyses*, *15th Edn.*, Vol. 2. Washington, DC: Association of Official Analytical Chemists.

Athès, V., Lillo, M. P. Y., Bernard, C., Pérez-Correa, R., and Souchon, I. (2004). Comparison of experimental methods for measuring infinite dilution volatilities of aroma compounds in water/ethanol mixtures. *J. Agric. Food Chem.* 52, 2021–2027. doi: 10.1021/jf0350257

Belisario-Sánchez, Y. Y., Taboada-Rodríguez, A., Marín-Iniesta, F., and López-Gómez, A. (2009). Dealcoholized wines by spinning cone column distillation: phenolic compounds and antioxidant activity measured by the 1,1-diphenyl-2-picrylhydrazyl method. *J. Agric. Food Chem.* 57, 6770–6778. doi: 10.1021/jf900387g

Bindon, K., Varela, C., Kennedy, J., and Herderich, H. H. M. (2013). Relationships between harvest time and wine composition in *Vitis vinifera* L. cv. Cabernet Sauvignon 1. Grape and wine chemistry. *Food Chem.* 138, 1696–1705. doi: 10.1016/j.foodchem.2012.09.146

Capozzi, V., Garofalo, C., Chiriatti, M. A., Grieco, F., and Spano, G. (2015). Microbial terroir and food innovation: The case of yeast biodiversity in wine. *Microbiol Res.* 181, 75–83. doi: 10.1016/j.micres.2015.10.005

Ciani, M., and Comitini, F. (2011). Non-*Saccharomyces* wine yeasts have a promising role in biotechnological approaches to winemaking. *Ann. Microbiol.* 61, 25–32. doi: 10.1007/s13213-010-0069-5

Ciani, M., and Ferraro, L. (1996). Enhanced glycerol content in wines made with immobilized *Candida stellata* cells. *Appl. Environ. Microbiol.* 62, 128–132.

Ciani, M., and Ferraro, L. (1998). Combined use of immobilized *Candida stellata* cells and *Saccharomyces cerevisiae* to improve the quality of wines. *J. Appl. Bacteriol.* 85, 247–254. doi: 10.1046/j.1365-2672.1998.00485.x

Ciani, M., and Maccarelli, F. (1998). Oenological properties of non-*Saccharomyces* yeasts associated with winemaking. *World J. Microbiol. Biol.* 14, 199–203.

Comitini, F., Gobbi, M., Domizio, P., Romani, C., Lencioni, L., Mannazzu, I., et al. (2011). Selected non-*Saccharomyces* wine yeasts in controlled multistarter fermentations with *Saccharomyces cerevisiae*. *Food Microbiol.* 28, 873–888. doi: 10.1016/j.fm.2010.12.001

Contreras, A., Curtin, C., and Varela, C. (2015a). Yeast population dynamics reveal a potential 'collaboration' between *Metschnikowia pulcherrima* and *Saccharomyces uvarum* for the production of reduced alcohol wines during Shiraz fermentation. *Appl. Microbiol. Biotechnol.* 99, 1885–1895. doi: 10.1007/s00253-014-6193-6

Contreras, A., Hidalgo, C., Schmidt, S., Henschke, P. A., Curtin, C., and Varela, C. (2014). Evaluation of non-*Saccharomyces* yeasts for the reduction

of alcohol content in wine. *Appl. Environ. Microbiol.* 80, 1670–1678. doi: 10.1128/AEM.03780-13

Contreras, A., Hidalgo, C., Schmidt, S., Henschke, P. A., Curtin, C., and Varela, C. (2015b). The application of non-*Saccharomyces* yeast in fermentations with limited aeration as a strategy for the production of wine with reduced alcohol content. *Int. J. Food Microbiol.* 205, 7–15. doi: 10.1016/j.ijfoodmicro.2015.03.027

Di Maio, S., Genna, G., Gandolfo, V., Amore, G., Ciaccio, M., and Oliva, D. (2012). Presence of *Candida zemplinina* in Sicilian musts and selection of a strain for wine mixed fermentations. *S. Afr. J. Enol. Vitic.* 33, 80–87.

Domizio, P., Romani, C., Comitini, F., Gobbi, M., Lencioni, L., Mannazzu, I., et al. (2011). Potential spoilage non-*Saccharomyces* yeasts in mixed cultures with *Saccharomyces cerevisiae*. *Ann. Microbiol.* 61, 137–144. doi: 10.1007/s13213-010-0125-1

EC (2000). *Commission Regulation (EC) no. 2870/2000 of 19 December 2000, Laying Down the Community Reference Methods for the Analysis of Spirits Drinks*. Brussels: Official Journal of the European Communities.

Ehsani, M., Fernandez, M. R., Biosca, J. A., Julien, A., and Dequin, S. (2009). Engineering of 2,3-butanediol dehydrogenase to reduce acetoin formation by glycerol-overproducing, low-alcohol *Saccharomyces cerevisiae*. *Appl. Environ. Microbiol.* 75, 3196–3205. doi: 10.1128/AEM.02157-08

Ehsani, M., Renault, P., and Puente-López, V. (2012). L'impact organoleptique de *Torulaspora delbrueckii* en conditions oenologiques lors d'une inoculation séquentielle avec *Saccharomyces cerevisiae*. *Rev. Oenologues. Tech. Vitivinicoles Oenologicques* 39, 23–26.

Gancedo, C., and Serrano, R. (1989). "Energy yielding metabolism," in *The Yeasts*, Vol. 3, 2nd Edn., eds A. H. Rose and J. S. Harrison (London: Academic Press), 205–259.

Gawel, R., Van Sluyter, S., and Waters, E. J. (2007). The effects of ethanol and glycerol on the body and other sensory characteristics of Riesling wines. *Aust. J. Grape Wine Res.* 13, 38–45. doi: 10.1111/j.1755-0238.2007.tb00070.x

Gobbi, M., De Vero, L., Solieri, L., Comitini, F., Oro, L., Giudici, P., et al. (2014). Fermentative aptitude of non-*Saccharomyces* wine yeast for reduction in the ethanol content in wine. *Eur. Food. Res. Technol.* 239, 41–48. doi: 10.1007/s00217-014-2187-y

Gonzalez, R., Quiròs, M., and Morales, P. (2013). Yeast respiration of sugars by non-*Saccharomyces* yeast species: a promising and barely explored approach to lowering alcohol content of wines. *Trend Food Sci. Technol.* 29, 55–61. doi: 10.1016/j.tifs.2012.06.015

Grant, M. (2010). "Who is listening?," in *Proceedings of the Fourteenth Australian Wine Industry Technical Conference; 3–8 July 2010; Adelaide, SA, Australia,* eds R. Blair, T. H. Lee, and S. Pretorius (Urrbrae, SA: The Australian Wine Industry Technical Conference), 25–27.

Guth, H., and Sies, A. (2001). "Flavour of wines: towards an understanding by reconstitution experiments and an analysis of ethanol's effect on odour activity of key compounds," in *Proceedings of the Eleventh Australian Wine Industry Technical Conference; 7–11 October 2001; Adelaide, SA, Australia,* eds R. J. Blair, P. J. Williams, and P. B. Hoj (Urrbrae, SA: Australian Wine Industry Technical Conference), 128–139.

Jolly, N. P., Varela, C., and Pretorius, I. S. (2014). Not your ordinary yeast: non-*Saccharomyces* yeasts in wine production uncovered. *FEMS Yeast Res.* 14, 215–237. doi: 10.1111/1567-1364.12111

Jones, G. V., White, M. A., Cooper, O. R., and Storchmann, K. (2005). Climate change and global wine quality. *Clim. Change* 73, 319–343. doi: 10.1007/s10584-005-4704-2

Kutyna, D. R., Varela, C., Henschke, P. A., Chambers, P. J., and Stanley, G. A. (2010). Microbiological approaches to lowering ethanol concentration in wine. *Trends Food Sci. Technol.* 21, 293–302. doi: 10.1016/j.tifs.2010.03.004

MacAvoy, M. G. (2010). "Wine – harmful or healthy? What is being considered in Australia and New Zealand?," in *Proceedings of the Fourteenth Australian Wine Industry Technical Conference; 3–8 July 2010; Adelaide, SA, Australia,* eds R. J. Blair, P. J. Williams, and S. Pretorius (Urrbrae, SA: Australian Wine Industry Technical Conference), 28–31.

Magyar, I., and Tóth, T. (2011). Comparative evaluation of some oenological properties in wine strains of *Candida stellata*, *Candida zemplinina*, *Saccharomyces uvarum* and *Saccharomyces cerevisiae*. *Food Microbiol.* 28, 94–100. doi: 10.1016/j.fm.2010.08.011

Morales, P., Rojas, V., Quirós, M., and Gonzalez, R. (2015). The impact of oxygen on the final alcohol content of wine fermented by a mixed starter culture. *Appl. Microbiol. Biotechnol.* 99, 3993–4003. doi: 10.1007/s00253-014-6321-3

Morata, A., Benito, S., Loira, I., Palomero, F., González, M. C., and Suarez-Lepe, J. A. (2012). Formation of pyranoanthocyanins by *Schizosaccharomyces pombe* during the fermentation of red must. *Int. J. Food Microbiol.* 159, 47–53. doi: 10.1016/j.ijfoodmicro.2012.08.007

Quirós, M., Rojas, V., Gonzalez, R., and Morales, P. (2014). Selection of non-*Saccharomyces* yeast strains for reducing alcohol levels in wine by sugar respiration. *Int. J. Food Microbiol.* 181, 85–91. doi: 10.1016/j.ijfoodmicro.2014.04.024

Renouf, V., Claisse, O., and Lonvaud-Funel, A. (2005). Understanding the microbial ecosystem on the grape berry surface through numeration and identification of yeast and bacteria. *Aust. J. Grape Wine Res.* 11, 316–327. doi: 10.1111/j.1755-0238.2005.tb00031.x

Renouf, V., Strehaiano, P., and Lonvaud-Funel, A. (2007). Yeast and bacteria analysis of grape, wine and cellar equipment by PCR-DGGE. *Int. J. Sci. Vin. Vigne* 41, 51–61.

Rossouw, D., Heyns, E. H., Setati, M. E., Bosch, S., and Bauer, F. F. (2013). Adjustment of trehalose metabolism in wine *Saccharomyces cerevisiae* strains to modify ethanol yields. *Appl. Environ. Microbiol.* 79, 5197–5207. doi: 10.1128/AEM.00964-13

Schmidtke, L. M., Blackman, J. W., and Agboola, S. O. (2012). Production technologies for reduced alcoholic wines. *J. Food Sci.* 77, R25–R41. doi: 10.1111/j.1750-3841.2011.02448.x

Stoll, M., Lafontaine, M., and Schultz, H. R. (2010). Possibilities to reduce the velocity of berry maturation through various leaf area to fruit ratio modifications in *Vitis vinifera* L. Riesling. *Progrès Agricole et Viticole.* 127, 68–71.

Tilloy, V., Ortiz-Julien, A., and Dequin, S. (2014). Biotechnology reduction of ethanol yield and improvement of glycerol formation by adaptive evolution of the wine yeast *Saccharomyces cerevisiae* under hyperosmotic conditions. *Appl. Environ. Microbiol.* 80, 2623–2632. doi: 10.1128/AEM.03710-13

Varela, C., Kutyna, D. R., Solomon, M. R., Black, C. A., Borneman, A., Henschke, P. A., et al. (2012). Evaluation of gene modification strategies for the development of low-alcohol-wine yeasts. *Appl. Environ. Microbiol.* 78, 6068–6077. doi: 10.1128/AEM.01279-12

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Simultaneous Alcoholic and Malolactic Fermentations by *Saccharomyces cerevisiae* and *Oenococcus oeni* Cells Co-immobilized in Alginate Beads

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Malolactic fermentation (MLF) usually takes place after the end of alcoholic fermentation (AF). However, the inoculation of lactic acid bacteria together with yeast starter cultures is a promising system to enhance the quality and safety of wine. In recent years, the use of immobilized cell systems has been investigated, with interesting results, for the production of different fermented foods and beverages. In this study we have carried out the simultaneous immobilization of *Saccharomyces cerevisiae* and *Oenococcus oeni* in alginate beads and used them in microvinifications tests to produce Negroamaro wine. The process was monitored by chemical and sensorial analyses and dominance of starters and cell leaking from beads were also checked. Co-immobilization of *S. cerevisiae* and *O. oeni* allowed to perform an efficient fermentation process, producing low volatile acidity levels and ethanol and glycerol concentrations comparable with those obtained by cell sequential inoculum and co-inoculum of yeast and bacteria cells in free form. More importantly, co-immobilization strategy produced a significant decrease of the time requested to complete AF and MLF. The immobilized cells could be efficiently reused for the wine fermentation at least three times without any apparent loss of cell metabolic activities. This integrated biocatalytic system is able to perform simultaneously AF and MLF, producing wines similar in organoleptic traits in comparison with wines fermented following traditional sequential AF and MLF with free cell starters. The immobilized-cell system, that we here describe for the first time in our knowledge, offers many advantages over conventional free cell fermentations, including: (i) elimination of non-productive cell growth phases; (ii) feasibility of continuous processing; (iii) re-use of the biocatalyst.

Keywords: wine fermentation, *Saccharomyces cerevisiae*, *Oenococcus oeni*, co-immobilization, biocatalyst for wine

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INTRODUCTION

Yeasts are the most important microorganisms responsible of alcoholic fermentation (AF), whereas lactic acid bacteria are able to perform malolactic fermentation (MLF) in winemaking (Diviès and Cachon, 2005). Several yeast commercial starter cultures are nowadays available for production of safe wines improved in desirable taste and aroma features (Romano et al., 2003). MLF is a secondary

process that can spontaneously occur several weeks after the AF, during storage of young wines. In fact, it consists of conversion, promoted by malolactic bacteria, of L-malic acid into L-lactic acid and CO_2, it is responsible of the acidity reduction and of the pH increase and it can contribute to the final wine flavor complexity (Bauer and Dicks, 2004). However, this bioprocess is unpredictable and very slow. Although starters have been selected also for the MLF, their use did not ensure that the process will start, occur, or be completed, especially due to the unfavorable conditions of the wine-environment for the bacterial growth (Alexandre et al., 2004).

In recent years, it is increasing the interest in using immobilized cells for fermentation processes, such as beer production (Masschelein et al., 1994), cider production (Nedovic et al., 2000), sparkling wine fermentation (Yokotsuka et al., 1997). Compared to the conventional free cell system, these strategies offer numerous technical and economic advantages (Nedović et al., 2011). Several immobilization systems have been studied for wine fermentation, such as calcium and sodium alginate, delignified cellulosic materials, Kissiris, DEAE–cellulose (Kosseva and Kennedy, 2004; Kourkoutas et al., 2004; Agouridis et al., 2008) and starchy materials (Nedović et al., 2015).

Immobilization approaches can influence yeast and bacteria metabolism producing effects on wine quality, aroma, and taste. It has also been demonstrated that these systems can improve AF and MLF productivity and economic efficiency, since immobilization can make easier to control the process and produces an acceleration of it (Melzoch et al., 1994; Sipsas et al., 2009; Vila-Crespo et al., 2010). Immobilization systems offer also the advantage to reuse the biocatalysts for several times without loss of fermentation activities, to perform continuous process and to decrease capital costs reducing bioreactor volumes (Pilkington et al., 1998; Bleve et al., 2011). It gives also the opportunity to co-immobilize different kind of microorganisms within the same porous matrix, allowing the accomplishment of the two fermentation steps in one integrated system.

In this study, for the first time in our knowledge, we have immobilized in a calcium alginate matrix a mixed AF/MLF starter i.e., a commercial strain of *S. cerevisiae* and a commercial strain of *O. oeni*. We have used this immobilized multistarter mix to promote the fermentation of Negroamaro must. The obtained wines were characterized for their fermentation kinetics, chemical profiles associated to AF and MLF and flavor profiles.

MATERIALS AND METHODS

Yeast, Bacterial Strains, and Media

Oenococcus oeni strain Lalvin VP41™ and *Saccharomyces cerevisiae* strain Lalvin ICV-D254® were supplied by Lallemand Fermented Beverages (Italy). Growth medium for *O. oeni* strain and for the propagation in alginate beads was FT80 medium (Cavin et al., 1989). The bacteria were grown at 30°C for 2–3 days under anaerobic conditions. The initial pH-values were adjusted to 5.2. Cycloheximide was added at a concentration of 100 mg/L in the solid and liquid FT80 media, in order to selectively count bacteria in wine samples inoculated also with *S. cerevisiae*. Growth medium for *S. cerevisiae* strain and for the propagation in alginate beads was YPD at 30°C for 16–18 h. Ampicillin was added at a concentration of 50 mg/L in the solid and liquid YPD media, in order to selectively count yeasts in wine samples inoculated also with *O. oeni*. Solid media were prepared with addition of 2% (w/v) agar.

Immobilization of *Oenococcus oeni* Whole Cells in Ca-Alginate Gel Beads

The following parameters of sodium alginate concentration and initial inoculum quantity were optimized for the preparation of beads. Different concentrations of sodium alginate (2, 3, and 4% w/v) were tested. The initial biomass to be packed in the beads was ascertained by incorporating in the aqueous sodium alginate solution different amount of cells corresponding to 10^4, 10^5, 10^6, or 10^7 CFU/mL. The *O. oeni* cells were grown in FT80 medium at 30°C for 24–48 h. When the turbidity of the culture reached an optical density at 600 nm = 0.8, the requested volume was harvested. Cells were washed with water and then suspended in Na-alginate (Sigma, USA) solution at the above indicated concentrations and then cured using a 0.1 M $CaCl_2$ solution. The beads were prepared following the procedure described by Bleve et al. (2008). Beads were then washed with saline solution, added with 250 mL of sterilized must (sugars 173.4 ± 0.7, malic acid 2.25 ± 0.4, pH 3.33) and subjected to fermentation at room temperature.

Viable counts of cells were periodically evaluated on agar plates by spreading 10-fold serial dilutions of the fermented must onto FT80 added with cycloheximide at 30°C for 2–3 days under anaerobic conditions.

Immobilization of *S. cerevisiae* and *O. oeni* Whole Cells in Ca-Alginate Gel Beads

The yeast cells were grown in YPD medium with shaking at 25°C for 16 h, whereas *O. oeni* cells were grown in FT80 medium at 30°C for 24–48 h. When the turbidity of the yeast and bacteria cultures reached an optical density of 0.8 at 600 nm, the requested volume was harvested. Cells were washed with water and then suspended in aliquot 3% Na-alginate (Sigma, USA) solution to obtain a final concentration of 10^6 CFU/mL for *O. oeni* and for *S. cerevisiae*. The beads were prepared following the procedure described by Bleve et al. (2008), using a 0.1 M $CaCl_2$ solution. The spherical beads (ca. 3 mm diameter) produced were cured in 0.1 M $CaCl_2$ solution for 4 h at 4°C.

Micro-Fermentation Assays

Micro-fermentation assays were conducted in Negroamaro grape must, as previously described (Tristezza et al., 2012). The must was filtered twice both through cheesecloth and a 0.22-μm membrane filter and, then, it was combined with 50 mg/L potassium metabisulphite. The must composition was: reducing sugars 220 ± 0.6 g/L, Brix 19.7, total acidity 6.12 ± 0.7 g/L, volatile acidity 0.22 ± 0.02 g/L, pH 3.3, malic acid 4.5 ± 0.3 g/L, tartaric acid 2.5 ± 0.08 g/L, glycerol 0.68 ± 0.04 g/L, density 1.07 g/ml. Two hundred milliliters of must were placed in sterile Erlenmeyer 250-mL flasks and then inoculated with yeast and bacteria in free and

immobilized form pre-cultured in specific media described above. The relative volume of beads inoculated in 200 ml of grape must was about 35–40 ml, corresponding to an additional 17.5–20% of the final volume. Alcoholic fermentations were carried out at 25°C and samples were weighted daily in order to follow the volatile CO_2 production until the weight was constant.

MLF was monitored at determined time intervals by the depletion of L-malic acid and this organic acid was detected using an enzymatic kit (La Roche, Basel, Switzerland). Each fermentation experiment was carried out by performing three simultaneous and independent tests.

An aliquot of fermented must (100 mL) was stored at −20°C, whereas the remaining wine was used for sensorial analysis. Each fermentation experiment was carried out by performing three simultaneous and independent tests.

The experimental plan included 10 trials based on different combinations of inocula:

a) beads containing co-immobilized *S. cerevisiae* and *O. oeni* cells, corresponding to an inoculum equivalent to 10^6 CFU/mL of *O. oeni* and of *S. cerevisiae* (sample C1);
b) a simultaneous inoculum of beads containing *S. cerevisiae* and *O. oeni* cells separately immobilized, corresponding to an inoculum equivalent to 10^6 CFU/mL of *O. oeni* or of *S. cerevisiae* (sample C2);
c) a simultaneous inoculum of beads containing *S. cerevisiae* corresponding to an inoculum equivalent to 10^6 CFU/mL and an inoculum of free 10^6 CFU/mL *O. oeni* cells (sample C3);
d) beads containing an inoculum of 10^6 CFU/mL *S. cerevisiae* cells, followed, at the end of AF, by beads containing an inoculum equivalent to 10^6 CFU/mL of *O. oeni* (sample C4);
e) a simultaneous inoculum of 10^6 CFU/mL free *S. cerevisiae* and 10^6 CFU/mL *O. oeni* cells (sample C5);
f) an inoculum of free 10^6 CFU/mL *S. cerevisiae* cells, followed, at the end of AF, by an inoculum equivalent to 10^6 CFU/mL free cells of *O. oeni* (sample C6);
g) an inoculum of free 10^6 CFU/mL *S. cerevisiae* cells, followed, at the end of AF, by beads containing an inoculum equivalent to 10^6 CFU/mL of *O. oeni* (sample C7);
h) an inoculum of free 10^6 CFU/mL *S. cerevisiae* cells to perform the only AF (sample C8);
i) control sample of must treated with uninoculated beads (sample C9);
j) uninoculated must control sample (sample C10).

Viable counts of cells were periodically evaluated on agar plates by spreading 10-fold serial dilutions of the fermented must onto YPD agar added with ampicillin and incubating at 28°C overnight for enumeration of yeasts and onto FT80 added with cycloheximide at 30°C for 2–3 days under anaerobic conditions for enumeration of oenococci. Also samples inoculated with free cells of *S. cerevisiae* and/or *O. oeni* were screened in the same conditions.

Chemical Analysis

General wine parameters (alcohol content, residual sugars, pH, titratable and volatile acidity, tartaric, citric, lactic acid, glycerol, and total sulfur dioxide) were determined using WineScan FT120 (Foss, Hillerød, Denmark) instrument. Malic acid was detected using an enzymatic kit (La Roche, Basel, Switzerland). The analyses were performed in triplicate.

Reagents and Standards

The standards of volatile compounds were purchased from Sigma–Aldrich (St. Louis, MO), with purity superior to 98%; methanol (HPLC gradient grade) and ethanol 96% were purchased from J.T. Baker (Phillipsburg, NJ, USA), and dichloromethane were purchased from Carlo Erba Reactive (Rodano, Italy). Pure water was obtained from a Milli-Q purification system (Millipore, USA).

Extraction of Volatile Compounds

The extraction of volatile compounds was carried out with a solid phase extraction (SPE) procedure, using polymeric sorbents and dichloromethane as elution sorbent (Ferreira et al., 1995; Piñeiro et al., 2006; Capone et al., 2013). A Vac Elut 20 station equipment from Varian (Palo Alto, USA) was used. The wine aroma compounds were separated by adsorption/desorption on cartridges. Strata polymeric SPE sorbents (styrene-divinylbenzene) prepacked in 500 mg/6 mL cartridges (Phenomenex) were first rinsed with 4 mL of dichloromethane, 4 mL of methanol and, finally, 4 mL of a water–ethanol mixture (12%, v/v).

To 50 mL of each wine sample and to each standard solution were added 300 μL of internal standard solution (2-octanol; 200 mg/L hydro-alcoholic solution). Each liquid sample was passed through the SPE cartridge at around 2 mL/min. Afterwards, the sorbent was dried by letting air pass through it. The volatile compounds were recovered by elution with 4 mL of dichloromethane, and concentrated to a final volume of 500 μL under a stream of pure nitrogen (N_2) (Shinohara, 1985; Vilanova and Sieiro, 2006; Gómez García-Carpintero et al., 2011a).

The sample (1 μL) was injected in the gas chromatographic system, the analyses were performed in triplicate and mean values were used in further data processing.

GC-MS Conditions and Quantitative Analysis

A 6890N series gas chromatograph (Agilent Technologies) with an Agilent 5973 mass spectrometer selective detector (MSD) and equipped with a DB-WAX capillary column (60 m*0.25 mm I.D., 0.25 μm film thickness, Agilent Technologies) was used. The carrier gas was helium at a flow rate of 1.0 mL/min. A split/splitless injector was used in the splitless mode, the injector temperature was 250°C and the injected volume was 2 μL. The column oven temperature was initially held at 40°C, then it was programmed to 200°C at 4°C/min, with a final holding time of 20 min. Spectra were recorded in the electron impact mode (ionization energy, 70 eV) in a range of 30–500 amu at 3.2 scans/s. A solvent delay time of 10 min was used to avoid overloading the mass spectrometer with solvent.

The identification of the volatile compounds was achieved by comparing mass spectra with those of the data system

library (NIST 98, $P > 90\%$), with the retention data of commercially available standards and MS data reported in the literature. Quantification analysis is based on the principle that the component area is proportional to the amount of the analyte present in the sample. The quantification was carried out following the internal standard quantification method.

Odor Activity Value

The specific contribution of each odorant compound to the overall wine aroma was determined by calculating the odor activity value (OAV) as the ratio of the concentration of each compound to its detection threshold concentration (Francis and Newton, 2005). An odor profile for the wines was obtained by grouping the volatile compounds with similar descriptor in specific aromatic series. The value of each aromatic series was obtained adding the OAVs of the compounds that form such a series. Therefore, it is possible to determine the contribution of a specific compound to each series. This procedure makes to relate quantitative information obtained by chemical analysis (GC-MS) to sensory perception, providing a hybrid chemical/sensory fingerprinting (Capone et al., 2013).

Statistical Analysis

Statistical analysis of the wine general parameters and wine volatile concentrations were performed using an analysis of variance (ANOVA) to determine statistically different values at a significance level of $P \leq 0.05$.

The comparison of volatile classes of compounds during fermentations was achieved by principal component analysis (PCA). All statistical analyses were carried out using the STATISTICA 7.0 software (StatSoft software package, Tulsa, OK, USA).

RESULTS

Optimization of *Oenoccus oeni* Immobilization in Calcium Alginate

The first step of this study consisted in the optimization of the following parameters for the *O. oeni* the immobilization, i.e., initial cell biomass to be loaded in the beads and sodium alginate concentration, in order to improve bead properties, such as permeability and rigidity. Different *O. oeni* cell concentrations, i.e., 10^4, 10^5, 10^6, and 10^7 CFU/mL, were individually immobilized in each beads preparation using 2,

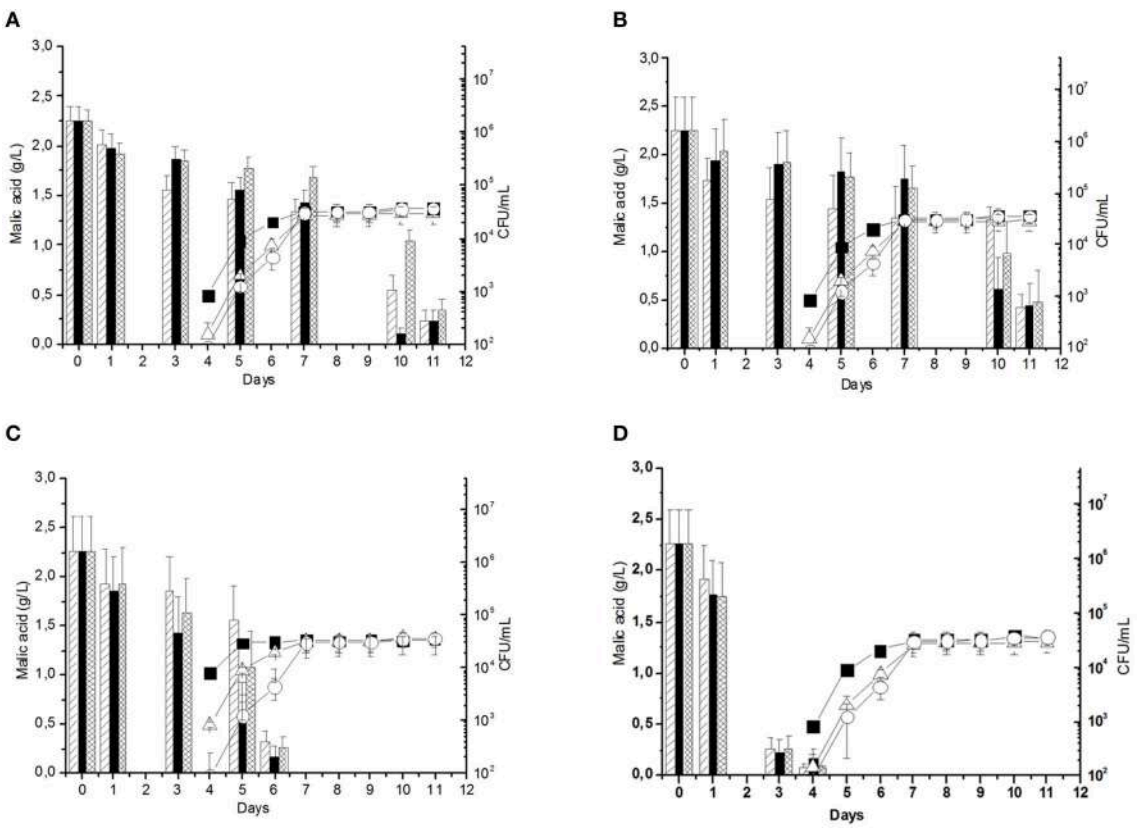

FIGURE 1 | Malic acid consumption in Negroamaro must inoculated with beads of 2%, 3%, and 4% calcium alginate containing *Oenococcus oeni* strain Lalvin VP41™. Panels **(A–D)** refer to results obtained using calcium alginate beads containing 10^4, 10^5, 10^6, 10^7 CFU/ml of *O. oeni*, respectively. Cell leaking and cell growth (CFU/ml) from beads containing 2%, 3%, and 4% (w/v) calcium alginate is indicated in each panel. The curves indicate the cell leaking.

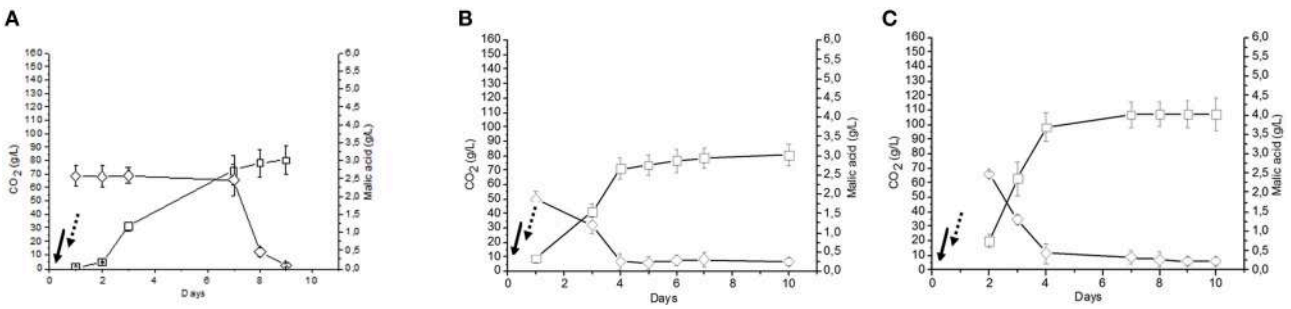

FIGURE 2 | Cycles of Negroamaro must fermentations inoculated with *Oenococcus oeni* strain Lalvin VP41™ and *Saccharomyces cerevisiae* strain Lalvin ICV-D254® co-immobilized in alginate beads (sample C1). Each fermentation cycle was performed for 10 days. At the end of AF and MLF, the fermentation cycle was considered completed, the wine was removed and submitted to quality assays. The beads were collected and, after a wash with sterile saline solution (0.9% NaCl), reinoculated for another fermentation cycle. Letters on the top of each panel indicate the three different cycles of fermentation **(A–C)**. CO_2 (g/l) production, Malic acid consumption. Inoculation time of *S. cerevisiae*, inoculation time of *O. oeni*.

3, and 4% (w/v) sodium alginate concentrations. Malolactic fermentation experiments were performed for each sample in filtered *Negroamaro* must. As reported in **Figure 1**, malic acid was completely consumed after 6 and 4 days of fermentation when inocula corresponding to 10^6 (**Figure 1C**) and 10^7 (**Figure 1D**) CFU/mL were used, respectively. Instead, the use of lower cell concentrations (10^4 and 10^5 CFU/mL, **Figures 1A,B**) did not produce a complete consumption of malic acid along the 11 days period of the experiment. In the used experimental conditions, comparable cell counts were recorded in all the analyzed samples, thus suggesting that the immobilization conditions and initial cell inocula did not influence final cell counts into the medium. The use of 3% (w/v) calcium alginate and of an inoculum of *O. oeni* cells corresponding to 10^6 CFU/mL resulted the minimum conditions to obtain a significant reduction in time required to completely reduce malic acid. In addition, the use of 3% (w/v) calcium alginate was suitable to perform co-immobilization experiments with yeasts, since it resulted the best conditions to immobilize yeasts (Bleve et al., 2008).

Microfermentations Using Different Yeast and Bacteria Inoculation Strategies

The fermentative performances of the two immobilization strategies, i.e., co-immobilized *S. cerevisiae* and *O. oeni* (C1 sample) and co-inoculation of beads containing separately immobilized *S. cerevisiae* and *O. oeni* (C2 sample) were analyzed. For both the utilized strategies, AF and MLF occurred simultaneously and resulted in a significant shortening of the time requested to complete the fermentation. In fact, the process was completed in a maximum of 10 days (**Figures 2A**, **3A**). The cell counts deriving from cell leakage from beads and their simultaneous growth in liquid ranged from 1.85 to12.5 $\times$ 10^6 CFU/ml for *S. cerevisiae* and from 0.012 to 0.036 $\times$ 10^6 CFU/ml for *O. oeni* in C1 and C2 samples. Moreover, the co-immobilized *S. cerevisiae* and *O. oeni* cells (C1 sample) was efficiently reused for the wine fermentation at least three times without any apparent loss of cell metabolic activities and cell viability (**Figures 2B,C**). As expected, the sequential inoculum of beads containing separately immobilized *S. cerevisiae* and *O. oeni* (C4 sample) produced a significant increase in time needed to obtain the end of MLF (28 days), whereas the AF was completed after 10 days (**Figure 3C**). Since the addition of beads (volume corresponding to about 35–40 ml) to grape must (200 ml) produced and increase in the final volume of about 15–20%, the use of beads to immobilize microorganisms reduced to about 80% of some initial metabolites (sugars, total acidity, malic acid, citric acid, glycerol, total SO2) concentrations (C9), compared to uninoculated control (C10) (**Table 1**). The AF and MLF processes, carried out following the traditional sequential inoculation strategy (C6 sample), were completed after a period of 30 days (**Figure 4A**).

The two trials consisting in co-inoculating immobilized *S. cerevisiae* and free *O. oeni* (C3 sample) or free *S. cerevisiae* and free *O. oeni* (C5 sample) completed the AF and MLF after about 18 days post inoculation. In terms of time, these approaches produced an intermediate behavior between the fermentation evolution observed by all the samples employing immobilized *S. cerevisiae* and *O. oeni* cells (C1, C2, and C4) and the traditional sequential inoculation system using free cells of *S. cerevisiae* and *O. oeni* (C6) (**Figures 2**–**4**). Moreover, the use of sequential inocula of free *S. cerevisiae* cells and of *O. oeni* immobilized cells (C7 sample) allowed the end of AF and MLF fermentations after 34 days.

In all must fermentations performed using *S. cerevisiae* free cells, final yeast cell concentrations ranged between 7 $\times$ 10^7 and 1.2 $\times$ 10^8 CFU/mL (**Figures 3D**, **4A–C**), whereas in samples inoculated with free *O. oeni* cells, bacterial concentrations ranged between 1.4 $\times$ 10^6 and 2.5 $\times$ 10^7 CFU/mL (**Figures 3B,D**, **4A**).

Fermentation Parameters

The biochemical analysis of main compounds was carried out in the wines produced in all the above trials. Sugar consumption rate of C1 and C5 samples scored the highest value (23.4 and 26.44 g/L d, respectively), followed by C8 (21.15 g/L d), C2 (20.6 g/L d), C4 (13.75 g/L d), C6 (13.23 g/L d), C3 (11.74 g/L d), and C7 (7.06 g/L

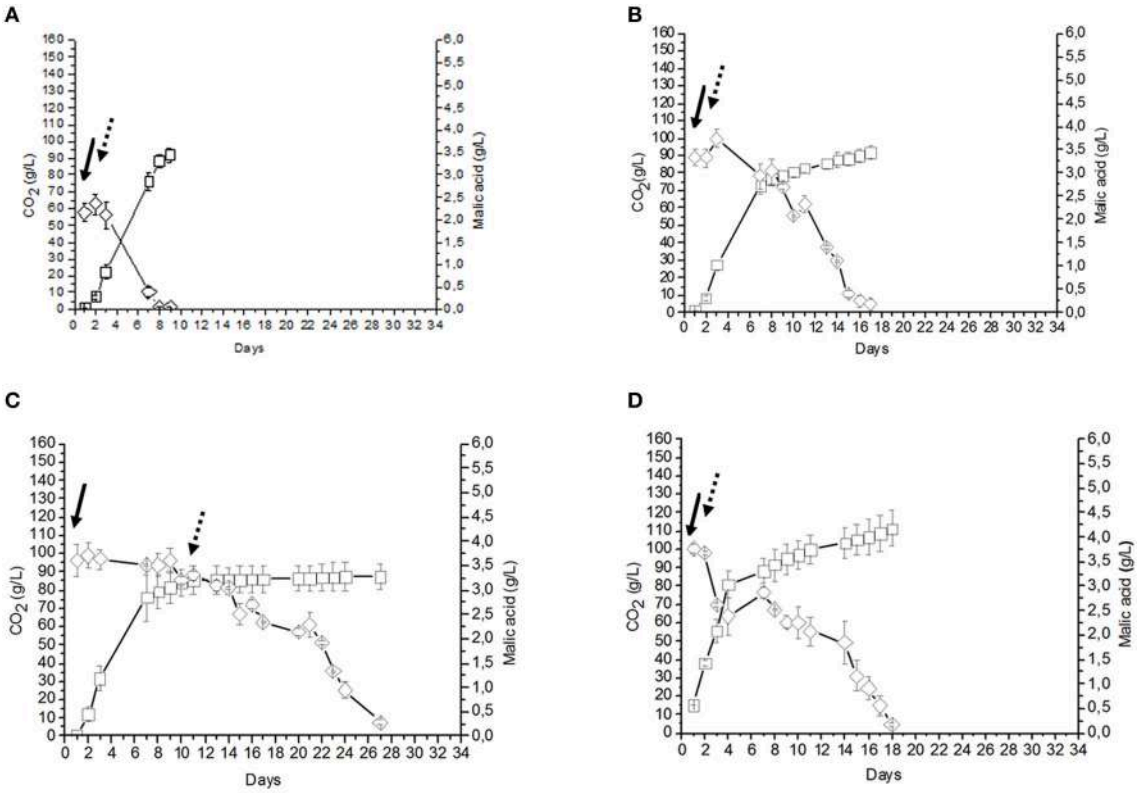

FIGURE 3 | (A) Must fermentation performed by simultaneous inoculum of beads containing *S. cerevisiae* and *O. oeni* cells separately immobilized, (sample C2). **(B)** Must fermentation performed by simultaneous inoculum of beads containing *S. cerevisiae* and an inoculum of free (10^6 CFU/ml) *O. oeni* cells (sample C3). **(C)** Fermentation using beads containing *S. cerevisiae* cells, followed, at the end of AF, by beads containing *O. oeni* (sample C4). **(D)** Fermentation performed by using simultaneous inoculum of (10^6 CFU/ml) free *S. cerevisiae* and (10^6 CFU/ml) *O. oeni* cells (sample C5); CO2 (g/l) production, Malic acid consumption. Inoculation time of *S. cerevisiae*, inoculation time of *O. oeni*.

d) samples. C1 sample showed also the highest rate of glycerol production (0.52 g/L d), malic acid consumption (0.93 g/L d) and lactic acid production (0.58 g/L d) and volatile acidity production (0.06 g/L d; **Table 2**).

Ethanol content in fermented must samples obtained employing immobilized *S. cerevisiae* and *O. oeni*, in co-inoculum or sequential inoculums (C1, C2, and C5), ranged from 7.90 to 10.58 ± 0.02% (v/v), with a yield of about 43–53% considering an initial sugar content corresponding to 162.85 g/L (**Table 1**). Analogously, must samples inoculated with *S. cerevisiae* and *O. oeni* following the traditional sequential inoculation approach (C6) contained 11.32 ± 0.01% (v/v) of ethanol, which corresponded to a yield of 53% considering the initial sugar content of 214.34 g/L (C10). The separate microbial immobilization of *S. cerevisiae* and *O. oeni* in alginate beads and their use in co-inoculum or in sequential inoculum approaches did not affect volatile acidity. In fact, very low levels of volatile acidity were produced in all fermented must samples (**Table 1**). All fermentations produced wines with pH values (3.23–3.47) and tartaric and citric acid concentrations were slightly different among samples. In particular, lactic acid was produced in a detectable quantity in all samples containing *O. oeni*, but not in C8 sample inoculated by only *S. cerevisiae*, thus indicating that it derived principally by bacterial malic acid decarboxylation. A corresponding high level of ethyl lactate was measured in samples inoculated with *O. oeni* immobilized in alginate beads (C1, C2, C4, and C7). Even low levels of glycerol ranging from 4.15 to 6.36 g/L were produced in all samples, as expected, this compound represented the second major product of AF. Moreover, a reduction of 15–20% of wine color was observed (Absorbance at 420, 520, 620 nm, and tonality).

Volatile Analyses

Quantitative data of the volatile compounds found in wines are shown in **Tables 3A,B**. These tables also show the perception thresholds of volatiles and their corresponding odor descriptors. Thirty-nine volatile components were identified and quantified in the analyzed wines. The main classes are alcohols, volatile fatty acids, esters, while aldehydes, terpens, sulfur compound, lactones, volatile phenols, and pyrazine were present in low concentrations.

Well-known by-products of yeast metabolism were the most abundant substances. In fact, alcohols are quantitatively the

TABLE 1 | Physicochemical parameters of wine samples.

Parameters	Wines									
	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10
Alcoholic degree (%vol)	$8.91^{e} \pm 0.21$	$7.90^{c} \pm 0.05$	$8.4^{d} \pm 0.01$	$7.14^{b} \pm 0$	$10.58^{g} \pm 0.02$	$11.32^{h} \pm 0.01$	$9.53^{f} \pm 0.31$	$11.35^{h} \pm 0.11$	$0.41a \pm 0.01$	$0.20^{a} \pm 0.04$
Reducing sugar (g/L)	$1.66^{a} \pm 0.08$	$1.29^{a} \pm 0.01$	$1.61^{a} \pm 0.04$	$1.01^{a} \pm 0.03$	$2.46^{a} \pm 0.04$	$2.28^{a} \pm 0.02$	$1.93^{a} \pm 0$	$2.41^{a} \pm 0.01$	$165.85b \pm 3.05$	$214.34^{c} \pm 4.05$
Total acidity (g/L)	$4.24^{b} \pm 0.08$	$3.81^{a} \pm 0.02$	$4.30^{b} \pm 0.01$	$3.90^{a} \pm 0.02$	$5.32^{c} \pm 0.02$	$5.48^{d} \pm 0.01$	$5.06^{c} \pm 0.03$	$7.18^{f} \pm 0.02$	$5.22^{c} \pm 0.2$	$6.99^{e} \pm 0.08$
Volatile acidity (g/L)	$0.57^{d} \pm 0.02$	$0.52^{c} \pm 0.01$	$0.56^{d} \pm 0$	$0.60^{e} \pm 0$	$0.55^{d} \pm 0.01$	$0.50^{c} \pm 0.01$	$0.53^{c} \pm 0$	$0.44^{b} \pm 0.01$	$0.20^{a} \pm 0.01$	$0.23^{a} \pm 0.02$
pH	$3.42^{c} \pm 0$	$3.40^{c} \pm 0$	$3.43^{c} \pm 0.01$	$3.42^{c} \pm 0$	$3.47^{d} \pm 0$	$3.38^{b} \pm 0.01$	$3.40^{c} \pm 0.01$	$3.23^{a} \pm 0.01$	$3.33^{b} \pm 0.01$	$3.33^{b} \pm 0.03$
Lactic acid (g/L)	$2.31^{e} \pm 0.04$	$2.17^{d} \pm 0.01$	$1.74^{b} \pm 0.01$	$1.59^{a} \pm 0.01$	$2.1^{c} \pm 0$	$2.78^{f} \pm 0.02$	$2.82^{f} \pm 0.01$	ND	ND	ND
Malic acid (g/L)	ND	ND	$0.22^{a} \pm 0.02$	$0.18^{a} \pm 0.04$	$0.25^{a} \pm 0.03$	$0.23^{a} \pm 0.04$	$0.17^{a} \pm 0.02$	$4.2^{c} \pm 0.40$	$3.7^{b} \pm 0.30$	$4.6^{c} \pm 0.04$
Tartaric acid (g/L)	$2.01^{d} \pm 0.03$	$2.04^{d} \pm 0.01$	$1.87^{c} \pm 0.01$	$1.63^{b} \pm 0.02$	$1.42^{a} \pm 0.01$	$1.79^{c} \pm 0.02$	$1.79^{c} \pm 0.01$	$1.73^{c} \pm 0.03$	$1.52^{b} \pm 0.06$	$2.39^{e} \pm 0.09$
Citric acid (g/L)	$0.22^{b} \pm 0.01$	$0.23^{b} \pm 0.01$	$0.22^{b} \pm 0.01$	$0.17^{a} \pm 0$	$0.36^{c} \pm 0$	$0.29^{b} \pm 0.01$	$0.25^{b} \pm 0$	$0.41^{c} \pm 0$	ND	ND
Density (g/mL)	$1.00^{a} \pm 0$	$1.00^{a} \pm 0$	$1.00^{a} \pm 0$	$1.00^{a} \pm 0$	$1.00^{a} \pm 0$	$0.99^{a} \pm 0$	$0.99^{a} \pm 0$	$0.99^{a} \pm 0$	$1.06^{b} \pm 0$	$1.08^{b} \pm 0$
Glycerol (g/L)	$5.19^{e} \pm 0.20$	$4.88^{d} \pm 0.02$	$4.82^{d} \pm 0.02$	$4.15^{c} \pm 0.02$	$5.68^{f} \pm 0.06$	$6.09^{g} \pm 0.03$	$5.60^{f} \pm 0.02$	$6.36^{h} \pm 0$	$0.25^{a} \pm 0.05$	$0.58^{b} \pm 0.17$
Total SO_2(mg/L)	$87.33^{c} \pm 1.66$	$78.83^{b} \pm 2.25$	$83.08^{c} \pm 0.29$	$64.67^{a} \pm 1.26$	$111.50^{e} \pm 2.50$	$94.83^{d} \pm 2.52$	$84.76^{c} \pm 2.00$	$123.17^{f} \pm 1.76$	ND	ND
A420 (nm)	$0.31^{a} \pm 0$	$0.30^{a} \pm 0$	$0.44^{b} \pm 0.01$	$0.30^{a} \pm 0$	$1.36^{d} \pm 0$	$0.57^{c} \pm 0$	$0.51^{c} \pm 0$	$0.42^{b} \pm 0$	ND	ND
A520 (nm)	$0.25^{a} \pm 0$	$0.25^{a} \pm 0$	$0.37^{b} \pm 0.01$	$0.26^{a} \pm 0$	$1.30^{b} \pm 0$	$0.49^{d} \pm 0$	$0.44^{b} \pm 0$	$0.37^{b} \pm 0$	ND	ND
A620 (nm)	$0.10^{a} \pm 0$	$0.12^{a} \pm 0$	$0.23^{b} \pm 0.01$	$0.15^{a} \pm 0$	$1.30^{c} \pm 0$	$0.24^{b} \pm 0$	$0.21^{b} \pm 0$	$0.10^{a} \pm 0$	ND	ND
Tonality	$1.29^{c} \pm 0.11$	$1.22^{b} \pm 0.03$	$1.19^{b} \pm 0.03$	$1.17^{b} \pm 0.06$	$1.05^{a} \pm 0.02$	$1.16^{b} \pm 0.04$	$1.17^{b} \pm 0.04$	$1.14^{b} \pm 0.05$	ND	ND

Values with different superscript roman letters (a–h) in the same row are significantly different according to the Tukey test ($p < 0.05$). ND. not determined.

largest group of volatile compounds and they were present in a higher amounts in C1 (152.44 mg/L), C3 (168.48 mg/L), and C6 (166.79 mg/L) samples, without any statistically significant difference among these samples. The highest concentrations were observed for isoamylic alcohols (73.55–99.05 mg/L), phenyl ethyl alcohol (4.35–56.56 mg/L), and 2-methyl-1-propanol (5.60–8.70 mg/L). Phenyl ethyl alcohol, key compound in the floral flavors of wines, was the second most abundant alcohol in C6 (56.56 mg/L), C3 (52.31 mg/L), and C1 (49.34 mg/L). In all fermented samples, isoamyl-alcohols and phenyl ethyl alcohol, the most important volatile compounds, were present in concentrations exceeding the odor threshold, producing positive impact in wine aroma (**Tables 3A,B**).

Eight different volatile fatty acids were identified and C1 (total amount 50.62 mg/L) and C3 (total amount 59.46 mg/L) samples showed the highest concentrations of these compounds. Statistically significant differences have been observed among the samples for all acids, except for decanoic acid. Acetic acid was the most abundant acid (1.64–14.57 mg/L), being present at levels lower than its perception threshold (200 mg/L), next followed by octanoic acid (8.59–16.79 mg/L) and hexanoic acid (0.47–13.09 mg/L). Ethyl esters of fatty acids and acetates were the second abundant group of volatile compounds in wines with 11 different identified components. Most of them are ethyl esters of fatty acids produced during the AF and the concentrations of many of them were significant different ($p < 0.05$), among wine samples. Since all of them (ethyl butanoate, hexanoate, octanoate, and decanoate) surpassed the detection threshold in all wines, except for ethyl lactate and 3-hydroxy ethyl butanoate, consequently, they are expected to have a great influence on the aroma of tested wines (**Tables 3A,B**). Isoamyl acetate and phenyl acetate (originating by the reaction of the acetyl-CoA with higher alcohols) showed high concentration levels, exceeding their odor threshold in all samples, whereas aldehydes remained quantitatively very limited (**Tables 3A,B**).

Among fatty acids, also produced during fermentation, butanoic, 3-methyl butanoic, hexanoic, octanoic, decanoic, and benzoic acids were quantified in concentrations exceeding the odor threshold and contributing with fruity, fatty, rancid, and cheese notes on wine odor profile. Also among terpenes, strongly influencing the varietal aroma, 2,6 dimethyl-7-octene 2,6 diol was identified in all wines with an OAV > 1. Since low levels of acetoin and 2,3 butanediol were detected in all wine samples, these compounds did not affect aroma with the unpleasant "*buttery*" attribute.

Volatile phenol (4-vinyl guaiacol) was detected with an OAV > 1 in all wine samples except in C6 and C7 wines. This compound can be responsible of spicy aromatic notes.

Principal Component Analysis

PCA was carried out on the 10 samples using the principal fermentation parameters reported in **Tables 1, 2**, in order to produce a multivariate analysis of the evolution of chemical compounds and the fermentation rates linked to production/consumption of main fermentation metabolites.

In **Figure 5** bi-plots displaying PC1 vs. PC2 indicated that the samples considered in this study were grouped into three main

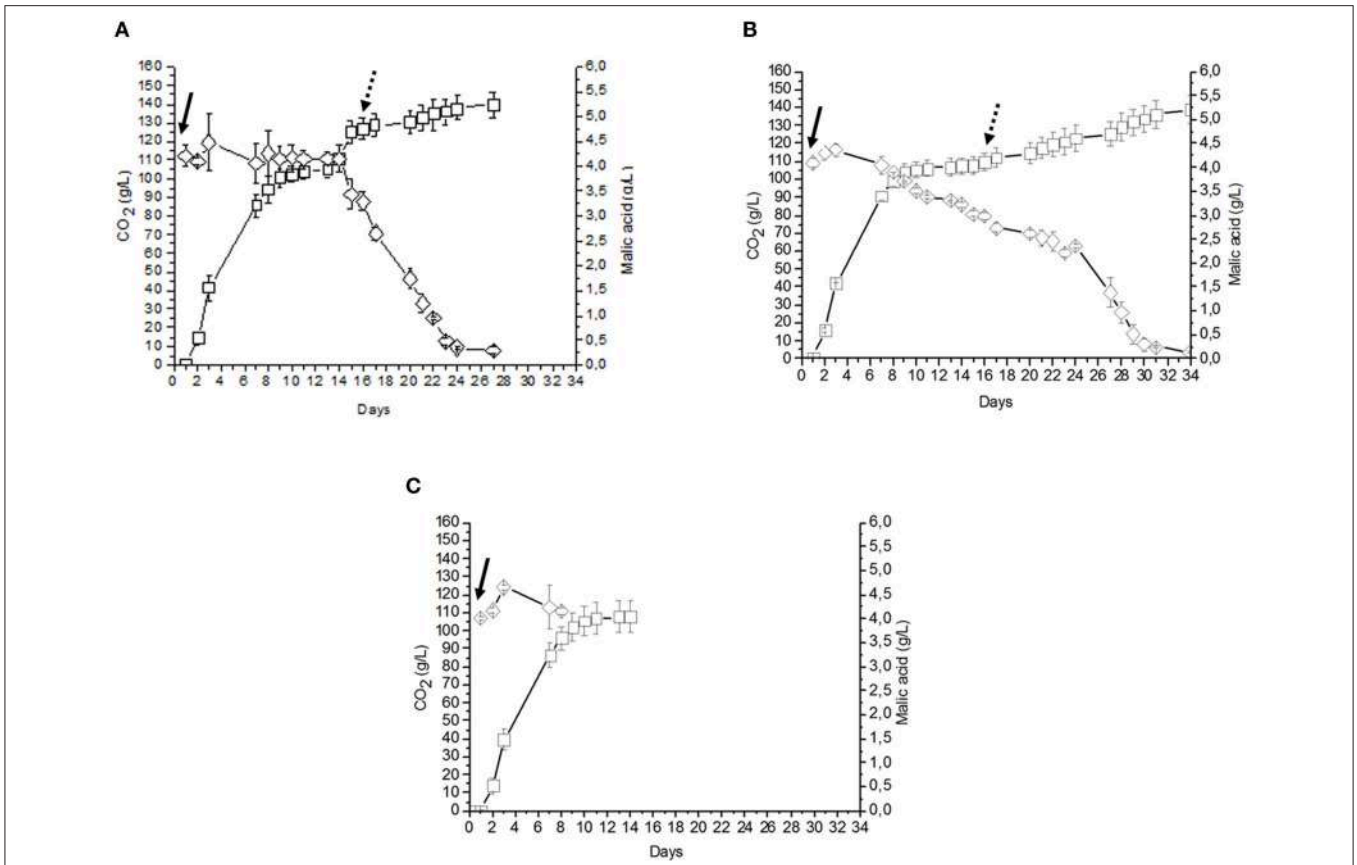

FIGURE 4 | (A) Fermentation performed using free (10^6 CFU/ml) *S. cerevisiae* cells, followed, at the end of AF, by free cells (10^6 CFU/ml) of *O. oeni* (sample C6). **(B)** Fermentation performed by free (10^6 CFU/ml) *S. cerevisiae* cells, followed, at the end of AF, by beads containing *O. oeni* (sample C7). **(C)** AF performed by an inoculum of free 10^6 CFU/ml *S. cerevisiae* cells (sample C8). CO_2 (g/l) production, Malic acid consumption. Inoculation time of *S. cerevisiae*, inoculation time of *O. oeni*.

TABLE 2 | Fermentation metabolites production/consumption rates.

	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10
	g/L Day									
Malic acid consumption rate	0.93	0.93	0.19	0.13	0.24	0.16	0.1	0	0	0
Lactic acid production rate	0.58	0.54	0.1	0.06	0.12	0.1	0.08	0	0	0
Sugar consumption rate	23.4	20.6	11.74	13.75	26.44	13.23	7.06	21.15	0	0
Volatile acidity	0.06	0.05	0.03	0.02	0.03	0.02	0.015	0.03	0	0
Glycerol production rate	0.52	0.49	0.27	0.15	0.32	0.22	0.16	0.45	0	0

clusters: the cluster 1 (C1 and C2) and 2 (C3, C4, C5, C6, C7, and C8) were divided by the PC2 and were localized in quadrants 3 and 4, respectively; the cluster 3 (C9 and C10 samples), separated from the two others group by PC1 localized in quadrant 2.

In order to correlate volatiles data with the different inoculum strategies used in this work, a PCA analysis was performed on the complete SPME/GC-MS data matrix of each wine sample (**Figure 6**). PC1 discriminated wine samples (C1–C8) which lied on the negative semi-axis of the first component form the two must controls (C9 and C10) for the high content of volatile compounds. The second dimension allowed to separate two clusters A and B: group A (C5, C6, C7, and C8) on the negative semi-axis of PC2 and group B (C1, C2, C3, and C4) on the positive semi-axis of PC2. Group A in the third quadrant along negative PC2 was characterized by the presence of esters, whereas, group B, localized in the fourth quadrant is mainly characterized by highly flavoring compounds such as acids, esters, terpens, alcohols, lactones, and aldehydes.

TABLE 3A | Quantification of volatile compounds identified in C1-C5 wines.

Compounds	OTH mg/L	Odor description	References	C1 mg/L ± sd	C2 mg/L ± sd	C3 mg/L ± sd	C4 mg/L ± sd	C5 mg/L ± sd
ALCOHOLS								
1-propanol	9	Pungent, harsh			1.47ab ± 0.44	1.53b ± 0.17	1.11ab ± 0.22	0.87ab ± 0.40
			Penaido et al., 2006	2.21b ± 0.80				
2-methyl-1-propanol	40	Fusel, spiritous	Gómez-Míguez et al., 2007	7.72b ± 1.70	5.60b ± 0.43	8.70b ± 1.11	7.18b ± 1.45	6.07b ± 2.85
1-butanol	150	Fusel, spiritous	Penaido et al., 2006	0.52a ± 0.26	0.28a ± 0.11	0.67b ± 0.26	0.45b ± 0.36	0.35b ± 0.12
Isoamylic alcohols	30	Harsh, nail polish	González Álvarez et al., 2011	85.66b ± 14.13	73.55b ± 7.22	99.05b ± 8.66	84.76b ± 6.54	75.79b ± 8.98
Hexanol	4	Herbaceous	Rocha et al., 2004	0.32b ± 0.10	0.28b ± 0.03	0.15*ab* ± 0.04	0.30b ± 0.07	0.10b ± 0.04
Heptanol	2.5	Oily		0.26b ± 0.05	0.21b ± 0.03	0.43a ± 0.18	0.45a ± 0.18	0.44a ± 0.03
(D,L)- butan-2,3-diol	150	Fruity	Penaido et al., 2006	4.63b ± 2.15	2.65*ab* ± 0.75	3.89ab ± 3.30	2.33ab ± 0.40	1.16ab ± 0.65
(R,S)- butan-2,3-diol	150	Fruity	Penaido et al., 2006	1.79a ± 0.22	0.89a ± 0.52	0.94a ± 0.23	0.64a ± 0.33	0.54a ± 0.20
Benzylalcohol	200	Flowery, sweet	González Álvarez et al., 2011	*ND*	1.39*b* ± 0.06	0.81a ± 0.24	1.57b ± 0.26	ND
Phenyl ethyl alcohol	10	Rose	Rocha et al., 2004	49.33c ± 9.41	38.64bc ± 6.60	52.31d ± 4.27	46.89c ± 5.90	49.13c ± 6.15
Total amounts				152.44 ± 28.82	124.96 ± 16.20	168.48 ± 18.46	145.68 ± 15.71	134.45 ± 19.42
FATTY ACIDS								
Acetic acid	200	Vinegar	Guth, 1997	9.56d ± 5.60	5.44c ± 0.84	14.57e ± 5.46	10.11d ± 3.52	3.31b ± 0.19
2-methyl propanoic acid	2.3	Fatty, rancid	Gómez García-Carpintero et al., 2011b	1.23b ± 0.36	0.92ab ± 0.12	1.62ab ± 0.17	1.13ab ± 0.29	1.07ab ± 0.51
Butanoic acid	2.2	Fatty-rancid, sweaty	Rocha et al., 2004	1.11b ± 0.18	0.90ab ± 0.12	1.39c ± 0.47	0.89ab ± 0.25	0.71ab ± 0.33
3-methyl butanoic acid	1.5	Fatty-rancid, cheesy	Moyano et al., 2002	3.13*b* ± 0.87	1.95ab ± 0.15	2.87b ± 0.78	2.37ab ± 0.71	2.40ab ± 0.63
Hexanoic acid	8	Rancid, grass, fruity	Rocha et al., 2004	10.79e ± 2.59	10.72e ± 1.30	13.09*f* ± 2.71	9.34d ± 1.32	8.64c ± 1.58
Octanoic acid	0.5	Fatty acid, dry, dairy	Rocha et al., 2004	14.98bc ± 5.42	15.02*bc* ± 3.56	16.79c ± 4.07	11.98bc ± 2.34	10.81bc ± 3.40
Decanoic acid	1.4	Fatty acid, dry, woody	Rocha et al., 2004	3.29a ± 0.46	3.01a ± 0.18	2.19a ± 0.22	2.88a ± 1.08	4.52a ± 0.75
Benzoic acid	1	Chemical		6.54ab ± 2.12	5.18ab ± 0.27	6.95b ± 0.64	5.22ab ± 0.41	3.59ab ± 0.46
Total amounts				50.62 ± 17.61	43.14 ± 5.71	59.46 ± 14.53	43.92 ± 9.91	35.06 ± 7.83
ESTERS								
Ethyl-butanoate	0.02	Floral, fruity	Guth, 1997	1.31b ± 0.54	1.07ab ± 0.03	1.20b ± 0.20	0.81ab ± 0.10	0.99ab ± 0.31
Isoamyl acetate	0.03	Banana, pear	Günata et al., 1985	4.33b ± 1.65	4.33b ± 0.32	5.07b ± 0.93	2.15ab ± 0.24	5.22b ± 0.74
Ethyl hexanoate	0.014	Green apple	Günata et al., 1985	1.70ab ± 0.43	1.30ab ± 0.07	1.95b ± 0.29	1.19ab ± 0.41	2.04b ± 0.21
Ethyl lactate	154.6	Lactic	Gómez-Míguez et al., 2007	6.03cd ± 1.16	5.89cd ± 0.05	4.41c ± 0.54	6.68d ± 0.93	2.29b ± 0.48
Ethyl octanoate	0.6	Sweet soap	Rocha et al., 2004	2.22b ± 0.87	3.35b ± 0.81	6.22c ± 0.20	3.10b ± 0.10	6.71c ± 0.64
3-hydroxy ethyl butanoate	20	Fruity	González Álvarez et al., 2011	0.61b ± 0.26	0.35ab ± 0.07	0.69b ± 0.20	0.33ab ± 0.07	0.39ab ± 0.12
Ethyl decanoate	0.2	Fruity		1.17ab ± 0.32	1.37ab ± 0.37	0.36ab ± 0.10	0.85ab ± 0.29	5.78b ± 2.26
Diethyl succinate	200	Fruity, melon	González Álvarez et al., 2011	0.46ab ± 0.23	0.38ab ± 0.05	0.54b ± 0.04	0.45b ± 0.08	1.30c ± 0.15
Ethyl-9-decenoate	ND			0.48ab ± 0.08	0.33ab ± 0.07	1.05ab ± 0.40	1.10ab ± 0.36	2.45b ± 0.81
Phenyl acetate	0.25	Flowery, rose, fruity	Guth, 1997	0.94abc ± 0.34	0.86abc ± 0.05	1.30bc ± 0.43	0.58abc ± 0.20	1.41c ± 0.12

(Continued)

TABLE 3A | Continued

Compounds	OTH mg/L	Odor description	References	C1 mg/L ± sd	C2 mg/L ± sd	C3 mg/L ± sd	C4 mg/L ± sd	C5 mg/L ± sd
Monoethyl succinate	ND	Chocolate		5.71abc ± 0.08	2.93ab ± 0.38	7.11bc ± 0.40	9.28bc ± 2.45	5.25abc ± 1.60
Total amounts				24.42 ± 5.97	22.17 ± 2.27	29.92 ± 3.73	26.48 ± 5.23	33.81 ± 7.44
ALDEHYDES/KETONS								
Acetoin	150		Moyano et al., 2002	0.49a ± 0.24	*ND*	1.01ab ± 0.10	0.47*a* ± 0.13	0.95ab ± 0.26
Furfural	3	Caramel		0.34 ± 0.08	0.23 ± 0.03	0.29 ± 0.12	0.23 ± 0.07	0.31 ± 0.07
Benzaldehyde	2	Bitter almond	Gómez García-Carpintero et al., 2011b	0.73a ± 0.25	0.63*a* ± 0.06	0.75a ± 0.24	0.49a ± 0.20	0.35*a* ± 0.06
Methyl-2-furoate	ND			0.87a ± 0.39	0.60a ± 0.09	0.81a ± 0.18	0.42a ± 0.13	0.38a ± 0.54
Total amounts				2.43 ± 0.96	1.46 ± 0.17	2.86 ± 0.64	1.61 ± 0.53	1.99 ± 0.93
LACTONES								
Butyrolactone	0.035	Sweet, toast, caramel	Moyano et al., 2002	0.53a ± 0.24	*ND*	1.78a ± 0.044	2.79a ± 0.61	ND
SULFUR COMPOUND								
3-methylthio-1-propanol	2	Potato, baked cabbage	Guth, 1997	1.55ab ± 0.70	0.71ab ± 0.11	2.32b ± 0.70	1.99ab ± 0.50	1.36ab ± 0.98
PHENOLS								
4-vinyl guaiacol	0.38-1.1	Black pepper, species	Rocha et al., 2004	3.68b ± 0.72	3.41ab ± 0.92	3.53ab ± 1.51	0.68ab ± 0.28	1.72ab ± 0.46
PYRAZINE								
2-methoxy-6-methyl pyrazine								
TERPENS								
2,6 dimethyl-7-octene 2,6 diol	0.250		Vilanova and Sieiro, 2006	0.64*a* ± 0.25	0.59*a* ± 0.06	0.86a ± 0.40	*ND*	0.30a ± 0.42
4H-piran-4-one-2,3 dihydro-3,5 dihydroxy	ND			2.50a ± 0.92	1.76a ± 0.22	3.55a ± 1.32	1.32a ± 0.45	1.20a ± 0.48
Total amounts				3.14 ± 1.17	2.35 ± 0.28	4.41 ± 1.72	1.32 ± 0.45	1.49 ± 0.90

Different letters in the same row indicate statistical differences ($p < 0.05$) using the Tukey test; ND, not determined; Data are average of three replicates ± standard deviation (SD); OTH, odor threshold.

TABLE 3B | Quantification of volatile compounds identified in C6-C10 wines.

Compounds	OTH mg/L	Odor description	Ref.	C6 mg/L ± sd	C7 mg/L ± sd	C8 mg/L ± sd	C9 mg/L ± sd	C10 mg/L ± sd
ALCOHOLS								
1-propanol	9	Pungent, harsh	Penaido et al., 2006	1.13a ± 0.53	0.99a ± 0.17	1.19a ± 0.17	Nd	Nd
2-methyl-1-propanol	40	Fusel, spiritous	Gómez-Míguez et al., 2007	7.74*a* ± 1.60	8.01a ± 1.02	8.34a ± 0.24	Nd	Nd
1-butanol	150	Fusel, spiritous	Penaido et al., 2006	0.43*a* ± 0.10	0.08a ± 0.11	0.39a ± 0.10	Nd	Nd
Isoamylic alcohols	30	Harsh, nail polish	González Álvarez et al., 2011	97.45b ± 7.80	93.52b ± 5.04	88.00b ± 9.71	34.11a ± 3.70	37.05a ± 4.11
Hexanol	4	Herbaceous	Rocha et al., 2004	0.11*a* ± 0.02	0.27b ± 0.04	Nd	Nd	Nd
Heptanol	2.5	Oily		0.42a ± 0.03	0.39a ± 0.03	0.46a ± 0.05	0.29a ± 0.08	0.24a ± 0.04
(D,L)- butan-2,3-diol	150	Fruity	Penaido et al., 2006	1.56a ± 1.03	1.40a ± 1.03	1.28a ± 0.21	Nd	Nd
(R,S)- butan-2,3-diol	150	Fruity	Penaido et al., 2006	0.96*a* ± 0.41	0.85a ± 0.11	0.54*a* ± 0.04	Nd	Nd
Benzylalcohol	200	Flowery, sweet	González Álvarez et al., 2011	0.43a ± 0.61	Nd	Nd	0.72a ± 0.25	0.98a ± 0.20
Phenyl ethyl alcohol	10	Rose	Rocha et al., 2004	56.56d ± 4.49	22.59b ± 3.10	49.48c ± 4.60	4.35a ± 1.12	5.66a ± 0.11
Total amounts				166.79 ± 16.62	128.1 ± 10.65	149.68 ± 15.12	39.47 ± 5.15	43.93 ± 4.46
FATTY ACIDS								
Acetic acid	200	Vinegar	Guth, 1997	6.21c ± 2.22	7.34*c* ± 1.15	4.52*c* ± 1.71	1.64a ± 0.70	Nd
2-methyl propanoic acid	2.3	fatty, rancid	Gómez García-Carpintero et al., 2011b	1.09ab ± 0.22	1.08ab ± 0.07	0.95ab ± 0.21	0.56a ± 0.13	0.70a ± 0.05
Butanoic acid	2.2	Fatty-rancid, sweaty	Rocha et al., 2004	0.84b ± 0.13	0.69b ± 0.06	0.83b ± 0.22	Nd	0.31a ± 0.06
3-methyl butanoic acid	1.5	Fatty-rancid, cheesy	Moyano et al., 2002	2.74b ± 0.35	2.61ab ± 0.07	2.47ab ± 0.23	0.45a ± 0.10	0.49a ± 0.06
Hexanoic acid	8	Rancid, grass, fruity	Rocha et al., 2004	10.35e ± 3.23	6.16b ± 0.57	9.53d ± 3.30	0.47a ± 0.10	0.77a ± 0.11
Octanoic acid	0.5	Fatty acid, dry, dairy	Rocha et al., 2004	14.12ab ± 4.82	8.59a ± 1.33	11.87ab ± 2.60	Nd	Nd
Decanoic acid	1.4	Fatty acid, dry, woody	Rocha et al., 2004	2.51a ± 0.66	4.59a ± 0.88	2.25*a* ± 0.26	Nd	Nd
Benzoic acid	1	Chemical		5.06ab ± 0.21	4.49ab ± 0.91	Nd	1.77ab ± 0.45	2.71ab ± 0.15
Total amounts				42.91 ± 9.84	35.54 ± 5.04	32.42 ± 8.53	4.89 ± 1.48	4.99 ± 0.43
ESTERS								
Ethyl-butanoate	0.02	Floral, fruity	Guth, 1997	1.04ab ± 0.35	0.69*a* ± 0.13	1.19*b* ± 0.35	Nd	Nd
Isoamyl acetate	0.03	Banana, pear	Günata et al., 1985	3.74ab ± 0.11	1.65a ± 0.24	3.99b ± 0.16	Nd	Nd
Ethyl hexanoate	0.014	Green apple	Günata et al., 1985	1.24b ± 0.50	0.55*a* ± 0.05	1.19b ± 0.30	Nd	Nd
Ethyl lactate	154.6	Lactic	Gómez-Míguez et al., 2007	4.65b ± 0.42	6.06b ± 0.27	0.60a ± 0.08	Nd	Nd
Ethyl octanoate	0.6	Sweet soap	Rocha et al., 2004	2.06a ± 0.34	1.33a ± 0.27	2.41a ± 0.80	Nd	2.31a ± 0.05
3-hydroxy ethyl butanoate	20	Fruity	González Álvarez et al., 2011	0.39a ± 0.05	0.36a ± 0.10	0.48a ± 0.06	Nd	Nd
Ethyl decanoate	0.2	Fruity		0.80a ± 0.04	1.03a ± 0.06	1.03a ± 0.04	Nd	Nd
Diethyl succinate	200	Fruity, melon	González Álvarez et al., 2011	0.47*b* ± 0.15	0.39a ± 0.07	0.38a ± 0.04	Nd	Nd
Ethyl-9-decenoate	Nd			0.92a ± 0.12	0.84a ± 0.08	1.17a ± 0.10	Nd	Nd
Phenyl acetate	0.25	Flowery, rose, fruity	Guth, 1997	0.82a ± 0.33	0.41a ± 0.05	0.72a ± 0.08	Nd	Nd
Monoethyl succinate	Nd	Chocolate		7.23b ± 0.32	10.20c ± 1.45	4.18a ± 0.56	Nd	Nd
Total amounts				23.38 ± 2.73	23.51 ± 2.77	17.35 ± 2.57		2.31 ± 0.05

(Continued)

TABLE 3B | Continued

Compounds	OTH mg/L	Odor description	Ref.	C6 mg/L ± sd	C7 mg/L ± sd	C8 mg/L ± sd	C9 mg/L ± sd	C10 mg/L ± sd
ALDEHYDES/KETONS								
Acetoin	150		Moyano et al., 2002	1.36b ± 0.23	0.89a ± 0.16	*Nd*	1.09a ± 0.05	1.17a ± 0.06
Furfural	3	Caramel		0.37a ± 0.07	0.47a ± 0.05	0.29a ± 0.05	1.56*a* ± 0.07	1.84*a* ± 0.04
Benzaldehyde	2	Bitter almond	Gómez García-Carpintero et al., 2011b	0.36a ± 0.08	0.34a ± 0.05	0.55a ± 0.05	0.47a ± 0.07	0.48*a* ± 0.05
Methyl-2-furoate	ND			0.93a ± 0.16	0.73a ± 0.14	0.60a ± 0.07	*Nd*	*Nd*
Total amounts				3.01 ± 0.54	2.43 ± 0.40	1.44 ± 0.17	3.13 ± 0.19	3.48 ± 0.15
LACTONES								
Butyrolactone	0.035	Sweet, toast, caramel	Moyano et al., 2002	0.45a ± 0.07	*Nd*	0.52a ± 0.05	1.07a ± 0.06	1.16a ± 0.07
SULFUR COMPOUND								
3-methylthio-1-propanol	2	Potato, baked cabbage	Guth, 1997	2.06ab ± 0.05	1.94ab ± 0.05	1.72ab ± 0.05	0.27a ± 0.04	0.32a ± 0.08
PHENOLS								
4-vinyl guaiacol	0.38-1.1	Black pepper, species	Rocha et al., 2004	*Nd*	*Nd*	0.96*a*	*Nd*	*Nd*
PYRAZINE								
2-methoxy-6-methyl pyrazine							4.80b ± 0.12	1.20a ± 0.05
TERPENS								
2,6 dimethyl-7-octene 2,6 diol	0.250		Vilanova and Sieiro, 2006	0.56a ± 0.05	0.53a ± 0.07	0.50a ± 0.04	0.47a ± 0.07	0.59a ± 0.10
4H-piran-4-one-2,3 dihydro-3,5 dihydroxy	ND			1.87a ± 0.15	1.24a ± 0.16	3.34a ± 0.12	1.64a ± 0.06	2.58a ± 0.06
Total amounts				2.44 ± 0.20	1.77 ± 0.23	3.84±	2.11 ± 0.13	3.16 ± 0.16

Different letters in the same row indicate statistical differences ($p < 0.05$) using the Tukey test; ND, not determined; Data are average of three replicates ± standard deviation (SD); OTH, odor threshold.

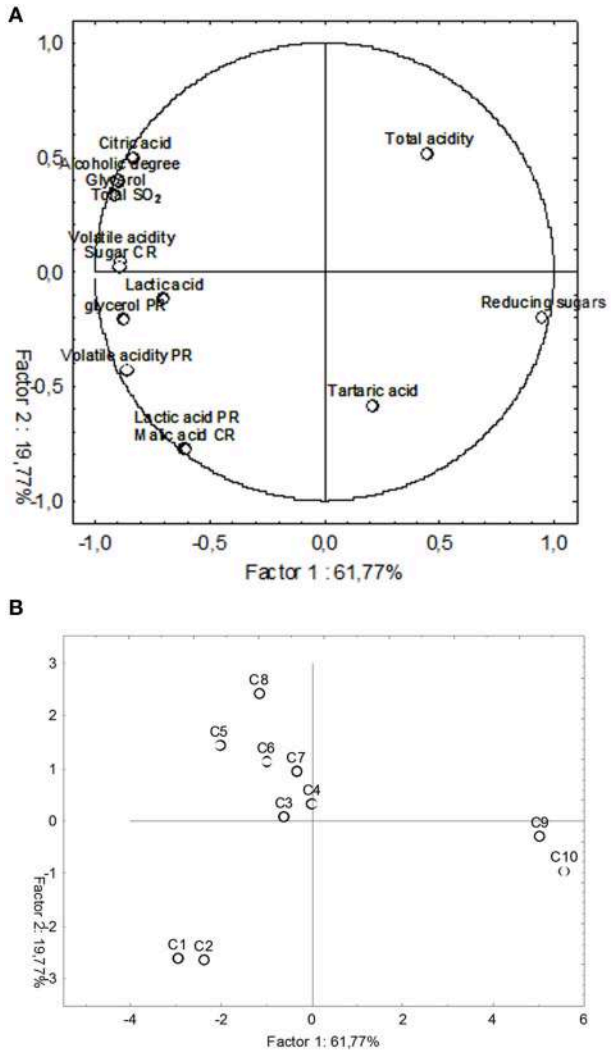

FIGURE 5 | PCA of fermentation parameters associated with all fermentation samples. PCA variables were the data obtained from the analysis of concentration and production rates (PR) and consumption rates (CR) of chemical compounds at the end of fermentation. The figure displays the sample scores and variable loadings in the planes formed by PC1–PC2. **(A)** Projection of the variables; **(B)** Projection of the cases.

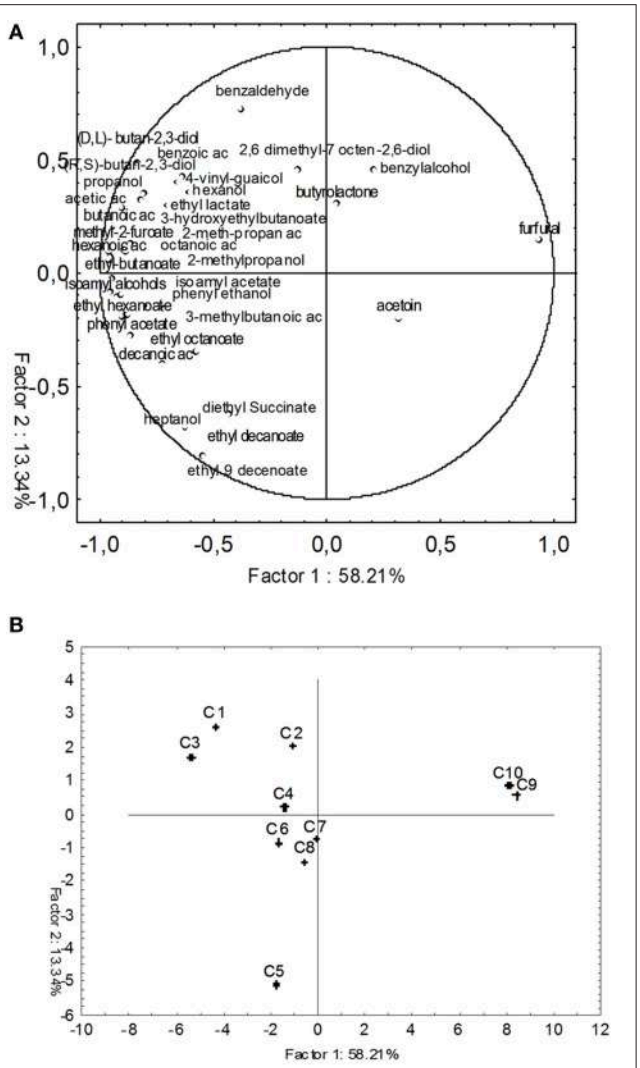

FIGURE 6 | PCA of volatile compounds associated with all tested fermentation samples. Score plot of variables (concentration of volatile molecules) and the end of fermentation in the plane formed by the first two principal components (PC1 against PC2). **(A)** Projection of the variables; **(B)** Projection of the cases.

Radar Plot

Odorous compounds detected in all analyzed wine and must samples with similar sensory descriptors were grouped into classes, denoted aromatic series (**Tables 3A,B**). In these Tables, one or more membership sensorial classes was affiliated to each compound. In this respect, solvent, floral, sweet, green, fatty, fruity, and spicy odor series were chosen for the description of wines aroma. **Figure 7** reports the radar plot representation of the odor series associated to C1 and C6 wine samples.

C1 wine sample, produced by yeast and bacteria co-immobilization strategy, showed a sensorial profile comparable with that produced by sequential inoculum strategy (C6 sample). In particular, C1 sample showed also spicy (phenols, 4 vinyl guaiacol) notes that are completely absent in C6 wine (**Tables 3A,B**).

DISCUSSION

In this study, for the first time the co-immobilization strategy in alginate beads of two commercial strains of *S. cerevisiae* and *O. oeni* was applied for the production of red wine. Although the parameters adopted for yeasts immobilization (initial cell biomass, $CaCl_2$, and the sodium alginate concentrations) were than those described by Bleve et al. (2008, 2011), the immobilization conditions for *O. oeni* were instead optimized. The best co-immobilization conditions for *S. cerevisiae* and *O. oeni* cells corresponded to an inoculum equivalent to 10^6 CFU/mL of each *O. oeni* and of *S. cerevisiae* in 3% (w/v)

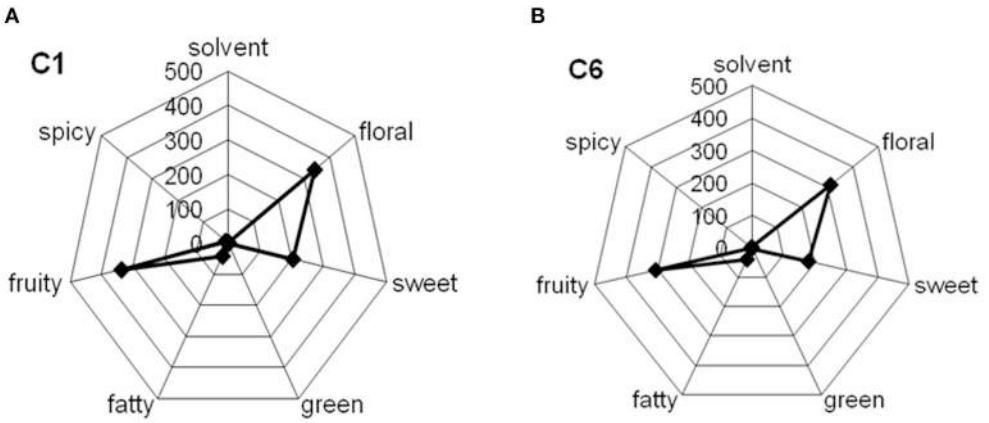

FIGURE 7 | Odor chemical profile of wines calculated by adding the odor descriptor of the total compounds concentration grouped in chemical class. Radar plot of the sensory descriptors associated to total concentrations of all classes of volatiles associated to Negroamaro wine obtained after **(A)** inoculation with *Oenococcus oeni* strain Lalvin VP41^{TM} and *Saccharomyces cerevisiae* strain Lalvin ICV-D254$^{®}$ co-immobilized in alginate beads (sample C1), **(B)** inoculation of free (10^6 CFU/ml) *S. cerevisiae* cells, followed, at the end of AF, by free cells (10^6 CFU/ml) of *O. oeni* (sample C6).

calcium alginate. The data reported in this study showed that, after immobilization, yeast and bacterial metabolic activities were enhanced in comparison with those inoculated in free cells form. In fact, the immobilization enhances biological stability, the tolerance to external stress conditions, the resistance to by-products deriving from cell metabolism and that (this can result toxic to the same cells; Bleve et al., 2008, 2011). This observation was particularly important for *O. oeni* since this microorganism is exposed to very difficult constraints (like pH, ethanol, SO_2, medium chain fatty acids, nutrient depletion, etc.) that can negatively affect its growth and metabolic activities and, consequently, the occurrence of MLF in wines (Alexandre et al., 2004). After comparison with the different supports (Kourkoutas et al., 2004), calcium alginate was chosen as material to encapsulate yeast and bacteria cells, since it is cheap and of food grade purity. This strategy offers the possibility to encapsulate cells mimicking the environment of large flocs, producing a high mass:surface ratio, protecting the inside aggregated cells from stress by an outer layer of sacrificial cells (Sun et al., 2007).

This effect cannot be obtained by the use of carrier materials (glass beads, wood chips, etc.) where cells adher to the matrix surface, leaving them to be directly exposed to the external stresses (Nedović et al., 2015).

Several examples of yeast immobilization in alginate beads have been proposed to obtain a suitable biocatalyst for mead production by diluted honey (Pereira et al., 2014), for making of pomegranate (Sevda and Rodrigues, 2011), and cagaita-derived (Oliveira et al., 2011) wines, for production of cabernet sauvignon and pinot noir young wines (Andrade Neves et al., 2014).

The high AF and MLF fermentation rates maintained in all the three different wine production cycles using co-immobilized *S. cerevisiae* and *O. oeni* in this study indicated that co-immobilization of yeast and bacterial cells did not induce alterations in physiology and metabolic activity and in cell growth. These evidences demonstrated that a good balance exists among fermentation rates, main fermentation metabolites in the final product, and suitable internal mass transfer in co-immobilization of yeasts and bacteria in calcium alginate, as suggested by Scott and O'Reilly (1996).

In a previous paper, Servetas et al. (2013) used tubular delignified cellulosic material and wheat starch gel to respectively entrap, in two overlapped layers, *O. oeni* and *S. cerevisiae* cells, which were able to carry out simultaneous AF and MLF at low temperature (10°C), without problems deriving from the biological competition in the same niche.

Co-immobilization strategy can be useful to significantly reduce the time necessary to obtain a complete AF and MLF. These results confirmed that when yeast cells are immobilized they showed a faster consume of glucose than free cells, due to a stable pattern of gene expression characterized by higher expression of genes involved in glycolysis, stress resistance and cell wall remodeling than planktonic cells (Nagarajan et al., 2014). In addition, Parascandola et al. (1997) and Junter et al. (2002) reported that immobilization produces significant changes in the cell proteome and gene expression that have relevant impact on cell wall and cytoplasmic membrane composition and architecture, finally producing deep impact on cell stress resistance.

According to what observed by Rodriguez-Nogales et al. (2012), where MLF was enhanced by the immobilization of *O. oeni* in Lentikats, the proposed approach in this paper can open interesting perspectives, since it speed up the wine production by shortening the time needed for MLF completion.

Statistical analyses used in this paper demonstrated that C1 and C2 wine samples were strongly distinguishable from all the other wines when metabolites production and/or their consumption rates were considered (**Figure 5**).

Although food grade alginate beads immersed in grape must can dilute metabolites and color of must and wines, as previously reported by Genisheva et al. (2013), the proposed approach produced final products with sensorial characteristics,

determined by OAVs, not different from the wine obtained using the traditional sequential inoculum procedure.

The perception thresholds and descriptors for each aroma compound studied as previously reported (Brugirard et al., 1991; Guth, 1997; López et al., 1999; Kotseridis and Baumes, 2000; Ferreira et al., 2001). Each compound was assigned to one or several aroma series, depending on its principal odor descriptors; the solvent, floral, sweet, green (vegetal or herbaceous), fatty, fruity, and species were chosen for this purpose on account of their extensive use for describing and distinguishing red wines in terms of aroma by specialized journals and tasters (Mijares, 1987; Peynaud, 1987; Peris and Masats, 2000).

However, the projection of the cases onto the first two axes of PCA, performed on volatiles, showed that wines produced using beads as support for yeasts and bacteria immobilization (C1, C2, C3, and C4) were more complex in terms volatile compounds concentrations (acids, esters, terpens, alcohols, lactones, and aldehydes) than wines obtained using free cells inocula (C5, C6, C7, and C8), mainly characterized by esters (**Figure 6**).

Higher alcohols, mainly formed during AF, are the largest group of aroma compounds, contributing, especially by the synergistic effect of the matrix (Verstrepen et al., 2003b) with fruity characters, when they are in optimal levels (<300 mg/L). They are also at the basis of volatile ester formation (Verstrepen et al., 2003a). These compounds did not exceed in any tested wines the threshold of 400 mg/L, avoiding to produce strong and pungent smell and taste and herbaceous notes (Ribereau-Gayon et al., 2000). Isoamyl alcohol may contribute to the complexity of aroma wine, although at very high levels, it can produce unpleasant notes. Among the aliphatic alcohols, 3-methyl-1-butanol showed the highest concentration in all studied wines; 2-Phenylethanol is formed principally by yeast metabolism (Etievant, 1991), has a floral aroma with roses notes. In all studied wines this compound exceeded its olfactory threshold (10 mg/L, Guth, 1997).

Although acetaldehyde is the most important volatile aldehyde for flavor in wines (Lambrechts and Pretorius, 2000), it resulted not detectable in all tested wines, probably because, after its production during the active yeast growth phase, it was sequestrated by the same yeast cells and used to furtherly produce ethanol (Verstrepen et al., 2003a).

The ethylic esters of the fatty acids (ethyl butanoate, hexanoate, octanoate, and decanoate) and the acetates of the higher alcohols (isoamyl acetate and phenyl acetate) are two groups of compounds of undoubted importance in the wine aroma, as their nuances coincide with the fruity, perfume-like, and candy descriptors of the wines. These compounds are important in young wine aroma and are among key compounds in the fruity flavors of wines (Rapp and Mandery, 1986). The presence of other esters, specifically ethyl acetate, phenylacetate, although exhibiting OAVs lower than one, also could contribute to the fruity character of analyzed wines. In fact, as already reported by Genisheva et al. (2014), immobilization system contribute to enhance the concentrations of isoamyl acetate, the ethyl esters ethyl hexanoate, ethyl octanoate, and ethyl decanoate in the final products above their perception thresholds, conferring to wines sweet and fruity flavors.

All volatile fatty acids detected were present at concentrations above 50 mg/L. Fatty acids have been described as giving rise to fruity, cheesy, fatty and rancid notes. Although, C6–C10 fatty acids are usually related to the appearance of negative odors, they are very important for aromatic equilibrium in wines because they oppose the hydrolysis of the corresponding esters (Torrens et al., 2008), and their presence plays an important role in the complexity of the aroma (Shinohara, 1985). Both esters and acetates have a key importance in the whole wine aroma impressing a characteristic fruity notes (Rapp and Versini, 1991; Swiegers and Pretorius, 2005).

The 4-vinyl-guaiacol was detected in all wines, with the exception of C6, C7, C9, and C10 samples. In white wines and at high concentrations, vinylphenols can be responsible for heavy "pharmaceutical" odors (Chatonnet et al., 1993), but at low and moderate concentration they can be related with pleasant spicy aroma. In this sense Grando et al. (1993), found that 4-vinil-guayacol was the main responsible for the spicy aroma of Gewurztraminer's wines.

Similar results were already obtained using yeast cells entrapped in sodium alginate and k-carrageenan for the production of rosé sparkling wine that resulted similar in sensory characteristics to the traditional products, but produced in reduced time (Tataridis et al., 2005).

In the presented screening of different inocula strategies of yeasts and bacteria for wine-making, aroma produced by free or immobilized cells in wine has been evaluated by gas chromatography: these chemical analyses of volatile compounds are suitable to produce important information about the compounds with odor-active potential. However, in agreement with the suggestions of Nedović et al. (2015), the actual sensory traits of wines produced by co-immobilization strategy will be evaluated employing trained panel and consumers, in order to obtain acceptable products to be proposed on the market.

Experiments are now under the way to set up a simple procedure to dry the co-immobilization system, in order to reduce the very limiting dilution effect on must metabolites, ensuring the maintenance of yeasts and bacteria viability and fermentation efficiency.

CONCLUSIONS

Co-immobilization of *S. cerevisiae* and *O. oeni* allowed to perform a efficient fermentation process, eliminating non-productive cell growth phase, producing a biocatalyst that can be reused several times, sensitively reducing the time of the process, opening in the future the possibility to develop continuous process. Co-immobilization strategy produced a wine with organoleptic profiles comparable with that produced with the co-inoculation and the sequential inoculation strategies in free form.

Co-immobilization of *S. bayanus* and *Leuconostoc oenos* in Ca- alginate matrix, has already been used to optimize a continuous fermentation process for cider production (Nedovic et al., 2000). Genisheva et al. (2014) developed a continuous process consisting in sequential AF and MLF by

the implementation of distinct packed-bed reactors containing immobilized *S. cerevisiae* on grape stems/skins, and *O. oeni* on grape skins, respectively.

This study individuates the most promising strategy to immobilize yeasts and *O. oeni* in a lab micro-vinification scale. The future step will be to test the suitability of co-immobilization strategy (in alginate or other matrices) to produce wines in pilot-scale, that can be more representative of actual conditions occurring in winemaking, and to obtain final products that can be submitted to sensory evaluation by panel of experts.

AUTHOR CONTRIBUTIONS

Fundamental contributions to the conception and design of the work (GB, FG), acquisition, analysis and interpretation of data (GB, MT, CV); drafting the work and revising it critically for intellectual content (GB, FG, GM). All authors approved the final version of the manuscript to be submitted for publication and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy and integrity of any part of the work are appropriately investigated and resolved.

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REFERENCES

Agouridis, N., Kopsahelis, N., Plessas, S., Koutinas, A., and Kanellaki, M. (2008). *Oenococcus oeni* cells immobilized on delignified cellulosic material for malolactic fermentation of wine. *Bioresour. Technol.* 99, 9017–9020. doi: 10.1016/j.biortech.2008.04.026

Alexandre, H., Costello, P. J., Remize, F., Guzzo, J., and Guilloux-Benatier, M. (2004). *Saccharomyces cerevisiae-Oenococcus oeni* interactions in wine: current knowledge and perspectives. *Int. J. Food Microbiol.* 93, 141–154. doi: 10.1016/j.ijfoodmicro.2003.10.013

Andrade Neves, N., Araújo Pantoja, L., and Santos, A. S. (2014). Thermovinification of grapes from the Cabernet Sauvignon and Pinot Noir varieties using immobilized yeasts. *Eur. Food Res. Technol.* 238, 79–84. doi: 10.1007/s00217-013-2062-2

Bauer, R., and Dicks, L. M. T. (2004). Control of malolactic fermentation in wine. A review. *S. Afr. J. Enol. Vitic.* 25, 74–88.

Bleve, G., Lezzi, C., Chiriatti, M. A., D'Ostuni, I., Tristezza, M., Di Venere, D., et al. (2011). Selection of non-conventional yeasts and their use in immobilized form for the bioremediation of olive oil mill wastewaters. *Bioresour. Technol.* 102, 982–989. doi: 10.1016/j.biortech.2010.09.059

Bleve, G., Lezzi, C., Mita, G., Rampino, P., Perrotta, C., Villanova, L., et al. (2008). Molecular cloning and heterologous expression of a laccase gene from *Pleurotus eryngii* in free and immobilized *Saccharomyces cerevisiae* cells. *Appl. Microbiol. Biotechnol.* 79, 731–741. doi: 10.1007/s00253-008-1479-1

Brugirard, A., Fanet, J., Seguin, A., and Torres, P. (eds.). (1991). *La Degustation et le Service des Vins Doux Naturels à Appellations d'origine Controlées*. Station Vitivinicole du Roussillon, Tresserre.

Capone, S., Tufariello, M., and Siciliano, P. (2013). Analytical characterisation of Negroamaro red wines by "Aroma Wheels". *Food Chem.* 141, 2906–2915. doi: 10.1016/j.foodchem.2013.05.105

Cavin, J. F., Prevost, H., Lin, J., Schmitt, P., and Divies, C. (1989). Medium for screening *Leuconostoc oenos* strains defective in malolactic fermentation. *Appl. Environ. Microb.* 55, 751–753.

Chatonnet, P., Dubourdieu, D., Boidron, J. N., and Lavigne, V. (1993). Synthesis of volatile phenols by *Saccharomyces cerevisiae* in wines. *J. Sci. Food Agric.* 62, 191.

Diviès, C., and Cachon, R. (2005). "Wine production by immobilized cell systems," in *Applications of Cell Immobilisation Biotechnology*, eds V. Nedović' and R. Willaert (Heidelberg: Springer), 285–293.

Etievant, P. X. (1991). "Wine," in *Volatile Compounds of Food and Beverages*, ed H. Maarse (New York, NY: Dekker), 483–546. doi: 10.1002/food.19910351027

Ferreira, V., Aznar, M., López, R., and Cacho, J. (2001). Quantitative gas chromatography-olfactometry carried out at different dilutions of an extract. Key differences in the odor profiles of four high quality Spanish aged red wines. *J. Agric. Food Chem.* 49, 4818–4824. doi: 10.1021/jf010283u

Ferreira, V., Fernandez, P., Pena, C., Escudero, A., and Cacho, J. F. (1995). Investigation on the role played by fermentation esters in the aroma of young spanish wines by multivariate analysis. *J. Sci. Food Agric.* 67, 381–392. doi: 10.1002/jsfa.2740670316

Francis, I. L., and Newton, J. L. (2005). Determining wine aroma from compositional data. Microbial modulation of wine aroma and flavour. *Aust. J. Grape Wine Res.* 11, 114–126. doi: 10.1111/j.1755-0238.2005.tb00283.x

Genisheva, Z., Mussatto, S. I., Oliveira, J. M., and Teixeira, J. A. (2013). Malolactic fermentation of wines with immobilised lactic acid bacteria – influence of concentration, type of support material and storage conditions. *Food Chem.* 138, 1510–1514. doi: 10.1016/j.foodchem.2012.11.058

Genisheva, Z., Teixeira, J. A., and Oliveira, J. M. (2014). Immobilized cell systems for batch and continuous winemaking. *Trends Food Sci. Tech.* 40, 33–47. doi: 10.1016/j.tifs.2014.07.009

Gómez García-Carpintero, E., Sánchez-Palomo, E., Gómez Gallego, M. A., and González-Viñas, M. A. (2011b). Effect of cofermentation of grape varieties on aroma profiles of La Mancha red wines. *J. Food Sci.* 76, 1169–1180. doi: 10.1111/j.1750-3841.2011.02374.x

Gómez García-Carpintero, E., Sánchez-Palomo, E., and Gonzalez-Viñas, M. A. (2011a). Aroma characterization of red wines from cv. Bobal grape variety grown in La Mancha region. *Food Res. Int.* 44, 61–70. doi: 10.1016/j.foodres.2010.11.013

Gómez-Míguez, M. J., Vicario, I. M., and Heredia, F. J. (2007). Assessment of colour and aroma in white wines vinifications: effects of grape maturity and soil type. *J. Food Eng.* 79, 758–764. doi: 10.1016/j.jfoodeng.2006.02.038

González Álvarez, M., González-Barreiro, C., Cancho-Grande, B., and Simal-Gándara, J. (2011). Relationships between Godello white wine sensory properties and its aromatic fingerprinting obtained by GC-MS. *Food Chem.* 129, 890–898. doi: 10.1016/j.foodchem.2011.05.040

Grando, M. S., Versini, G., Nicoli, G., and Mattivi, F. (1993). Vitis. 32, 43.

Günata, Y. Z., Bayonove, C., Baumes, R., and Cordonnier, R. (1985). The aroma of grapes. Extraction and determination of free and glycosidically bound fractions of some grapes aroma components. *J. Chromatogr. Sci.* 331, 83–90. doi: 10.1016/0021-9673(85)80009-1

Guth, H. (1997). Identification of character impact odorants of different white wine varieties. *J. Agric. Food Chem.* 45, 3022–3026. doi: 10.1021/jf9608433

Junter, G. A., Coquet, L., Vilain, S., and Jouenne, T. (2002). Immobilized-cell physiology: current data and the potentialities of proteomics. *Enzyme Microb. Technol.* 31, 201–212. doi: 10.1016/S0141-0229(02)00073-X

Kosseva, M., and Kennedy, J. F. (2004). Encapsulated lactic acid bacteria for control of malolactic fermentation in wine. *Artif. Cells Blood Substit. Immobil. Biotechnol.* 32, 55–65. doi: 10.1081/BIO-120028668

Kotseridis, Y., and Baumes, R. (2000). Identification of impact odorants in Bordeaux red grape juice, in the commercial yeast used for its fermentation and in the produced. *J. Agric. Food Chem.* 48, 400–406. doi: 10.1021/jf990565i

Kourkoutas, Y., Bekatorou, A., Banat, I. M., Marchant, R., and Koutinasa, A. A. (2004). Immobilization technologies and support materials suitable in alcohol beverages production: a review. *Food Microbiol.* 21, 377–397. doi: 10.1016/j.fm.2003.10.005

Lambrechts, M. G., and Pretorius, I. S. (2000). Yeast and its importance to wine aroma – a review. *S. Afr. J. Enol. Vitic.* 21, 97–129. doi: 10.1016/S0065-2164(05)57005-9

López, R., Ferreira, V., Hernández, P., and Cacho, J. F. (1999). Identification of impact odorants of young red wines made with Merlot, Cabernet Sauvignon and Grenache grape varieties: a comparative study. *J. Sci. Food Agric.* 79, 1461–1467.

Masschelein, C. A., Ryder, D. S., and Simon, J. P. (1994). Immobilized cell technology in beer production. *Crit. Rev. Biotechnol.* 14, 155–177. doi: 10.3109/07388559409086966

Melzoch, K., Rychtera, M., and Habova, V. (1994). Effects of immobilization upon the properties and behavior of *Saccharomyces cerevisiae* cells. *J. Biotechnol.* 32, 59–65. doi: 10.1016/0168-1656(94)90120-1

Mijares, M. J. (1987). *Wine Tasting. Wine Enciclopedia, Vol. 1*. Barcelona: Barcelona.

Moyano, L., Zea, L., Moreno, J., and Medina, M. (2002). Analytical study of aromatic series in sherry wines subjected to biological aging. *J. Agric. Food Chem.* 50, 7356–7361. doi: 10.1021/jf020645d

Nagarajan, S., Kruckeberg, A. L., Schmidt, K. H., Kroll, E., Hamiltona, M., McInnerney, K., et al. (2014). Uncoupling reproduction from metabolism extends chronological lifespan in yeast. *Proc. Natl. Acad. Sci. U.S.A.* 111, E1538–E1547. doi: 10.1073/pnas.1323918111

Nedović, V., Gibson, B., Mantzouridou, T. F., Bugarski, B., Djordjeviæ, V., Kaluševiæ, A., et al. (2015). Aroma formation by immobilized yeast cells in fermentation processes. *Yeast* 32, 173–216. doi: 10.1002/yea.3042

Nedović, V., Kalusevic, A., Manojlovic, V., Levica, S., and Bugarskib, B. (2011). An overview of encapsulation technologies for food applications. *Proc. Food Sci.* 1, 1806–1815. doi: 10.1016/j.profoo.2011.09.265

Nedovic, V. A., Durieuxb, A., Van Nedervelde, L., Rosseels, P., Vandegans, J., Plaisant, A., et al. (2000). Continuous cider fermentation with co-immobilized yeast and *Leuconostoc oenos* cells. *Enzyme Microb. Technol.* 26, 834–839. doi: 10.1016/S0141-0229(00)00179-4

Oliveira, M. E. S., Pantoja, L., Duarte, W. F., Collela, C. F., Valarelli, L. T., Schwan, R. F., et al. (2011). Fruit wine produced from cagaita (Eugenia dysenterica DC) by both free and immobilised yeast cell fermentation. *Food Res. Int.* 44, 2391–2400. doi: 10.1016/j.foodres.2011.02.028

Parascandola, P., de Alteriis, E., Sentandreu, R., and Zueco, J. (1997). Immobilization and ethanol stress induce the same molecular response at the level of the cell wall in growing yeast. *FEMS Microbiol. Lett.* 150, 121–126. doi: 10.1016/S0378-1097(97)00107-9

Penaido, R. A., Mauricio, J. C., and Moreno, J. (2006). Aromatic series in sherry wines with gluconic acid subjected to different biological aging conditions by *Saccharomyces cerevisiae var. capensis. Food Chem.* 94, 232–239. doi: 10.1016/j.foodchem.2004.11.010

Pereira, A. P., Mendes-Ferreira, A., Oliveira, J. M., Estevinhob, L. M., and Mendes-Faia, A. (2014). Effect of *Saccharomyces cerevisiae* cells immobilisation on mead production. *LWT- Food Sci. Technol.* 56, 21–30. doi: 10.1016/j.lwt.2013.11.005

Peris, A., and Masats, J. (2000). *El Libro Del Vino. Guia de Los Vinos de Espana*. Barcelona: Ediciones Primera Plana.

Peynaud, E. (1987). *The Taste of Wine*. London: Macdonald & Co.

Pilkington, P. H., Margaritis, A., Mensour, N. A., and Russell, I. (1998). Fundamentals of immobilised yeast cells for continuous beer fermentation: a review. *J. Inst. Brew.* 104, 19–31. doi: 10.1002/j.2050-0416.1998.tb00970.x

Piñeiro, Z., Natera, R., Castro, R., Palma, M., Puertas, B., and Barroso, C. G. (2006). Characterisation of volatile fraction of monovarietal wines: influence of winemaking practices. *Anal. Chim. Acta* 563, 165–172. doi: 10.1016/j.aca.2005.11.016

Rapp, A., and Mandery, H. (1986). Wine aroma. *Experientia* 42, 873–883. doi: 10.1007/BF01941764

Rapp, A., and Versini, G. (1991). "Influence of nitrogen compounds in grapes on aroma compounds of wine," in *Proceedings of the International Symposium on Nitrogen in Grapes and Wines*, ed J. M. Rantz (Davis, CA: American Society for Ecology and Viticulture), 156–164.

Ribereau-Gayon, P., Glories, Y., Maujean, A., and Dubourdieu, D. (2000). *Handbook of Enology, Vol. 2: The Chemistry of Wine and Stabilization and Treatments*. New York, NY: John Wiley & Sons.

Rocha, S. M., Rodrigues, F., Coutinho, P., Delgadillo, I., and Coimbra, M. A. (2004). Volatile composition of Baga red wine assessment of the identification of the would-be impact odourants. *Anal. Chim. Acta* 513, 257–262. doi: 10.1016/j.aca.2003.10.009

Rodriguez-Nogales, J. M., Fernandez-Fernández, Gómez, E. M., and Vila-Crespo, J. (2012). Antioxidant properties of sparkling wines produced with β-glucanases and commercial yeast preparations. *J. Food Sci.* 77, 1005–1010. doi: 10.1111/j.1750-3841.2012.02857.x

Romano, P., Fiore, C., Paraggio, M., Caruso, M., and Capece, A. (2003). Function of yeast species and strains in wine flavour. *Int. J. Food Microbiol.* 86, 169–180. doi: 10.1016/S0168-1605(03)00290-3

Scott, J. A., and O'Reilly, A. M. (1996). Co-immobilization of selected yeast and bacteria for controlled flavour development in an alcoholic cider beverage. *Proc. Biochem.* 31, 111–117.

Servetas, I., Berbegal, C., Camacho, N., Bekatorou, A., Ferrer, S., Nigam, P., et al. (2013). *Saccharomyces cerevisiae* and *Oenococcus oeni* immobilized in different layers of a cellulose/starch gel composite for simultaneous alcoholic and malolactic wine fermentations. *Process Biochem.* 48, 1279–1284. doi: 10.1016/j.procbio.2013.06.020

Sevda, S. B., and Rodrigues, L. (2011). The making of pomegranate wine using yeast immobilized on sodium alginate. *Afr. J. Food Sci.* 5, 299–304.

Shinohara, T. (1985). Gas chromatographic analysis of volatile fatty acids in wines. *Agric. Biol. Chem.* 49, 2211–2212.

Sipsas, V., Kolokythas, G., Kourkoutas, Y., Plessas, S., Nedovic, V. A., and Kanellaki, M. (2009). Comparative study of batch and continuous multi-stage fixed-bed tower (MFBT) bioreactor during wine-making using freeze-dried immobilized cells. *J. Food Eng.* 90, 495–503. doi: 10.1016/j.jfoodeng.2008.07.016

Sun, Z. J., Lv, G. J., Li, S. Y., Yu, W. T., Wang, W., Xie, Y. B., et al. (2007). Differential role of microenvironment in microencapsulation for improved cell tolerance to stress. *Appl. Microbiol. Biotechnol.* 75, 1419–1427. doi: 10.1007/s00253-007-0960-6

Swiegers, J. H., and Pretorius, I. S. (2005). Yeast modulation of wine flavor. Review article. *Adv. Appl. Microbiol.* 57, 131–175. doi: 10.1016/S0065-2164(05)57005-9

Tataridis, P., Ntagas, P., Voulgaris, I., and Nerantzis, E. T. (2005). Production of sparkling wine with immobilized yeast fermentation. *Electron J. Sci. Technol.* 1, 1–21.

Torrens, J., Urpi, P., Riu-Aumatell, M., Vichi, S., López-Tamames, E., and Buxaderas, S. (2008). Different commercial yeast strains affecting the volatile and sensory profile of cava base wine. *Int. J. Food Microbiol.* 124, 48–57. doi: 10.1016/j.ijfoodmicro.2008.02.023

Tristezza, M., Vetrano, C., Bleve, G., Grieco, F., Tufariello, M., Quarta, A., et al. (2012). Autochtonous fermentation starters for the industrial production of Negroamaro wines. *J. Ind. Microbiol. Biotechnol.* 39, 81–92. doi: 10.1007/s10295-011-1002-z

Verstrepen, K. J., Derdelinckx, G., and Delvaux, F. R. (2003b). Esters in beer– part 3: why do yeast cells produce fruity flavours: the physiological role of acetate ester synthesis. *Cerevisiae* 29, 19–29. doi: 10.1016/S1389-1723(03)90112-5

Verstrepen, K. J., Derdelinckx, G., Dufour, J. P., Winderickx, J., Thevelein, J. M., Pretorius, I. S., et al. (2003a). Flavor-active esters: adding fruitiness to beer. *J. Bio. Bioeng.* 96, 110–118. doi: 10.1016/S1389-1723(03)90112-5

Vila-Crespo, J., Rodriguez-Nogales, J. M., Fernandéz-Fernandéz, E., and Hernanz-Moral, M. C. (2010). "Strategies for the enhancement of malolactic fermentation in the new climate conditions," in *Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology,* ed A. Méndez-Vilas (Badajoz: Formatex Research Center), 920–929.

Vilanova, M., and Sieiro, C. (2006). Determination of free and bound terpene compounds in Albarino wine. *J. Food Compos. Anal.* 19, 694–697. doi: 10.1016/j.jfca.2005.10.002

Yokotsuka, K., Yajima, M., and Matsudo, T. (1997). Production of bottle-fermented sparkling wine using yeast immobilized in double-layer gel beads or strands. *Am. J. Enol. Vitic.* 48, 471–481.

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MINI REVIEW
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Metabolites of Microbial Origin with an Impact on Health: Ochratoxin A and Biogenic Amines

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Safety and quality are significant challenges for food; namely, safety represents a big threat all over the world and is one of the most important goal to be achieved in both Western Society and Developing Countries. Wine safety mainly relies upon some metabolites and many of them are of microbial origin. The main goal of this review is a focus on two kinds of compounds (biogenic amines and mycotoxins, mainly Ochratoxin A) for their deleterious effects on health. For each class of compounds, we will focus on two different traits: (a) synthesis of the compounds in wine, with a brief description of the most important microorganisms and factors leading this phenomenon; (b) prevention and/or correction strategies and new trends. In addition, there is a focus on a recent predictive tool able to predict toxin contamination of grape, in order to perform some prevention approaches and achieve safe wine.

Keywords: safety, biogenic amines, Ochratoxin A, prevention, correction

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INTRODUCTION

Safety is a challenge for consumers; wine safety relies upon a complex equilibrium from good manufacturing practices, quality of raw materials, fermentation, and post-fermentation events. An outbreak in wine safety generally results in the recovery of a wide variety of harmful compounds with a strong biological activity on human health (carbamate, amines, mycotoxins, heavy metals and residues from wrong production practices, methanol etc.). This paper offers an overview on wine safety with a special focus on some metabolites of microbial origin, namely Ochratoxin A (OTA) and biogenic amines (BA), as the number of papers dealing with these compounds has significantly increased in the last decade, due to an increased awareness on the effects on health and well-being.

Moreover, the choice of focusing on OTA and BA also relies on some other factors, i.e., (i) the origin (OTA from fungi and BA generally from bacteria); (ii) the time of production (pre-harvest and/or pre-fermentation for OTA and throughout fermentation or in the post-fermentation phase for BA); (iii) the molecular weight (lower for BA and higher for OTA); (iv) the increasing prevalence in human outbreaks (Spano et al., 2010; Petruzzi et al., 2014e).

Both BA and OTA are used as topics to address safety issues raised by microorganisms and show how uncontrolled microbial dynamics both on grape and must/wine could lead to significant threats. BA and OTA are described in relation to some traits, like origin, effects on health and correction/prevention strategies, in order to offer a deep insight on the literature and pinpoint the most recent advances on these compounds.

PRE-HARVEST CHALLENGE: GRAPE AND WINE CONTAMINATION BY OCHRATOXIN A

Ochratoxin A is produced by *Aspergillus* spp. and *Penicillium* spp. and derives from 3,4-dihydrocumarin linked to an amide bond with an amino group of L-β-phenylalanine (Peraica et al., 1999). It can be recovered in a variety of foods, including cereals, grapes, cocoa, coffee, and spices; its presence in alcoholic beverages is mainly in red wine followed by rosé and white wines (Battilani et al., 2006; Bellver Soto et al., 2014). Although it is found throughout the world, European and African regions are the most affected by this compound (Bellver Soto et al., 2014). Several authors conducted a survey to asses OTA contamination in European wines (Italy, Spain, Greece, Hungary, Croatia) and found it in 60–80% samples, although the median value was strongly variable (from 0.007 to 2.79 ng/ml; Bellver Soto et al., 2014).

Ochratoxin A is a great threat for humans, because it accumulates in several tissues in the body and is classified by the International Agency for Research on Cancer [IARC] (1993) in the group 2B (possible human carcinogen). Kidney is its main target, and hereby causes Balkan endemic nephropathy (BEN), chronic interstitial nephritis, and karyomegalic interstitial nephritis (Simon, 1996). In the kidney, OTA mainly impairs proximal tubular functions and causes glucosuria, enzymuria, and a decrease in the transport of *p*-aminohippuric acid (PAH), a prototypical renal organic anion (Gekle and Silbernagl, 1993, 1994; Dahlmann et al., 1998). The presence of OTA in blood from healthy humans confirms a continuous and widespread exposure (Thuvander et al., 2001; Sangare-Tigori et al., 2006), thus the Scientific Panel on Contaminants in the Food Chain from the European Food Safety Authority [EFSA] (2006) set OTA Tolerable Weekly Intake (TWI) to 120 ng/kg body weight.

A new tool for risk assessment of OTA is predictive mycology, focusing on the development of some model to predict fungal growth and inactivation, such as the theory of the Design of Experiments (Dagnas et al., 2014; Ioannidis et al., 2015; Burgain and Dantigny, 2016), equations to predict the germination of fungal spores (Kalai et al., 2014) or the production of mycotoxins (Aldars-García et al., 2015).

Recently, Battilani and Camardo Leggieri (2015) developed and designed a conceptual model for the dynamic simulation of OTA production on grape berries by *Aspergillus carbonarius*; they used some primary variables (overwinter inoculum, spores on berries, germinated spores, growth on berries, infected berries, colonized berries, and OTA index), intermediate variables (berry status; growth stage of berries), parameters (air temperature, relative humidity, rainfall, a_w, pest and disease) and rates (dispersal, germination, growth, infection, colonization, and OTA production). The variables represent the status of the fungus at any time and OTA-index represents the most important output (OTA contamination); the flow from a status to another is driven by known parameters (temperature, relative humidity etc...) or by intermediate variables derived from crop or weather data. The rates were described by Battilani and Camardo Leggieri (2015) as a kind of valve and are represented by a mathematical function ranging from 0 to 1, with 1 intended as the rate occurring at the optimal conditions and the flow to the next status stopped when the rate is 0. The most important output of this function is a risk model able to build a cumulative function, showing OTA accumulation on berries at any stage and predict if grape is under or over the legal limit and a correction strategy is required. The model was based on the data of a survey conducted in some Italian regions (Apulia, Emilia Romagna) and was preliminary validated with some confirmatory surveys. To our knowledge, this is the only attempt of predictive mycology applied to OTA; therefore, a possible way for innovation could be the design of simple software/Excel file able to predict the risk associated with OTA in grape, must and wines, in order to avoid economic losses due to the toxin.

Prevention and Correction Strategies

The presence of OTA in the grape can be shifted from grain grapes to wine during fermentation. OTA levels depend on different factors such as vineyard location (latitude), weather (rain, temperature, and relative humidity in the vineyards), period of harvest, pesticide treatments, and wine fermentation, with a strong impact of the duration of grape maceration. The European Union allows a maximum limit for OTA in wine of 2 ng/g (Bellver Soto et al., 2014).

Quintela et al. (2013) extensively reviewed physical, chemical and biological approaches to perform the decontamination of OTA. The dilution of contaminated must is strictly forbidden by EU (Commission Regulation 1881/2006; European Commission [EC], 2006) and few approaches are feasible at industrial levels, like the removal of mouldy grapes or bunches (Rosseau, 2004), the repassage of contaminated must or wines over grape pomaces having no or little OTA contamination (Solfrizzo et al., 2010), pressing the pomace at 80 atm, filtration, heat treatment on a hot plate at 55°C (Gambuti et al., 2005). Chemical removal relies upon the use of some fining agents, like activated carbon, bentonite, chitin and chitosan, egg albumin, gelatin, oak wood pieces, potassium caseinate, and PVPP (Bellver Soto et al., 2014). Each method shows benefits (cost, simple use, etc...) and drawbacks (effect on color and phenols, etc...), with a removal efficiency ranging from 2 to 98%. Moreover, some agents could cause an adverse reaction in susceptible wine consumers; therefore, the European Commission Directive 2007/68/EC (European Commission [EC], 2007) establishes that all the wines placed on European market, it is compulsory to indicate on the label if they have been treated with adjuncts derived from eggs, fish, and milk.

A promising way for wine decontamination could be the bioremediation (Quintela et al., 2013; Petruzzi et al., 2014e). There two main routes for the bioremediation, i.e., toxin degradation and adsorption. Recently, Abrunhosa et al. (2014) isolated and characterized some strains of *Pediococcus parvulus* able to hydrolize OTA bond by a putative peptidase and produce Ochratoxin α (OT α), a not toxic moiety

synthesized by animals and humans as the most important detoxification pathway. A similar mechanism was recovered by De Bellis et al. (2015) for *Acinetobacter calcoaceticus*, isolated from soil. Previously, a OTA-detoxification way was found in *Phenylobacterium immobile* (Wegst and Lingens, 1983), *Trichosporon mycotoxinivorans* (Schatzmayr et al., 2006), *Brevibacterium* spp. (Rodriguez et al., 2011), *Aspergillus* spp. (Bejaoui et al., 2006; Abrunhosa and Venâncio, 2007), and *Rhizophus* spp. (Varga et al., 2005). This pathway is promising; however, the production of OTα could be a threat, because we do not know the implication of the accumulation of this compound in the body.

A second way is OTA adsorption on yeast cell wall throughout fermentation. Toxin could be absorbed by several bacteria (*Lactobacillus rhamnosus*, *L. acidophilus*, *L. plantarum*, *Oenococcus oeni*, *L. brevis*), and fungi (*A. niger*, *A. carbonarius*, *A. japonicus*) as a result of ionic and non-covalent interaction with the cell wall. Petruzzi et al. (2013, 2014a–d, 2015a,b) and Bevilacqua et al. (2014) proposed the yeasts as adsorbing tools both under *in vitro* and *in vivo* conditions. OTA adsorption by yeasts is the result of complex interactions with glucans, and mannoproteins. Moreover, this phenomenon could be strongly affected by some factors, like pH, temperature, sugar, nitrogen supplementation, and to some extent was found partly reversible, as toxin could be released back into wine. An interesting implementation of this approach is yeast entrapment into alginate beads to design a re-usable biocatalyst (Farbo et al., 2016); entrapped yeasts were able to remove the 80% of OTA in 48 h and toxin release by beads could be better controlled than in free cells.

FERMENTATION AND POST-FERMENTATION THREATS: BIOGENIC AMINES

Biogenic amines are low-molecular-weight organic molecules originated in fermented foods from the microbial catabolism of the corresponding amino acids. Wine BA include histamine (from histidine), tyramine (from tyrosine), tryptamine (from tryptophane), cadaverine (from lysine), and putrescine (from arginine and ornithine). The production of BA is a strategy to obtain metabolic advantages to face certain stress conditions (Wolken et al., 2006). However, this feature seems to be strain-dependent rather than species-specific, suggesting that it could be disseminated by horizontal gene transfer (Lucas et al., 2008; Coton and Coton, 2009).

Although BA are degraded in the human gut lumen by the activity of amino oxidases, adverse health implications can occur in susceptible individuals. Therefore, the intake of high amounts of dietary BA can determine several disorders, from mild symptoms resembling allergic reactions until death in severe cases of histaminosis or tyraminosis (Spano et al., 2010). Moreover, it is crucial to consider the synergistic effect of inhibitors of the amino oxidases such as some drugs, alcohol, or putrescine that act as histamine enhancers. On the other hand, putrescine and other polyamines are involved in cell proliferation, and they have been correlated with cancer events (Spano et al., 2010).

Arginine and histidine are found among the most abundant amino acids in grapes. Therefore, histamine production in wines is a critical concern, since its toxicity could be amplified by the concomitant occurrence of alcohol and high levels of putrescine (Beneduce et al., 2010). Based on this issue, histamine content in wines is differently regulated among European countries, thereby foreclosing certain commercial opportunities for winemakers. Moreover, high levels of putrescine and cadaverine negatively affect the aromatic bouquet of the wine (Beneduce et al., 2010). Finally, less obvious is the threat posed by the intake of live BA-producing bacteria that could contribute to increase the risk of BA formation in the gut environment (Russo et al., 2012).

The production of BA in wine is related to the metabolic activity of lactic acid bacteria (LAB) responsible for the malolactic fermentation (MLF; Lonvaud-Funel, 2001) or associated to spoilage and/or contaminant microorganisms (Benavent-Gil et al., 2016). Since MLF is especially wanted in red wine production, higher BA amounts are usually found in red wines, then in rosè, white, or sparkling wines. *O. oeni* is the main LAB species carrying out the MLF, and some authors reported its capability to produce histamine (Lonvaud-Funel and Joyeux, 1994; Landete et al., 2007; Lucas et al., 2008). However, this metabolic trait of *O. oeni* is controversial and has been recently questioned (García-Moruño and Muñoz, 2012), suggesting that other LAB may be involved in BA formation. Among typical inhabitants of the wine, *Pd. parvulus*, *L. mali,* and *Leuconostoc mesenteroides* are reported to produce histamine, *L. brevis*, *L. hilgardii,* and *L. buchneri* were mainly associated with tyramine and putrescine formation; moreover, some strains could produce two or more BA (Moreno-Arribas et al., 2003; Landete et al., 2007; Coton et al., 2010). Recently, *Enterococcus* spp. isolated from must and wine have been described as tyramine, putrescine, and histamine producers (Capozzi et al., 2011; Pérez-Martín et al., 2014).

Biogenic amines formation during the alcoholic fermentation is considered irrelevant. Accordingly, enological yeasts were found unable to produce BA (Landete et al., 2007). Nonetheless, some strains can produce low amounts of polyamines (Caruso et al., 2002). Moreover, some non-*Saccharomyces* strains were able to synthesize histamine and cadaverine during must fermentation suggesting the importance of a correct yeast management during wine-making (Tristezza et al., 2013).

Controlling BA in Wine

In fermented foods, different levels of BA can be achieved depending from the occurrence of BA-forming bacteria, the availability of free amino acids and the expression of the corresponding BA biosynthetic pathways under favorable environmental conditions (Spano et al., 2010).

Actually, the control of these toxic compounds in wine is mainly based on the adoption of strategy to prevent their formation than on their elimination from the beverage (Mohedano et al., 2014).

A correct management of all the factors related to the increase of precursor amino acids should be implemented. The abundance of free amino acids is strictly related to agronomical techniques (i.e., nitrogenous fertilization, irrigation, and vintage), environmental conditions, grape variety and/or geographical origin, maturation degree of the grape (Landete et al., 2005; Ancín-Azpilicueta et al., 2008; Del Prete et al., 2009; Cecchini and Morassut, 2010; Ortega-Heras et al., 2014; Smit et al., 2014).

Amino acids are key precursors and contribute to aroma and organoleptic profile of wines. Therefore, it seems to be more advisable to intervene controlling the microflora responsible for the vinification in order to avoid potential BA-producers. Common practices in wine-making are the addition of sulphite and the inoculation of starter cultures in order to inhibit the growth of unknown and uncharacterized indigenous microorganisms. Accordingly, organic wines obtained by spontaneous fermentation and lower levels of sulphite showed higher contents of BA (García-Marino et al., 2010; Comuzzo et al., 2013). In contrast, concurrent yeast/bacteria inoculation of musts has been proposed as an interesting practice to obtain a significant reduction of BA (Izquierdo-Cañas et al., 2012; Smit et al., 2012). Therefore, a remarkable criterion to select oenological starters should be the absence of the genetic determinants to produce BA (Landete et al., 2011; Capozzi et al., 2014). Nonetheless, an underestimated issue is the contamination of commercial yeast starters with BA-producing LAB (Costantini et al., 2009), as well as the interactions between natural yeasts and LAB able to form BA from short peptides (Bonnin-Jusserand et al., 2012).

Wine environment enhance BA formation, since the genes responsible for BA production were induced at low pH (Arena et al., 2011), and BA content could be increased under wine poor nutritional conditions (Aredes-Fernández et al., 2010). Winemaking practices (maceration, aging, and storage) can influence the levels of BA in wine, probably due to an increase of the amino acid concentration from grape skin, yeast autolysis or contact with lees (Marques et al., 2008; Ancín-Azpilicueta et al., 2010; Smit and du Toit, 2013; Smit et al., 2013).

Therefore, prevent BA biosynthesis in wine is not always possible since a number of microbiological, chemical, and physical conditions should be addressed in a way that may affect the organoleptic properties of the wine or result incompatible with specific productions such as spontaneous fermented, organic, or sulphite-free wines. An attractive strategy to correct the occurrence of BA in wines could be the employment of BA-degrading microorganisms (Alvarez and Moreno-Arribas, 2014). Grapevine ecosystem fungi and some wine LAB belonging to *Lactobacillus* and *Pediococcus* genera were able to degrade histamine, tyramine, and putrescine in culture media (García-Ruiz et al., 2011; Cueva et al., 2012). However, the ability of these microorganisms to reduce BA was negatively affected by wine matrix, suggesting that the effectiveness of the amino oxidase activity could be modulated by the physico-chemical wine composition (García-Ruiz et al., 2011; Cueva et al., 2012). With a similar approach, two wine *L. plantarum* were able to reduce tyramine and putrescine in media containing BA or in presence of specific chemical precursors and BA-producers LAB (Capozzi et al., 2012). These strains showed promising technological aptitudes, suggesting that the ability to degrade BA could be a driver to select a new generation of MLF starter cultures (Capozzi et al., 2012). In a recent study, multicopper oxidases from wine-associated LAB have been purified and identified as responsible for the reduction of histamine, tyramine, and putrescine in wine (Callejón et al., 2014). The same authors further investigated this enzyme with a recombinant approach, indicating that oenological LAB or their purified enzymes could solve the problem of high amine concentrations in wine (Callejón et al., 2016). This strategy could be particularly interesting if the employment of a microbial strain is not recommended as starter culture for wine production, as reported for the yeast *Debaryomyces hansenii* although this microorganism was able to degrade a broad spectrum of BA (Bäumlisberger et al., 2015). Accordingly, it was demonstrated that a flavin-dependent oxidase from *Kocuria varians*, a bacterial starter for the manufacture of fermented meat, degraded putrescine and cadaverine even under the harsh wine conditions (Callejón et al., 2015).

FUTURE PERSPECTIVES

Although the biological degradation of BA and the removal of OTA offer interesting perspectives for wine industry, prevention is the most important strategy to control the threat of these toxic compounds. Nowadays, advances in molecular and ohmic approaches may ensure considerable advantages to select safe microbial starter cultures. At the same time, progresses in analytical tools can provide an early detection of BA and OTA encouraging their monitoring during winemaking and storage. However, it is presumable that the risk of these compounds in wine is still underestimated, due to a poor awareness of the consumer, misdiagnosis, and discrepant surveillances across the world countries. In this regard, it is crucial to emphasize the effort of regulatory agencies, such as recently EFSA, to propose a standardized and harmonious framework for BA and OTA risk assessment and detection (European Food Safety Authority [EFSA], 2011).

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PR, VC, GS, MC, MS, and AB performed an accurate research in the literature and planned paper. PR and AB wrote the paper.

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REFERENCES

Abrunhosa, L., Inês, A., Rodrigues, A. I., Guimarães, A., Pereira, V. L., Paropt, P., et al. (2014). Biodegradation of Ochratoxin A by *Pediococcus parvulus* isolated from Douro wines. *Int. J. Food Microbiol.* 188, 45–52. doi: 10.1016/j.ijfoodmicro.2014.07.019

Abrunhosa, L., and Venâncio, A. (2007). Isolation and purification of an enzyme hydrolyzing Ochratoxin A from *Aspergillus niger*. *Biotechnol. Lett.* 29, 1909–1914. doi: 10.1007/s10529-007-9479-2

Aldars-García, L., Ramos, A. J., Sanchis, V., and Marín, S. (2015). An attempt to model the probability of growth and aflatoxin B1 production of *Aspergillus flavus* under non-isothermal conditions in pistachio nuts. *Food Microbiol.* 51, 117–129. doi: 10.1016/j.fm.2015.05.013

Alvarez, M. A., and Moreno-Arribas, M. V. (2014). The problem of biogenic amines in fermented foods and the use of potential biogenic amine-degrading microorganisms as a solution. *Trends Food Sci. Technol.* 39, 146–155. doi: 10.1016/j.tifs.2014.07.007

Ancín-Azpilicueta, C., González-Marco, A., and Jiménez-Moreno, N. (2008). Current knowledge about the presence of amines in wine. *Crit. Rev. Food Sci. Nutr.* 48, 257–275. doi: 10.1080/10408390701289441

Ancín-Azpilicueta, C., González-Marco, A., and Jiménez-Moreno, N. (2010). Comparative study of the amine concentration in wines obtained from the traditional fermentation and from a more anaerobic fermentation method. *LWT-Food Sci. Technol.* 43, 771–776. doi: 10.1016/j.lwt.2009.12.013

Aredes-Fernández, P. A., Farias, M. E., and Manca de Nadra, M. C. (2010). Interaction between *Oenococcus oeni* and *Lactobacillus hilgardii* isolated from wine. Modification of available nitrogen and biogenic amine production. *Biotechnol. Lett.* 32, 1095–1102. doi: 10.1007/s10529-010-0260-6

Arena, M. P., Russo, P., Capozzi, V., Beneduce, L., and Spano, G. (2011). Effect of abiotic stress conditions on expression of the *Lactobacillus brevis* IOEB 9809 tyrosine decarboxylase and agmatine deiminase genes. *Ann. Microbiol.* 61, 179–183. doi: 10.1007/s13213-010-0089-1

Battilani, P., and Camardo Leggieri, M. (2015). OTA-grapes: a mechanistic model to predict Ochratoxin A risk in grapes, a step beyond the systems approach. *Toxins* 7, 3012–3029. doi: 10.3390/toxins7083012

Battilani, P., Giorni, P., Bertuzzi, T., Formenti, S., and Pietri, A. (2006). Black aspergilli and Ochratoxin A in grapes in Italy. *Int. J. Food Microbiol.* 111, S53–S60. doi: 10.1016/j.ijfoodmicro.2006.03.006

Bäumlisberger, M., Moellecken, U., König, H., and Claus, H. (2015). The potential of the yeast *Debaryomyces hansenii* H525 to degrade biogenic amines in food. *Microorganisms* 3, 839–850. doi: 10.3390/microorganisms3040839

Bejaoui, H., Mathieu, F., Taillandier, P., and Lebrihi, A. (2006). Biodegradation of Ochratoxin A by *Aspergillus* section Nigri species isolated from French grapes: a potential means of Ochratoxin A decontamination in grape juice and musts. *FEMS Microbiol. Lett.* 255, 203–208. doi: 10.1111/j.1574-6968.2005.00073.x

Bellver Soto, J., Fernández-Franzón, M., Ruiz, M. J., and García, A. J. (2014). Presence of Ochratoxin A (OTA) mycotoxin in alcoholic drinks from southern european countries: wine and beer. *J. Agric. Food Chem.* 62, 7643–7651. doi: 10.1021/jf501737h

Benavent-Gil, Y., Berbegal, C., Lucio, O., Pardo, I., and Ferrer, S. (2016). A new fear in wine: isolation of *Staphylococcus epidermidis* histamine producer. *Food Control* 62, 142–149. doi: 10.1016/j.foodcont.2015.10.026

Beneduce, L., Romano, A., Capozzi, V., Lucas, P., Barnavon, L., Bach, B., et al. (2010). Biogenic amines in regional wines. *Ann. Microbiol.* 60, 573–578. doi: 10.1007/s13213-010-0094-4

Bevilacqua, A., Petruzzi, L., Corbo, M. R., Baiano, A., Garofalo, C., and Sinigaglia, M. (2014). Ochratoxin A released back into the medium by *Saccharomyces cerevisiae* as a function of the strain, washing medium, and fermentative conditions. *J. Sci. Food Agric.* 94, 3291–3295. doi: 10.1002/jsfa.6683

Bonnin-Jusserand, M., Grandvalet, C., Rieu, A., Weidmann, S., and Alexandre, H. (2012). Tyrosine-containing peptides are precursors of tyramine produced by *Lactobacillus plantarum* strain IR BL0076 isolated from wine. *BMC Microbiol.* 12:199. doi: 10.1186/1471-2180-12-199

Burgain, A., and Dantigny, P. (2016). Inoculation of airborne conidia of *Penicillium chrysogenum* on the surface of a solid medium. *Food Microbiol.* 54, 162–166. doi: 10.1016/j.fm.2015.10.002

Callejón, S., Sendra, R., Ferrer, S., and Pardo, I. (2014). Identification of a novel enzymatic activity from lactic acid bacteria able to degrade biogenic amines in wine. *Appl. Microbiol. Biotechnol.* 98, 185–198. doi: 10.1007/s00253-013-4829-6

Callejón, S., Sendra, R., Ferrer, S., and Pardo, I. (2015). Ability of *Kocuria varians* LTH 1540 to degrade putrescine: identification and characterization of a novel amine oxidase. *J. Agric. Food Chem.* 63, 4170–4178. doi: 10.1021/jf5026967

Callejón, S., Sendra, R., Ferrer, S., and Pardo, I. (2016). Cloning and characterization of a new laccase from *Lactobacillus plantarum* J16 CECT 8944 catalyzing biogenic amines degradation. *Appl. Microbiol. Biotechnol.* 100, 3113–3124. doi: 10.1007/s00253-015-7158-0

Capozzi, V., Ladero, V., Beneduce, L., Fernández, M., Alvarez, M. A., Benoit, B., et al. (2011). Isolation and characterization of tyramine-producing *Enterococcus faecium* strains from red wine. *Food Microbiol.* 28, 434–439. doi: 10.1016/j.fm.2010.10.005

Capozzi, V., Russo, P., Ladero, V., Fernandez, M., Fiocco, D., Alvarez, M. A., et al. (2012). Biogenic amines degradation by *Lactobacillus plantarum*: towards a potential application in wine. *Front. Microbiol.* 3:122. doi: 10.3389/fmicb.2012.00122

Capozzi, V., Russo, P., Lamontanara, A., Orrù, L., Cattivelli, L., and Spano, G. (2014). Genome sequences of five *Oenococcus oeni* strains isolated from Nero Di Troia wine from the same terroir in Apulia, Southern Italy. *Genome Announc.* 2, e1077-14. doi: 10.1128/genomeA.01077-14

Caruso, M., Fiore, C., Contursi, M., Salzano, G., Paparella, A., and Romano, P. (2002). Formation of biogenic amines as criteria for the selection of wine yeasts. *World J. Microbiol. Biotechnol.* 18, 159–163. doi: 10.1023/A:1014451728868

Cecchini, F., and Morassut, M. (2010). Effect of grape storage time on biogenic amines content in must. *Food Chem.* 123, 263–268. doi: 10.1016/j.foodchem.2010.04.026

Comuzzo, P., Rauhut, D., Werner, M., Lagazio, C., and Zironi, R. (2013). A survey on wines from organic viticulture from different European Countries. *Food Control* 34, 274–282. doi: 10.1016/j.foodcont.2013.04.039

Costantini, A., Vaudano, E., Del Prete, V., Danei, M., and García-Moruño, E. (2009). Biogenic amine production by contaminating bacteria found in starter preparations used in winemaking. *J. Agric. Food Chem.* 57, 10664–10669. doi: 10.1021/jf9025426

Coton, E., and Coton, M. (2009). Evidence of horizontal transfer as origin of strain to strain variation of the tyramine production trait in *Lactobacillus brevis*. *Food Microbiol.* 26, 52–57. doi: 10.1016/j.fm.2008.07.009

Coton, M., Romano, A., Spano, G., Ziegler, K., Vetrana, C., Desmarais, C., et al. (2010). Occurrence of biogenic amine-forming lactic acid bacteria in wine and cider. *Food Microbiol.* 27, 1078–1085. doi: 10.1016/j.fm.2010.07.012

Cueva, C., García-Ruiz, A., González-Rompinelli, E., Bartolome, B., Martín-Álvarez, P. J., Salazar, O., et al. (2012). Degradation of biogenic amines by vineyard ecosystem fungi. Potential use in winemaking. *J. Appl. Microbiol.* 112, 672–682. doi: 10.1111/j.1365-2672.2012.05243.x

Dagnas, S., Onno, B., and Membré, J.-M. (2014). Modeling growth of three bakery product spoilage molds as a function of water activity, temperature and pH. *Int. J. Food Microbiol.* 186, 95–104. doi: 10.1016/j.ijfoodmicro.2014.06.022

Dahlmann, A., Dantzler, W. H., Silbernagl, S., and Gekle, M. (1998). Detailed mapping of Ochratoxin A reabsorption along the rat nephron in vivo: the nephrotoxin can be reabsorbed in all nephron segments by different mechanisms. *J. Pharmacol. Exp. Ther.* 286, 157–162.

De Bellis, P., Tristezza, M., Haidukowski, M., Fanelli, F., Sisto, A., Mulè, G., et al. (2015). Biodegradation of Ochratoxin A by bacterial strains isolated from vineyard soils. *Toxins* 7, 5079–5093. doi: 10.3390/toxins7124864

Del Prete, V., Costantini, A., Cecchini, F., Morassut, M., and García-Moruño, E. (2009). Occurrence of biogenic amines in wine: the role of grapes. *Food Chem.* 112, 474–481. doi: 10.1016/j.foodchem.2008.05.102

European Commission [EC] (2006). Commission regulation (EC) N. 1881/2006 of 19 December 2006 setting levels. (for)certain contaminants in food-stuffs. *Off. J. Eur. Un.* L364, 5–24.

European Commission [EC] (2007). Commission directive 2007/68/EC of 27 November 2007 amending Annex IIIa to directive. (2000)/13/EC of the European parliament and of the council as regards certain food ingredients. *Off. J. Eur. Un.* L310, 11–14.

European Food Safety Authority [EFSA] (2006). Opinion of the scientific panel on contaminants in the food chain on a request. (from)commission related to Ochratoxin A in food. *EFSA J.* 365, 1–56.

European Food Safety Authority [EFSA] (2011). Scientific opinion on risk based control of biogenic amine formation in fermented foods. *EFSA J.* 9:2393.

Farbo, M. G., Urgeghe, P. P., Fiori, S., Marceddu, S., Jaoua, S., and Migheli, Q. (2016). Adsorption of Ochratoxin A from grape juice by yeast cells immobilised in calcium alginate beads. *Int. J. Food Microbiol.* 217, 29–34. doi: 10.1016/j.ijfoodmicro.2015.10.012

Gambuti, A., Strollo, D., Genovese, A., Ugliano, M., Ritieni, A., and Moio, L. (2005). Influence of enological practices on Ochratoxin A concentration in wine. *Am. J. Enol. Viticul.* 56, 155–162.

García-Marino, M., Trigueros, Á., and Escribano-Bailón, T. (2010). Influence of oenological practices on the formation of biogenic amines in quality red wines. *J. Food Compos. Anal.* 23, 455–462. doi: 10.1016/j.jfca.2010.02.003

García-Moruño, E., and Muñoz, R. (2012). Does *Oenococcus oeni* produce histamine? *Int. J. Food Microbiol.* 157, 121–129. doi: 10.1016/j.ijfoodmicro.2012.05.013

García-Ruiz, A., González-Rompinelli, E. M., Bartolomé, B., and Moreno-Arribas, M. V. (2011). Potential of wine-associated lactic acid bacteria to degrade biogenic amines. *Int. J. Food Microbiol.* 148, 115–120. doi: 10.1016/j.ijfoodmicro.2011.05.009

Gekle, M., and Silbernagl, S. (1993). Mechanism of Ochratoxin A-induced reduction of glomerular filtration rate in rats. *J. Pharmacol. Exp. Ther.* 267, 316–321.

Gekle, M., and Silbernagl, S. (1994). The role of the proximal tubule in Ochratoxin A nephrotoxicity in vivo: toxicodynamic and toxokinetic aspects. *Ren. Physiol. Biochem.* 17, 40–49.

International Agency for Research on Cancer [IARC] (1993). *Monographs on the Evaluation of Carcinogenic Risks to Humans. Some Naturally Occurring Substances: Food Items and Constituents, Heterocyclic Aromatic Amines and Mycotoxins*. Lyon: IARC.

Ioannidis, A.-G., Kogkaki, E. A., Natskoulis, P. I., Nychas, G. J. E., and Panagou, E. Z. (2015). Modelling the influence of temperature, water activity and sodium metabisulphite on the growth and OTA production of *Aspergillus carbonarius* isolated from Greek wine grapes. *Food Microbiol.* 49, 12–22. doi: 10.1016/j.fm.2015.01.006

Izquierdo-Cañas, P. M., Pérez-Martín, F., García-Romero, E., Seseña-Prieto, S., and Palop-Herreros, M. L. (2012). Influence of inoculation time of an autochthonous selected malolactic bacterium on volatile and sensory profile of Tempranillo and Merlot wines. *Int. J. Food Microbiol.* 156, 245–254. doi: 10.1016/j.ijfoodmicro.2012.03.033

Kalai, S., Bensoussan, M., and Dantigny, P. (2014). Lag time for germination of *Penicillium chrysogenum* conidia is induced by temperature shifts. *Food Microbiol.* 42, 149–153. doi: 10.1016/j.fm.2014.03.016

Landete, J. M., De Las Rivas, B., Marcobal, A., and Muñoz, R. (2011). PCR methods for the detection of biogenic amine-producing bacteria on wine. *Ann. Microbiol.* 61, 159–166. doi: 10.1007/s13213-010-0068-6

Landete, J. M., Ferrer, S., and Pardo, I. (2007). Biogenic amine production by lactic acid bacteria, acetic bacteria and yeast isolated from wine. *Food Control* 18, 1569–1574. doi: 10.1016/j.foodcont.2006.12.008

Landete, J. M., Ferrer, S., Polo, L., and Pardo, I. (2005). Biogenic amines in wines from three Spanish regions. *J. Agric. Food Chem.* 53, 1119–1124. doi: 10.1021/jf049340k

Lonvaud-Funel, A. (2001). Biogenic amines in wines: role of lactic acid bacteria. *FEMS Microbiol. Lett.* 199, 9–13. doi: 10.1111/j.1574-6968.2001.tb10643.x

Lonvaud-Funel, A., and Joyeux, A. (1994). Histamine production by wine lactic acid bacteria: isolation of a histamine-producing strain of *Leuconostoc* oenos. *J. Appl. Bacteriol.* 77, 401–407. doi: 10.1111/j.1365-2672.1994.tb03441.x

Lucas, P. M., Claisse, O., and Lonvaud-Funel, A. (2008). High frequency of histamine-producing bacteria in the enological environment and instability of the histidine decarboxylase production phenotype. *Appl. Environ. Microbiol.* 74, 811–817. doi: 10.1128/AEM.01496-07

Marques, A. P., Leitão, M. C., and San Romão, M. V. (2008). Biogenic amines in wines: influence of oenological factors. *Food Chem.* 107, 853–860. doi: 10.1016/j.foodchem.2007.09.004

Mohedano, M. L., Spano, G., López, P., and Russo, P. (2014). "Controlling the formation of biogenic amines in fermented foods," in *Advances in Fermented Foods and Beverages. Improving Quality, Technologies and Health Benefits*, ed. W. Holzapfel (Cambridge: Woodhead Publishing), 273–310.

Moreno-Arribas, M. V., Polo, M. C., Jorganes, F., and Muñoz, R. (2003). Screening of biogenic amine production by lactic acid bacteria isolated from grape must and wine. *Int. J. Food Microbiol.* 84, 117–123. doi: 10.1016/S0168-1605(02)00391-4

Ortega-Heras, M., Pérez-Magariño, S., Del-Villar-Garrachón, V., González-Huerta, C., Moro-Gonzalez, L. C., Guadarrama-Rodríguez, A., et al. (2014). Study of the effect of vintage, maturity degree, and irrigation on the amino acid and biogenic amine content of a white wine from the *Verdejo* variety. *J. Sci. Food Agric.* 94, 2073–2082. doi: 10.1002/jsfa.6526

Peraica, M., Radic, B., Lucicć, P., and Pavlovic, M. (1999). Toxic effects of mycotoxins in humans. *Int. J. Public Health* 77, 754–766.

Pérez-Martín, F., Seseña, S., Izquierdo, P. M., and Palop, M. L. (2014). Are *Enterococcus* populations present during malolactic fermentation of red wine safe? *Food Microbiol.* 42, 95–101. doi: 10.1016/j.fm.2014.03.001

Petruzzi, L., Baiano, A., De Gianni, A., Sinigaglia, M., Corbo, M. R., and Bevilacqua, A. (2015a). Differential adsorption of Ochratoxin A and anthocyanins by inactivated yeasts and yeast cell walls during simulation of wine aging. *Toxins* 7, 4350–4365. doi: 10.3390/toxins7104350

Petruzzi, L., Bevilacqua, A., Baiano, A., Beneduce, L., Corbo, M. R., and Sinigaglia, M. (2014a). Study of *Saccharomyces cerevisiae* W13 as a functional starter for the removal of Ochratoxin A. *Food Control* 35, 373–377. doi: 10.1016/j.foodcont.2013.07.033

Petruzzi, L., Bevilacqua, A., Baiano, A., Beneduce, L., Corbo, M. R., and Sinigaglia, M. (2014b). In vitro removal of Ochratoxin A by two strains of *Saccharomycers cerevisiae* and their performances under fermentative and stress conditions. *J. Appl. Microbiol.* 116, 60–70. doi: 10.1111/jam.12350

Petruzzi, L., Bevilacqua, A., Corbo, M. R., Garofalo, C., Baiano, A., and Sinigaglia, M. (2014c). Selection of authochtonous *Saccharomyces cerevisiae* as strains as wine starters using a polyphasic approach and Ochratoxin A removal. *J. Food Protect.* 77, 1168–1177. doi: 10.4315/0362-028X.JFP-13-384

Petruzzi, L., Corbo, M. R., Baiano, A., Beneduce, L., Sinigaglia, M., and Bevilacqua, A. (2015b). In vivo stability of the complex Ochratoxin A - *Saccharomyces cerevisiae* starter strains. *Food Control* 50, 516–520. doi: 10.1016/j.foodcont.2014.09.042

Petruzzi, L., Corbo, M. R., Sinigaglia, M., and Bevilacqua, A. (2014d). Yeasts as adsorbing tools to remove Ochratoxin A in a model wine. *Int. J. Food Sci. Technol.* 49, 936–940. doi: 10.1111/ijfs.12380

Petruzzi, L., Sinigaglia, M., Corbo, M. R., Beneduce, L., and Bevilacqua, A. (2013). Ochratoxin A removal by *Saccharomyces cerevisiae* strains: effects of wine-related physico-chemical factors. *J. Sci. Food Agric.* 93, 2110–2115. doi: 10.1002/jsfa.6010

Petruzzi, L., Sinigaglia, M., Corbo, M. R., Campaniello, D., Speranza, B., and Bevilacqua, A. (2014e). Decontamination of Ochratoxin A by yeasts: possible approaches and factor leading to toxin removal in wine. *Appl. Microbiol. Biotechnol.* 98, 6555–6567. doi: 10.1007/s00253-014-5814-4

Quintela, S., Villaràn, M. A. C., López de Armentia, I., and Elejalde, E. (2013). Ochratoxin A removal in wine: a review. *Food Control* 30, 439–445. doi: 10.1016/j.foodcont.2012.08.014

Rodriguez, H., Reveron, I., Doria, F., Costantini, A., de Las Rivas, B., Muñoz, R., et al. (2011). Degradation of Ochratoxin A by *Brevibacterium* species. *J. Agric. Food Chem.* 59, 10755–10760. doi: 10.1021/jf203061p

Rosseau, J. (2004). Ochratoxina A en los vinos: estado de los conocimientos. *Wine Internet Tech. J.* 17, 1–5.

Russo, P., Fernández de Palencia, P., Romano, A., Fernández, M., Lucas, P., Spano, G., et al. (2012). Biogenic amine production by the wine *Lactobacillus brevis* IOEB 9809 in systems that partially mimic the gastrointestinal tract stress. *BMC Microbiol.* 12:247. doi: 10.1186/1471-2180-12-247

Sangare-Tigori, B., Moukha, S., Kouadio, J. H., Dano, D. S., Betbeder, A. M., Achour, A., et al. (2006). Ochratoxin A in human blood in Abidjan. *Côte d'Ivoire. Toxicon.* 47, 894–900. doi: 10.1016/j.toxicon.2006.03.001

Schatzmayr, G., Zehner, F., Täubel, M., Schatzmayr, D., Klimitsch, A., Loibner, A., et al. (2006). Microbiologicals for deactivating mycotoxins. *Mol. Nutr. Food Res.* 50, 543–551. doi: 10.1002/mnfr.200500181

Simon, P. (1996). Ochratoxin and kidney disease in the human. *J. Toxicol.* 15, 239–249.

Smit, A. Y., and du Toit, M. (2013). Evaluating the influence of malolactic fermentation inoculation practices and ageing on lees on biogenic amine

production in wine. *Food Bioprocess Tech.* 6, 198–206. doi: 10.1007/s11947-011-0702-8

Smit, A. Y., du Toit, W. J., Stander, M., and du Toit, M. (2013). Evaluating the influence of maceration practices on biogenic amine formation in wine. *LWT-Food Sci. Technol.* 53, 297–307. doi: 10.1016/j.lwt.2013.01.006

Smit, A. Y., Engelbrecht, L., and du Toit, M. (2012). Managing your wine fermentation to reduce the risk of biogenic amine formation. *Front. Microbiol.* 3:76. doi: 10.3389/fmicb.2012.00076

Smit, I., Pfliehinger, M., Binner, A., Großmann, M., Horst, W. J., and Löhnertz, O. (2014). Nitrogen fertilisation increases biogenic amines and amino acid concentrations in *Vitis vinifera* var. Riesling musts and wines. *J. Sci. Food Agric.* 94, 2064–2072. doi: 10.1002/jsfa.6525

Solfrizzo, M., Avantaggiato, G., Panzarini, G., and Visconti, A. (2010). Removal of Ochratoxin A from contaminated red wines by repassage over grape pomaces. *J. Agric. Food Chem.* 58, 317–323. doi: 10.1021/jf9030585

Spano, G., Russo, P., Lonvaud-Funel, A., Lucas, P., Alexandre, H., Grandvalet, C., et al. (2010). Biogenic amines in fermented foods. *Eur. J. Clin. Nutr.* 64, 95–100. doi: 10.1038/ejcn.2010.218

Thuvander, A., Paulsen, J. E., Axberg, K., Johansson, N., Vidnes, A., Enghardt-Barbieri, H., et al. (2001). Levels of Ochratoxin A in blood from Norwegian and Swedish blood donors and their possible correlation with food consumption. *Food Chem. Toxicol.* 39, 1145–1151. doi: 10.1016/S0278-6915(01)00080-1

Tristezza, M., Vetrano, C., Bleve, G., Spano, G., Capozzi, V., Logrieco, A., et al. (2013). Biodiversity and safety aspects of yeast strains characterized from vineyards and spontaneous fermentations in the Apulia Region, Italy. *Food Microbiol.* 36, 335–342. doi: 10.1016/j.fm.2013.07.001

Varga, J., Péteri, Z., Tábori, K., Téren, J., and Vágvölgyi, C. (2005). Degradation of Ochratoxin A and other mycotoxins by *Rhizopus* isolates. *Int. J. Food Microbiol.* 99, 321–328. doi: 10.1016/j.ijfoodmicro.2004.10.034

Wegst, W., and Lingens, F. (1983). Bacterial degradation of Ochratoxin A. *FEMS Microbiol. Lett.* 17, 341–344. doi: 10.1111/j.1574-6968.1983.tb00433.x

Wolken, W. A. M., Lucas, P. M., Lonvaud-Funel, A., and Lolkema, J. S. (2006). The mechanism of the tyrosine transporter TyrP supports a proton motive tyrosine decarboxylation pathway in *Lactobacillus brevis*. *J. Bacteriol.* 188, 2198–2206. doi: 10.1128/JB.188.6.2198-2206.2006

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