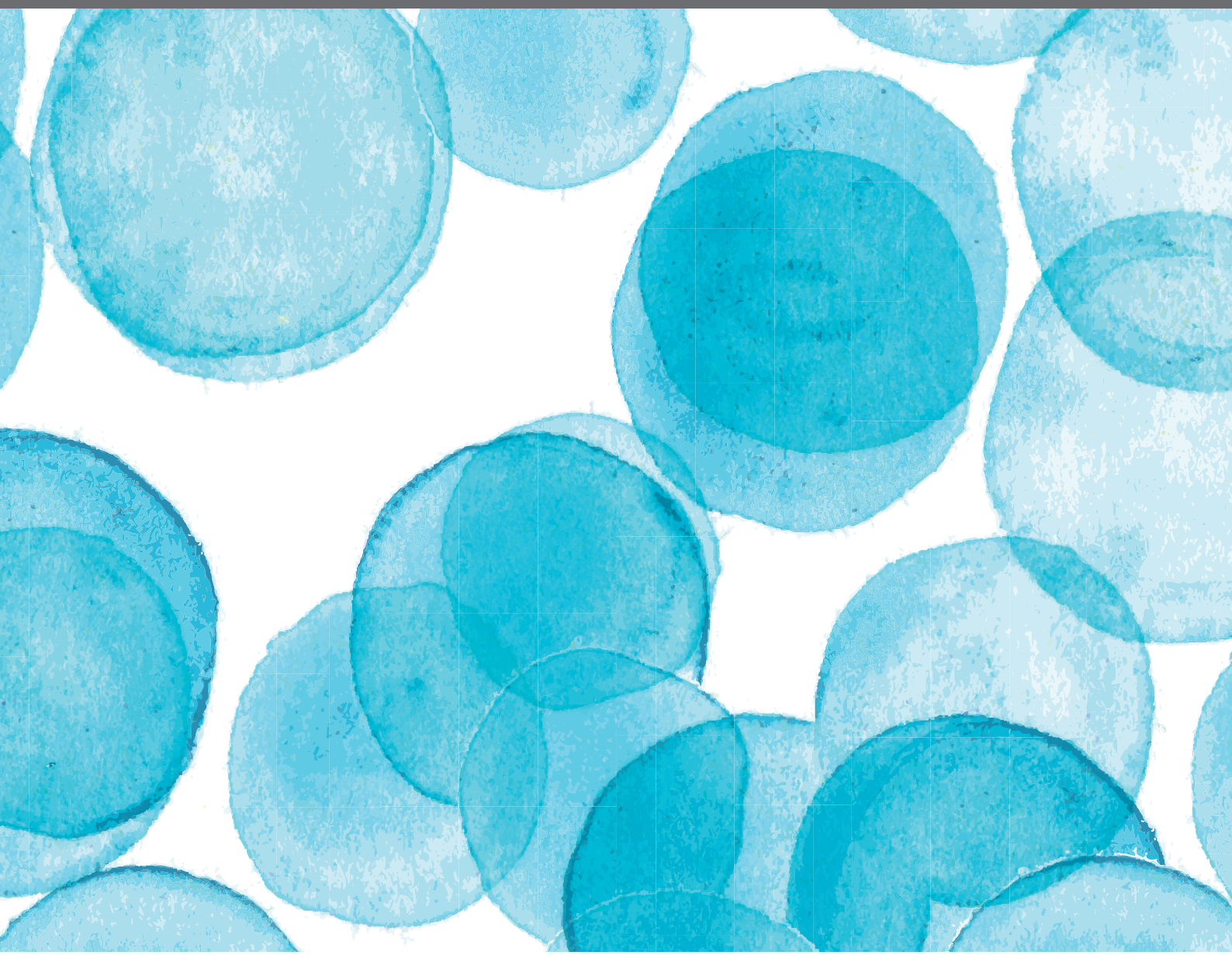
UNDERSTANDING WINE MICROBIOTA: CHALLENGES AND OPPORTUNITIES

EDITED BY: Aline Lonvaud and Linda F. Bisson
PUBLISHED IN: Frontiers in Microbiology



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ISSN 1664-8714
ISBN 978-2-88945-959-9
DOI 10.3389/978-2-88945-959-9

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UNDERSTANDING WINE MICROBIOTA: CHALLENGES AND OPPORTUNITIES

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Wine yeast and bacteria have been extensively characterized in terms of physiological and metabolic traits largely in pure culture analyses. Winemaking practices derived from this basic knowledge have undoubtedly improved wine quality. Phylogenetic studies and genome comparisons in extensive collections have revealed the processes of evolution and adaptation of the two main microbial species, *Saccharomyces cerevisiae* and *Oenococcusoeni*, present in wine. However, grapes and grape juice contain a variety of microorganisms and these principal agents of fermentation are in fact part of a complex microbial community that evolves dynamically in a special niche. Thanks to the new methods of analysis, the complexity of the microbiota can be measured in any sample of must or wine. In addition, there is greater appreciation of diversity within the main species present in wine. Intraspecific diversity has been evaluated in yeast and bacteria species and strains can be typed even in the mixture of selected or indigenous strains. Descriptions of microbial profiles in all the regions of the world suggest that the microbiota is a significant element of terroir or regional signature. It is no longer enough to simply describe what is present. It is important to consider evolution, physiology and metabolism taking into account microbial interactions within the community.

Research in wine microbiology has also expanded our understanding of the participation and role of non-*Saccharomyces* organisms in winemaking, and refined knowledge on microbial spoilage. However, it is challenging to go from the simple description of these phenomena to their interpretation. The greatest difficulty lies in analyzing the functioning of the extraordinary complex system of yeast and bacteria present during different stages of the fermentation. Interactions in the very particular environment of fermenting grape induce alternations of relative populations' dominances and declines with subsequent impacts on wine composition. Some mechanisms have been identified or suggested, but much remains to be done. The recent advent of inoculation with non-*Saccharomyces* in oenological practice, sometimes leading to inconstant results, reflects the profound gaps that exist in knowledge of the complexity of fermentation and wine microbial ecosystems. Understanding how the microbial community works is expected to provide a sound basis before using fermentation helpers and starters, taking into account the indigenous microbiota. It will also aid in monitoring and understanding native or uninoculated fermentations that rely on the complex interactions of grape, winery and fermentation biota for their aroma and flavor profile.

The aim of this Research Topic was to bring together current knowledge on several key aspects of wine microorganism biology:

i) Evolution / co-evolution of yeasts and bacteria in their process of domestication and adaptation to the oenological niche.
ii) Mechanisms of interactions between species and strains, both on grapes and in grape must.
iii) Metabolism and physiology of yeast and bacteria in interactions with each other and with the environment, considering to what extent expected objectives (typicity, lower alcohol, etc.,) can be reached by using selected strains.
iv) Development of novel technologies or approaches for the assessment of changes in a dynamic microbial community and the linking of such changes to wine flavor and aroma properties.
v) Diversity, ecology, physiology and metabolism of *B. bruxellensis*. Damage from this spoilage agent is not effectively prevented because we do not fully understand the biology of this species, particularly in interaction with other yeast and bacteria.

Each chapter presents advances in these areas of study. Research in wine microbiology, particularly in the wine microbiome and its impacts on wine composition is enhancing our understanding of the complexities and dynamics of microbial food and beverage ecosystems.

Citation: Lonvaud, A., Bisson, L. F., eds. (2019). Understanding Wine Microbiota: Challenges and Opportunities. Lausanne: Frontiers Media. doi: 10.3389/978-2-88945-959-9

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frontiers in Microbiology

REVIEW
published: 16 May 2018
doi: 10.3389/fmicb.2018.00991

Metagenomic Approaches to Investigate the Contribution of the Vineyard Environment to the Quality of Wine Fermentation: Potentials and Difficulties

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The winemaking is a complex process that begins in the vineyard and ends at consumption moment. Recent reports have shown the relevance of microbial populations in the definition of the regional organoleptic and sensory characteristics of a wine. Metagenomic approaches, allowing the exhaustive identification of microorganisms present in complex samples, have recently played a fundamental role in the dissection of the contribution of the vineyard environment to wine fermentation. Systematic approaches have explored the impact of agronomical techniques, vineyard topologies, and climatic changes on bacterial and fungal populations found in the vineyard and in fermentations, also trying to predict or extrapolate the effects on the sensorial characteristics of the resulting wine. This review is aimed at highlighting the major technical and experimental challenges in dissecting the contribution of the vineyard and native environments microbiota to the wine fermentation process, and how metagenomic approaches can help in understanding microbial fluxes and selections across the environments and specimens related to wine fermentation.

Keywords: wine, metagenomics, bacteria, fungi, vineyard, environment

OPEN ACCESS

Edited by:
Linda F. Bisson, University of California, Davis, United States

Reviewed by:
Masneuf-Pomarede Isabelle, BSA, France
Lucía González-Arenzana, Instituto de Ciencias de la Vid y del Vino (ICVV), Spain

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

Received: *30 December 2017*
Accepted: *27 April 2018*
Published: *16 May 2018*

Citation:
Stefanini I and Cavalieri D (2018) Metagenomic Approaches to Investigate the Contribution of the Vineyard Environment to the Quality of Wine Fermentation: Potentials and Difficulties. Front. Microbiol. 9:991. doi: 10.3389/fmicb.2018.00991

INTRODUCTION

Wines made from identical vine cultivars and under the same conditions can be recognized for their distinctive features encompassing chemical composition (Son et al., 2009; Perestrelo et al., 2014; Ziółkowska et al., 2016) and sensory characteristics (Van Leeuwen and Seguin, 2006; Van Leeuwen, 2010; Robinson et al., 2012; Hopfer et al., 2015) due to the different regional origins. The French word for "soil" (also "land"), *terroir,* was adopted to refer to the interaction between the plants, the environment and human factors (Gladstones, 2011) and nowadays it is frequently used to relate wine sensory attributes to its geographic origin (Van Leeuwen and Seguin, 2006). Recently, several studies have shown that the differences between grapes or fermenting musts from different regions are mirrored by geographic variation of the microbial community compositions (Bokulich et al., 2014; Gilbert et al., 2014; Taylor et al., 2014; Morrison-Whittle and Goddard, 2015; Pinto et al., 2015; Belda et al., 2017). In addition, the differences among microbial populations have been shown to be correlated with the organoleptic characteristics of fermenting musts (Knight et al., 2015)

(Bokulich et al., 2016). The reasons why microbial communities differ among geographic locations are still far to be fully understood. However, recent studies shaded some light on this topic, highlighting that microbial populations found in musts may originate from the native environment surrounding the vineyard (Morrison-Whittle and Goddard, 2018) and that the geographical differences among populations were more evident for fungi than for bacteria (Miura et al., 2017). Because of the observation of a putative microbial terroir, the role and persistence of environmental microbial species in the wine fermentative process gained a renewed interest.

The compositions of microbial populations present in the vineyard, in the winery, and in fermenting musts have been extensively investigated by means of traditional microbiological methods (Morgan et al., 2017). Culture-based approaches relied on the isolation of microbes on laboratory media, and their identification and characterization through biochemical assays, microscopy, and molecular biology. Nevertheless, culture-based methods often failed to identify microorganisms present at low frequency in the sample and non-culturable cells. In 1999, Ampe and collaborators showed that at least 25–50% of the microbial community could not be cultured in laboratory conditions, hence clearly highlighting the drawbacks of culture-based approaches (Ampe et al., 1999). In addition, the use of only biochemical and phenotypic characteristics to identify microbes was shown to be inadequate, likely because parallelism and reversals of phenotypes occurred in species evolution (Kurtzman and Robnett, 1994; Guzmán et al., 2013). As an example, the *Candida* genus, initially intended to include all the "asexual yeasts that divide by multilateral budding but have no distinctive cellular morphology" (Daniel et al., 2014), is now recognized as a polyphyletic genus and undergoing revision to make species grouping consistent with phylogenetic affinities (Daniel et al., 2014). These limitations highlighted the need to develop culture-independent techniques enabling the rapid, accurate, and exhaustive description of microbial populations. Next Generation Sequencing (NGS) approaches fulfilled these needs, allowing the identification of both bacteria and fungi present in complex samples such as grapes, musts, and fermentations (Morgan et al., 2017).

METAGENOMIC APPROACHES

Thanks to the advent of NGS, several metagenomic approaches are nowadays available to dissect the composition of microbial populations. The available sequencing techniques have already been reviewed by Morgan et al. (2017), and this review is aimed at highlighting the potentials of different metagenomic approaches grouped as amplicon-based and whole-genome sequencing. The first group is based on the sequencing of target sequences known to be able to distinguish microbes, the latter group allows the sequencing of the complete pool of DNA extracted from a given sample.

Amplicon-Based Metagenomic Approaches

Amplicon-based approaches, also called metabarcoding, rely on the contemporary sequencing of the same DNA sequence shared by all the microbes present in a given sample, but different enough to allow the identification of different microorganisms (**Table 1**).

Back in 1977, Woese and Fox proposed the use of ribosomal RNA (rRNA) sequences to determine the phylogenetic relations among organisms (Woese and Fox, 1977). rRNA sequences fulfilled the requirements for a good molecular marker: they are present in all the living organisms, their sequences present conserved regions suitable as targets for primers used in polymerase chain reactions (PCR) but differ enough between species to discriminate them (Woese and Fox, 1977). The pioneering proposal of Woese and Fox is still relevant, as the rRNA sequences are currently used for amplicon-based metagenomics analyses. The typical target for bacterial metabarcoding is the 16S rRNA gene (Liu et al., 2007), while three regions are usually targeted in fungi: the ITS1-5.8S rRNA-ITS2, the 26S rRNA gene, and a region of the 18S rRNA gene (Xu, 2016).

Choosing the Target Region

By sequencing specific genes (or regions) one can identify the microbes at the genus or even at the species level. However, there are some limits in using the complete gene/region sequences for metagenomic analyses. First of all, the average reads length of NGS ranges from 150 to 300 bp, far shorter than the length of the target genes/regions which are ∼1,500 bp for the 16S rRNA gene (Liu et al., 2007), 400–900bp for the ITS1-5.8S rRNA-ITS2 region (Esteve-Zarzoso et al., 1999), and >1,300 bp for the 26S rRNA gene (Pinto et al., 2014). The use of the whole IT1-5.8S rRNA-ITS2 fungal region for metagenomic purposes has an additional problem: the length of this region is not conserved among fungi (i.e., 400 bp in *Metschnikowia pulcherrima*, 880 bp in *Saccharomyces cerevisiae*) (Esteve-Zarzoso et al., 1999), and a preferential amplification may occur for shorter fragments. Hence, for metagenomics purposes, shorter regions have been selected from the full length of the target genes.

Nine hypervariable regions of the 16S rRNA gene sequence (V1-V9) have been targeted for the assessment of bacterial diversity (Liu et al., 2007). Unfortunately, the choice of partial sequence regions can significantly affect the results because the 16S rRNA gene regions have different divergence (**Table 1;** Youssef et al., 2009). Recent *in silico* studies showed the V4-V6 regions as the most reliable for the phylogenetic study of new phyla (Yang et al., 2016) and the V4, V5-V6, and V6-V7 regions as the most suitable regions for metagenomic purposes because providing estimates comparable to those obtained with the complete 16S rRNA gene sequence (Youssef et al., 2009). The sequencing of the V1-V2 region and the V6 region overestimated the species richness, while the sequencing of the V3, V7, and V7-V8 regions underestimated the species richness (Youssef et al., 2009). However, experiments did not confirm the results

TABLE 1 | Advantages and drawbacks of amplicon-based and whole-genomics sequencing approaches.

	Advantages	Drawbacks	Organism	Region	Advantages	Drawbacks
Amplicon-based sequencing	Large and comprehensive reference databases are available Several pipelines available for bioinformatics analysis Detection of rare taxa Taxonomy to the genus level (species at best)	Biased relative quantification of bacterial communities: bacterial species bear various number of copies of 16S rRNA genes Functional annotation can only be inferred Sequencing of matrix (e.g., grape ITS, chloroplast 16S) Low confidence for taxonomic assignment at the species level	Bacteria	V1-V2 V6 (*16S rRNA)*		Overestimate richness
				V3, V7 V7-V8 (16S rRNA)		Underestimate richness
				V4, V5-V6 V6-V7 (16S rRNA)	Provide estimates comparable to those obtained with the complete 16S rRNA gene sequence	
			Fungi	ITS1	Detects more OTUs than D2 region	
				ITS2	Detects more OTUs than D2 region	
				D1-D2		
				18S rRNA gene		
Whole-genome sequencing	All microbes detected at once Taxonomic assignment at the species or strain level Functional annotations can be carried out by gene enrichment	May need available reference genomes Relative organism abundances vary significantly depending on the protocols adopted for DNA extraction and sequencing Generally, not deep enough to detect taxa present at low frequency in complex communities Amplification of sequencing of the matrix (e.g., grape)				

obtained with *in silico* analyses: the sequencing of the V3-V4 and V5-V6 from the same samples showed poor overlap in the lists of identified bacteria (Campanaro et al., 2014).

As for bacterial metabarcoding, even for fungal amplicon-based metagenomics choosing the proper fragment to be sequenced is pivotal. Again, comparative analyses have been carried out to assess which region is the most suitable for fungal metabarcoding (**Table 1**). For instance, Pinto and colleagues showed that the taxonomies identified in the same samples by sequencing the ITS2 region and the D2 domain of the 26S rRNA gene were only partially shared and that the ITS2 region identified a higher number of taxa than the D2 region (Pinto et al., 2014). In addition, the ITS1 and ITS2 region performances were compared by means of *in silico* and experimental analyses, revealing that the two regions gave highly similar results, but the ITS1 region allowed the identification of a greater number of taxa (Blaalid et al., 2013; Bokulich and Mills, 2013).

It is worth to mention that another problem raises when using metabarcoding for the dissection of the composition of microbial populations present in grapes and musts. In fact, being *Vitis vinifera* (and hence grapes) a eukaryote, it also bears the ITS1-5.8S rRNA-ITS2 region and 26S rRNA gene. Similarly, *V. vinifera* chloroplasts, being originated from cyanobacteria (Gray, 1989), bear the 16S rRNA gene. This implies that reads belonging to

the matrix (grape or must) will be amplified and sequenced in metabarcoding, thus reducing the coverage for the associated microbial population. Hence, a particular care should be adopted in the extraction of microbial DNA, reducing at the minimum the amount of DNA from the matrix.

Available Reference Databases

An additional factor influencing the choice of the target to be used for microbial metabarcoding should be the availability of an exhaustive and curated reference database of annotated sequences. In fact, the taxonomic assignment is carried out through the comparison (e.g., alignment) of the sequenced regions with a database of annotated sequences. In principle, public repositories of sequences (i.e., GenBank) could be used as a source for reference sequences. Nevertheless, these repositories also encompass sequences amalgamated into the pseudo-divisions "environmental samples" and "unclassified," worthless for taxonomic assignment in metabarcoding (DeSantis et al., 2006).

Several curated 16S rRNA databases are available, among which the most frequently used are RDP, Greengenes, SILVA, and LTP (Santamaria et al., 2012). Such resources, in addition to offering a curated list of annotated 16S rRNA sequences, also show additional functionalities. For instance, the RDP reference database can be used with the standalone program RDP Classifier for phylogenetic classification, and with LibCompare for comparison of taxa abundances between samples (Wang et al., 2007). Similarly, SILVA (Pruesse et al., 2007), LTP (Yarza et al., 2008), and Greengenes (DeSantis et al., 2006) reference databases can be used with the standalone program ARB for phylogenetic classification (Ludwig et al., 2004). While RDP, LTP, and Greengenes databases include complete 16S rRNA bacterial gene sequence, the SILVA database encompasses aligned sequences of the small (16S/18S, SSU) and large (23S/28S, LSU) rRNA subunits for all three domains of life.

While several reference databases are available for 16S rRNA bacterial sequences, just a few databases are available for fungal metabarcoding: UNITE (User-friendly Nordic ITS Ectomycorrhiza Database) (Abarenkov et al., 2010), ITS2 Database (Ankenbrand et al., 2015), and ITSoneDB (Santamaria et al., 2017). The lack of a wider range of available databases and tools specific for fungal metabarcoding can be ascribed to the relatively recent interest in fungal metagenomics and to the lack of a consensus in the selection of the target used for metabarcoding. While UNITE encompasses entire ITS1-5.8S rRNA-ITS2 sequences (Abarenkov et al., 2010), the ITS2 database includes sequences of the ITS2 region (Ankenbrand et al., 2015), and the ITSoneDB includes sequences of the ITS1 region (Santamaria et al., 2017).

Analytic Tools and Pipelines

The great success of amplicon-based metagenomic approaches encouraged researchers with various backgrounds to approach a technique that strongly relies on bioinformatics. Despite the collaboration of an expert bioinformatician being highly recommended to choose the best procedures, overcome with eventual unexpected outcomes of the analysis, and interpret the data, nowadays the availability of pipelines allows non-specialized researchers to handle and analyze metagenomic data. Such pipelines have been built by combining pre-existing tools and allowing the user to rapidly proceed through the steps of data processing without i.e., incurring the data conversion to meet the requirements of the used tool.

Once sequenced, amplicons need to be handled in a consequential series of steps: *i*- trim bases that have been flagged as low-quality by the sequencing platform; *ii*- (in case of paired-end sequencing) match and stitch paired reads; *iii*- remove artifacts such as chimeras (merged sequences wrongly paired); *iv*- filter out contaminant sequences (i.e., non 16S sequences); *v*- identify the Operational Taxonomic Units (OTUs) in the samples (e.g., clustering the entire set of sequences and then selecting a representative sequence for each cluster); *vi*- assign taxonomic identities to the OTUs by comparing the sequences to these present in reference databases. Such a set of processes has been variously implemented in the most frequently used pipelines such as mothur (Schloss et al., 2009), MICCA (Albanese et al., 2015), QIIME and QIIME2 (Caporaso et al., 2010), BioMaS (Fosso et al., 2015), the RDP's Pyrosequencing Pipeline (Cole et al., 2009), CloVR (Angiuoli et al., 2011), and CloVR-ITS (White et al., 2013) (the latter designed for fungal populations analyses).

Thanks to metagenomic analyses it is possible to describe and compare the compositions of microbial populations in almost every kind of sample. A step forward consists of the understanding of how changes in the composition of microbial communities impact the population's biological functions. Under the assumption that a given microbial taxon is uniformly able to perform specific biological functions [i.e., *Bacteroides* spp. might be inferred to contain genes encoding glycoside hydrolase activity (Xu et al., 2007)], it is possible to predict the functional profile of a given population from the taxon composition obtained by means of metabarcoding. Some tools have been generated with this aim, i.e., Tax4Fun (Aßhauer et al., 2015), PICRUSt (phylogenetic investigation of communities by reconstruction of unobserved states; Langille et al., 2013), and PanFP (pangenome-based functional profiles; Jun et al., 2015). All these tools are designed to infer functional profiles for bacterial populations. PICRUSt is based on the use of the Greengenes reference database (DeSantis et al., 2006) and the functional composition of reference genomes described in IMG (Markowitz et al., 2012). Briefly, OTUs are identified according to their clustering with taxa of the Greengenes database, and the biological function profile of the sample is inferred by the combination of functions described for the reference genomes corresponding to the taxa identified in the sample. Thus, PICRUSt predictions depend on the topology of the tree and on the distance to the next sequenced organism, limiting the analysis to well-characterized phyla (Aßhauer et al., 2015). Even Tax4Fun relies on the taxa identification by means of clustering against a reference database (SILVA Pruesse et al., 2007), but the SILVA-based 16S rRNA profiles are converted into taxonomic profiles based on the prokaryotic organisms in the KEGG database (Kanehisa and Goto, 2000) and finally the functions are inferred. PanFP works similarly to Tax4Fun, but in addition to KEGG, allows the inclusion of other gene annotation databases, e.g., Gene Ontology (Ashburner et al.,

2000), Pfam (Punta et al., 2012), and TIGRFAMs (Haft et al., 2003).

However, it must be considered that those softwares cannot cope with lateral gene transfer or gene gain and loss, which may affect the ability to predict biological functions from taxonomy based on a single gene (**Table 1**). A further drawback of using DNA-based metagenomic data to infer the biological functions potentially exploited by microbial populations is that the detected DNA may belong to dead organisms. A few studies on the dynamics of microbial populations in fermentations reported the disappearance of DNAs belonging to microbes reasonably dying during the process (Marzano et al., 2016; Stefanini et al., 2016), hence suggesting a rapid degradation of DNA in this chemically hostile environment. However, an approach based on RNA sequencing would give a direct report of the functions achievable by the viable microbial populations.

Whole-Genome Sequencing

Another NGS approach used to study the composition of microbial communities is whole genome sequencing. Instead of sequencing target DNA regions allowing microbial identification, whole-genome sequencing consists in the sequencing of all the DNA extracted from a given sample. The obtained sequences can be handled in various ways to identify the organisms present in the samples or to obtain other information. Hence, the composition of both fungal and bacterial populations can be dissected with a single round of whole-genome sequencing (**Table 1**).

Despite being unaffected by the problems highlighted for amplicon-based approaches, whole-genome sequencing has disadvantages. Indeed, it has been shown that, differently from amplicon-based sequencing, the relative organism abundances inferred from whole-genome sequencing may significantly vary according to the protocols used for DNA extraction and sequencing (**Table 1**; Gomez-Alvarez et al., 2009). In addition, whole-genome sequencing usually does not allow the identification of organisms present at low frequency in the sample (**Table 1**; Shah et al., 2011). However, a few direct comparisons of amplicon-based and whole-genome sequencing techniques revealed that the two approaches identify highly similar microbial populations, with the whole-genome sequencing approach capturing a higher level of diversity (more phyla and genera; Poretsky et al., 2014).

The reads obtained by means of whole-genome sequencing can be used not only to identify the microorganisms present in the sample but also to compare the relative abundances of bacteria and fungi (Cao et al., 2017). The main advantage of whole-genome sequencing over amplicon-based sequencing is its potential to characterize microbes at the species or even strain level (Cao et al., 2017). This topic is detailed in section Future Challenges for Metagenomic Approaches: the Sub-Species Level. Furthermore, the whole-genome sequencing approach also allows the direct identification of genes having relevant functional roles, whose presence could be only inferred with the amplicon-based metagenomic approach (see the previous section for further details), and thus, it is not affected by lateral gene transfer or deletion. In addition, this approach potentially allows the identification of functions previously unknown in certain organisms, even if the organisms do not have their genomes sequenced.

Several tools have been generated to obtain the microbial taxonomy profile from whole-genome sequencing data, among which the most used are Kraken (Wood and Salzberg, 2014), MetaPhlAn2 (Truong et al., 2015), riboFrame (Ramazzotti et al., 2015), and CLARK (Ounit et al., 2015). Other tools are available (e.g., TETRA, CompostBin, MEGAN, GRAMMY) and have been previously reviewed in Alaimo et al. (2018). Kraken and CLARK identify the percentages of reads aligning against a set of references genomes. riboFrame identifies reads overlapping the 16S rDNA genes through Hidden Markov Models and carries out the taxonomic assignment thanks to a naïve Bayesian classification. Hence, all reads identified as ribosomal are coherently positioned in the 16S rDNA gene, allowing the use of the topology of the gene to guide the abundance analysis. MetaPhlAn2 allows the species-level and strain-level profiling of bacteria, eukaryotes, and viruses, by means of sequence matching against a set of unique clade-specific marker genes identified from reference genomes (**Table 1**).

CHARACTERIZING AND COMPARING POPULATIONS

In metagenomic analyses, populations are generally compared among samples having defined and known differences (i.e., the stage of grape maturation or the stage of must fermentation). Aiming to this, several measures are available to describe and compare the structure and composition of populations measuring the alpha biodiversity (within sample diversity) and the beta biodiversity (between samples diversity).

In metagenomics, three estimators are generally used to estimate the alpha biodiversity: richness, Simpson index, and Shannon index. The taxa richness is the number of different taxa present in the population, not considering their abundances. For example, the richness of the populations shown in **Figure 1A** is the number of different taxa (letters in the figure) present in the three populations (**Figure 1B**). The Simpson index is a measure of the population evenness, indicating the probability that two randomly sampled individuals belong to two different taxa (i.e., species) (Lugtenberg and Kamilova, 2009). Hence, it considers both the richness and the abundances of the identified taxa: the more equal the proportions for each of the taxa, the more homogeneous, or even, they are (Simpson, 1949). As the Simpson index, the Shannon index combines both evenness and richness, but it quantifies the uncertainty in the taxon identity of a randomly chosen individual (Tuomisto, 2012). In plain terms, if the population is composed by many taxa present at the same frequency, all the randomly chosen individuals will have the same (low) probability of being assigned to the correct taxon, hence, the uncertainty (Shannon index) will be high. On the contrary, if a large part of the population belongs to a given taxon, the probability of correctly assigning the randomly chosen individual will be high, thus reducing the Shannon index (Shannon, 1948). The major difference between the Shannon and the Simpson

indexes is that the first gives a higher weight to rare taxa. Hence, the population with a low richness (s1 in **Figure 1A**) will have a lower Shannon index compared to a population with a higher richness (s2 in **Figure 1A**) if the first population encompasses more rare taxa than the second (**Figure 1B**). On the contrary, the Simpson index of the first population will be comparable or higher than the Simpson index of the second population (**Figure 1B**).

Two beta diversities are usually used in metagenomic analyses: the Bray-Curtis dissimilarity and the UniFrac distance. The Bray-Curtis dissimilarity is a measure of the differences in composition between two samples based on taxa abundances in each sample (Tuomisto, 2012). The UniFrac distance, devised by the Knight group at the University of Colorado, incorporates the phylogenetic distances between taxa, and can include the information on the abundance of taxa (weighted UniFrac) or simply consider the presence/absence of taxa (unweighted UniFrac; Borcard et al., 2011).

When delving into the details of the components of the microbial population, it is worth to make a consideration of abundances. Usually, the abundances of taxa are reported as relative abundances (the percentage of counts of the given taxon on the total of counts in the sample) (**Figure 1C**). This measure is fairly used to indicate how the proportion of taxa changes in different samples. However, when comparing bacterial populations, it must be considered that the 16S copy number varies greatly among different bacteria (Lozupone et al., 2007; Kembel et al., 2012) and this obviously affects the quantification of bacterial abundances in different samples (Větrovský and Baldrian, 2013). To cope with this problem and properly compare the abundances of bacteria, tools such as CopyRighter (Angly et al., 2014) and rrnDB (Klappenbach et al., 2001) have been created to scale the abundances according to the known number of 16S copies in different bacteria.

In addition, a further care should be used especially when analyzing dynamic processes such as must fermentations. The amount of microbes present in grapes before harvesting, is known to exponentially increase during the late phases of grape maturation (from 10^2-10^4 cells per grape before maturation to 10^7-10^9 per grape in damaged, ripen grapes; Mortimer and Polsinelli, 1999; Kembel et al., 2012) and even more during the early phases of fermentation, when free sugars are available and microbes find a more suitable environment (Mortimer and Polsinelli, 1999; Barata et al., 2012). On the other hand, the increasing amount of ethanol produced by fermenting yeasts progressively selects the most sensitive species, reducing the biodiversity of the sample and potentially modifying the total amount of present microbes (see further details in section Metagenomic From Vineyard to Wine; Goddard, 2008). Because of these fluctuations of the size of microbial populations, the use of proportions to compare the abundances of taxa in different samples might not be suitable. For instance, the same amount of a taxon in populations of different sizes (i.e., taxon E in samples s1 and s2, **Figure 1D**) will result in different relative abundances (20 and 10% in sample s1 and s2, respectively). Hence, it is not possible to obtain information on the individual fitness (or persistence) of taxa during the process from relative abundances. To help in this comparison, we recently applied an approach allowing us to scale the relative abundances, obtained through amplicon-based metagenomics, according to the total amount of microbes identified in the sample (Stefanini et al., 2016). This approach, based on the quantification of the total amount of fungal or bacterial DNA in a given sample through quantitative real-time PCR (qRT-PCR) allowed us to gain insightful information on the evolution of microbial populations before and during the fermentation process (Stefanini et al., 2016).

METAGENOMIC FROM VINEYARD TO WINE

Despite wine fermentation is usually associated to the process of sugar conversion into ethanol, the production of wine is nowadays known to be influenced also by the characteristics of the vineyard (Gladstones, 2011; Bokulich et al., 2014). These observations opened a new branch of investigations aimed at the identification of environmental factors impacting on the composition of microbial communities and eventually on the organoleptic characteristics of the wine. Microbes can have both positive and detrimental effects on the wine fermentation process and on the organoleptic characteristics of the final product. Loureiro, Malfeito-Ferreira, and Barata proposed to group the microbes found in musts in classes highlighting their effects on fermentation: *i*- "spoilage sensu stricto" species, responsible of wine spoilage even when good practices are adopted; *ii*- "innocent species", unable to spoil wine because controllable through the application of good manufacturing practices; *iii*- fermenting species, able to convert sugars and lactic acids, and whose presence needs to be preserved in order to achieve the fermentation (Barata et al., 2012).

The following sections will review the information obtained thanks to metagenomic approaches used to disclose the composition of microbial populations in the vineyard, in its surroundings, and in the winery, the influence of such communities on the fermentation process, and the effects of environmental factors and human intervention on microbial communities' composition.

The Vineyard

It is well known that microorganisms on and inside plant organs have an impact on the plant health, as they are involved in functions such as plant nutrition and resistance to stresses (Mendes et al., 2013). Microorganisms can promote plant growth by supplying the plants with nutrients, i.e., nitrogen, or by solubilizing substances, i.e. soluble phosphate (Lugtenberg and Kamilova, 2009). On the other hand, microbes can also have detrimental effects on plants, e.g., *Botrytis cinerea* infecting vine grapes, or saprophytic molds responsible for grape tors or mycotoxin production (e.g., *Aspergillus* spp., *Cladosporium* spp., and *Penicillium* spp.) (Barata et al., 2012). Hence, it is well known that the plant microbiota is composed of a large variety of microorganisms. However, only some of these microbes can

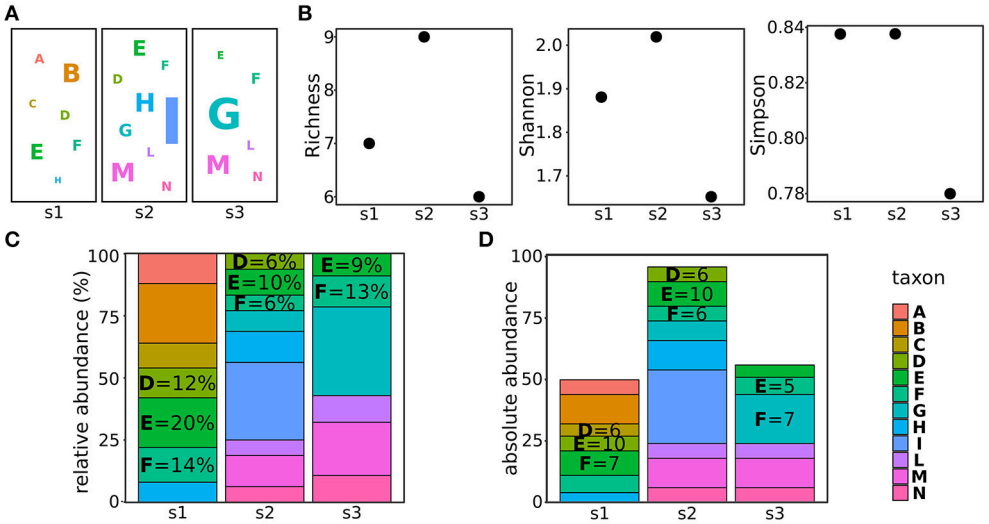

FIGURE 1 | Characterizing and comparing populations. **(A)** Examples of three populations, letters refer to different taxa, the letter size indicating the abundance of the taxon in the sample. **(B)** alpha diversities (richness, Shannon index, and Simpson index) calculated for the populations shown in **(A)**. **(C)** taxa relative abundances of the taxa composing the populations shown in **(A)**. **(D)** taxa absolute abundances of the taxa composing the populations shown in panel **(A)**.

grow in musts, and only a portion of these has a direct effect on wine production (Barata et al., 2012).

The microorganisms found in musts originate from various components of the vineyard, encompassing soil (Burns et al., 2015), air, other plants (Morrison-Whittle and Goddard, 2018), and insects (Stefanini et al., 2012; Stefanini, 2018; **Table 2**). The vineyard soil is one of the natural source of fungi associated with wine-related environments, with the most abundant genera being known to have an environmental origin (e.g., *Amniculicola*, *Doratomyces*, *Endocarpon*, and *Tricellulortus* (for the complete list refer to **Table 2**) (Morrison-Whittle and Goddard, 2018). Notably, the most abundant fungi in vineyard soil do not bear features relevant for wine production e.g., spoilage or fermentation (Barata et al., 2012). Contrarily, bacteria having various impacts on the fermentative process have been found in the vineyard soil. Among these, the most abundant are Firmicutes (encompassing fermenting, innocent and spoilage sensu-stricto species), spoiling Acidobacteria and Proteobacteria, and other bacteria having unknown effects on the fermentation such as Actinobacteria, Bacteroidetes, Chloroflexi, Gemmatimonadetes, Plactomycetes, and Verrucomicrobia (**Table 2**; Burns et al., 2015).

Another environmental source of microbes relevant for wine fermentation is the vine, in particular the bark, leaves, and obviously the grapes. An assessment carried on Portuguese vineyards (Bairrada appellation, Cantanhede) over a year revealed that leaves fungal communities were dominated by fungi belonging to the *Rhizopus*, *Mucor*, *Zoophthora*, and *Pandora* genera (**Table 2**; Pinto et al., 2014). While the first two genera are associated with post-harvest diseases of table grapes (Hocking et al., 2007), the two latter genera are insect-pathogenic fungi (Xu et al., 2009), and their effects on wine fermentation are unknown or absent. The presence of Ascomycota and Basidiomycota on vine leaves widely changed over time, with the most abundant being *Aureobasidium*, and *Guignardia* (a phytopathogen) (Pinto et al., 2014). The fermenting species *Saccharomyces*, *Hanseniaspora*, and *Metschnikowia* have also been identified on vine leaves, though at low frequencies (Pinto et al., 2014). The most abundant bacterial families on vine leaves are Streptococcaceae, Enterobacteriaceae, Pseudomonadaceae, and Moraxellaceae (Pinto et al., 2014). Only a few Lactic Acid Bacteria (LAB), responsible for malolactic fermentation, have been identified at low frequencies on vine leaves (*Lactobacillaceae* family; **Table 2**; Pinto et al., 2014). Furthermore, Acetic Acid Bacteria (AAB), known to spoil wine fermentations (Drysdale and Fleet, 1988), such as the genera *Acidisoma*, *Gluconoacetobacter,* and *Roseomonas*, are predominantly present on leaves (Pinto et al., 2014). Noticeably, the fungal biodiversity on vine leaves show a tendency to decrease over time (Pinto et al., 2014). This reduction of biodiversity can be ascribed to various factors: repeated chemical treatments, routinely used in conventional viticulture (see section Anthropogenic Factors Influencing Microbial Populations; Pinto et al., 2014); seasonal/climatic changes (see section Environmental Factors Influencing Microbial Populations); the emergence of fruit, a potentially more suitable habitat than leaves for molds and fungi because rich in sugars (Bokulich et al., 2014; Grangeteau et al., 2017).

In spring vine fertilized flowers start to develop a seed and a grape berry to protect it. Grape growth and maturation occur in the following months, with a duration changing according to the climate (Perrot et al., 2015). While growing and ripening, grapes are exposed to microbes originating from the surrounding environment, and the microbial communities on grape skins are subjected to dynamic changes due to environmental factors and anthropogenic interventions. Being the only ingredient

TABLE 2 | Most abundant microorganisms found in vineyard and winery environments.

Source	Fungi	Bacteria
Vineyard-soil	**Absent/unknown effect:** *Amniculicola*[M], *Ascobolus*[M], *Ascodesmis*[M], *Byssonectria*[M], *Boudiera*[M], *Chalara*[M], *Chytridium*[M], *Cordyceps*[M], *Doratomyces*[M], *Emericellopsis*[M], *Endocarpon*[M], *Flagelloscypha*[M], *Gaertneriomyces*[M], *Glomus*[M], *Lamprospora*[M], *Lasiobolidium*[M], *Lipomyces*[M], *Massarina*[M], *Melastiza*[M], *Microbotryum*[M], *Olpidium*[M], *Scolecobasidiella*[M], *Sorocybe*[M], *Spizellomyces*[M], *Tricellulortus*[M], *Valsonectria*[M]	**Fermenting:** Firmicutes[4,§], **Innocent:** Firmicutes[4,§], **Spoilage sensu-stricto:** Acidobacteria[4], Firmicutes[4,§], Proteobacteria[4], **Absent/unknown effect:** Actinobacteria[4], Bacteroidetes[4], Chloroflexi[4], Gemmatimonadetes[4], Planctomycetes[4], Verrucomicrobia[4]
Vineyard-Leaves	**Absent/unknown effect:** *Aureobasidium*[8], *Guignardia*[8], *Mucor*[8], *Rhizopus*[8], *Pandora*[8], *Zoophthora*[8], Dothideomycetes[7]	**Fermenting:** Firmicutes[7,§], **Innocent:** Actinobacteria[7], Firmicutes[7,§], Proteobacteria[7,***] **Spoilage sensu-stricto:** *Acidisoma*[8], Enterobacteriaceae[8], Firmicutes[7,§], *Gluconoacetobacter*[8], Proteobacteria[7****], Pseudomonadaceae[8], *Roseomonas*[8]; **Absent/unknown effect:** Streptococcaceae[8], Moraxellaceae[8],
Vineyard-grapes	**Fermenting:** *Hanseniaspora*[1,5,**], *Saccharomyces*[1,5]; **Innocent:** *Candida*[1,5,*], *Debaryomyces*[1], *Hanseniaspora*[1,5,**], *Metschnikowia*[1,5,9], *Pichia*[1,***]; **Spoilage sensu-stricto:** *Botryotinia*[5], *Cladosporium*[5], *Pichia*[1,***], *Torulaspora*[1], *Zygosaccharomyces*[1], Saccharomycodaceae[6]; **Absent/unknown effect:** *Alternaria*[5], *Aureobasidium*[1,5,9], *Brettanomyces*[1], *Cryptococcus*[5], *Erysiphe*[5], *Issatchenkia*[1], *Itersonilia*[5], *Monilinia*[5], *Mucor*[5], *Phoma*[5], *Sporidiobolus*[5], Starmerella[9], Dothioraceae[6], Pleosporaceae[6], Dothideomycetes[7]	**Fermenting:** Firmicutes[7,§], Lactobacillales[6], **Innocent:** *Bacillales*[6], *Bacillus*[6], *Enterobacteriales*[6], Firmicutes[7,§], Proteobacteria[7****], *Pseudomonadales*[6]; **Spoilage sensu-stricto:** Firmicutes[7,§], Proteobacteria[7****], Rhodospirillales[6], **Absent/unknown effect:** *Lysinibacillus*[6], *Sporosarcina*[6], Pasteurellales[6], Bacteroidales[6], Actinobacteria[7],
Musts	**Fermenting:** *Hanseniaspora*[2,**], *Saccharomyces*[2]; **Innocent:** *Candida*[2,10], *Hanseniaspora*[2,**], *Lachancea*[M], *Metschnikowia*[M], *Pichia*[M,***]; **Spoilage sensu-stricto:** *Aspergillus*[M], *Botryotinia*[2], *Cladosporium*[2], *Saccharomycodes*[M], *Penicillium*[2,10], *Pichia*[M,***]; **Absent/unknown effect:** *Aureobasidium*[2,10], *Davidiella*[2], *Erysiphe*[M], *Saccharomycopsis*[M], *Saturnispora*[M], *Sphingomonas*[10], *Starmerella*[10] *Yarrowia*[M]	**Fermenting:** Lactobacillales[2], *Oenococcus oeni*[P1]; **Spoilage sensu stricto:** Rhodospirillales[2], **Innocent:** Bacillales[2], Enterobacteriales[2], Pseudomonadales[2]; **Absent/unknown effect:** Propionibacter[10], Corynebacterium[10]
Winery surfaces (prior to harvest)	**Fermenting:** *Saccharomyces cerevisiae*[3]; **spoilage sensu-stricto:** *Aspergillus* spp.[3]; **Absent/unknown effect:** *Cryptococcus* spp.[3], *Aureobasidium pullulans*[3]	**Innocent:** *Bacillus*[3], *Enterobacteriaceae*[3], *Pseudomonas*[3]; **Absent/unknown effect:** *Comamonadaceae*[3], *Flavobacterium*[3], *Brevundimonas*[3],

Microbes were classified as "fermenting," "spoilage sensu stricto," and "innocent" according to the (Barata et al., 2012) definition. [1]*(Barata et al., 2012);* [2]*(Bokulich et al., 2014);* [3]*(Bokulich et al., 2013);* [4]*(Burns et al., 2015);* [5]*(Grangeteau et al., 2017);* [6]*(Mezzasalma et al., 2017);* [7]*(Miura et al., 2017);* [M]*(Morrison-Whittle and Goddard, 2018);* [8]*(Pinto et al., 2014);* [P1]*(Portillo Mdel and Mas, 2016);* [9]*(Setati et al., 2015);* [10]*(Stefanini et al., 2016); *considering the multi-phyletic nature of the Candida phylum further characterization at the species level is required. **encompassing innocent and fermenting species. ***encompassing spoilage and innocent species. ****encompassing spoilage and fermenting species. §encompassing fermenting, innocent, and spoilage species. Taxa are listed at the level indicated in the referenced study.*

for wine production, harvested grapes are the major source of microbes contributing and affecting the fermentation. *Mucor* and *Aureobasidium* have been identified among the most abundant fungal genera in grapes (**Table 2**; Grangeteau et al., 2017). In addition, grape fungal populations also show high levels of fungal genera known to variously affect the fermentation process: fermenting genera (*Saccharomyces*), "innocent" genera (e.g., *Debaryomyces*), spoilage *sensu stricto* genera

(*Brettanomyces, Cladosporium*, Saccharomycodaceae), genera encompassing spoilage and fermenting yeasts (*Torulaspora*, and *Zygosaccharomyces*), and also genera whose impact on fermentation is unknown (e.g., *Alternaria*)(full list of genera in **Table 2**; Barata et al., 2012; Grangeteau et al., 2017; Mezzasalma et al., 2017). Acetic acid bacteria have been found at low frequencies in grape samples (Portillo Mdel et al., 2016), but still potentially affecting the outcome of fermentation.

The composition of fungal populations in grapes has been found to be associated with the geographical location of the vineyard, thus further supporting the concept of microbial terroir (Pinto et al., 2014; Bokulich et al., 2016; Miura et al., 2017; Morrison-Whittle and Goddard, 2018). The geographical diversification of fungi has been observed when comparing the complete population structure, and none of the identified fungal species had a geographic specificity, being either more abundant or present in only one of the compared locations (Bokulich et al., 2016; Miura et al., 2017). The geographical diversification observed for grape fungal populations has been observed also for grape bacterial populations (Portillo Mdel et al., 2016; Mezzasalma et al., 2017). However, some bacteria have been constantly found at high frequencies in grapes: Lactobacillales (fermenting), Bacillales, Enterobacteriales, and Pseudomonadales (innocent), Actinomycetales and Rhodospirillales (Portillo Mdel et al., 2016; Mezzasalma et al., 2017). Bacillales have been identified at high frequencies in all the grape samples analyzed in both the Mezzasalma et al. (2017) and Portillo Mdel et al. (2016) studies, encompassing various vine varietals, geographical locations, and vineyard orientations (Portillo Mdel et al., 2016; Mezzasalma et al., 2017). Contrarily, the presence and abundance of other bacterial genera and families have been found to be associated with either the vineyard orientation (South, East, or flat) or the vine varietal (further details in section Environmental Factors Influencing Microbial Populations; Portillo Mdel et al., 2016).

The Winery and the Fermentation Process

The conversion of must into wine is a dynamic process involving numerous transformations carried out by a complex succession of yeast and bacterial species. The process is achieved in two steps: alcoholic fermentation, generally carried out by yeasts, followed by malolactic fermentation, conducted by bacteria (Cappello et al., 2017). It is well known that alcoholic fermentation is carried out by a few yeast species, which eventually overcome the microbial population present in must because of the sensitivity to high ethanol concentrations and temperature of most microorganisms (Goddard, 2008). In general, the increase of temperature and ethanol concentration during fermentation induces a decrease in population complexity (e.g., richness), while the size of the population continues to increase, due to the overcome of the resistant species (Stefanini et al., 2016). In a recent study, Morrison-Whittle and Goddard highlighted the high similarity of vineyard and must fungal populations, with approximately the 40% of the fungal communities present in musts and during fermentation being also present in vineyard samples (soil, vine bark, and ripe fruit) (Morrison-Whittle and Goddard, 2018). The clear majority of fungi found in musts are also found in grapes, such as the genera *Aureobasidium, Botryotinia, Candida, Cladosporium*, *Columnosphaeria*, *Davidiella*, *Hanseniaspora*, and *Saccharomyces* (Bokulich et al., 2014; Morrison-Whittle and Goddard, 2018; **Table 2**). In addition, approximately the 30% of species present during fermentation were also present in samples (soil and fruit) collected from native conservation reserves located nearby the studied vineyards (**Figure 2**), thus highlighting the relevance of preserving uncultivated areas nearby the vineyards to safeguard the maintenance of fungal biodiversity in fermentations (Morrison-Whittle and Goddard, 2018).

Other fungal genera can be found in must samples, but rarely persist during the fermentation, both because of the environmental changes and thanks to the adoption of techniques aimed at the control of spoiling species. Among the species found in musts and rarely persisting, the most abundant are usually *Pichia* (encompassing both "innocent" and "spoilage *sensu stricto*" species), *Aspergillus* (considered a spoiling fungus as producing ochratoxins), *Saturnispora*, *Saccharomycopsis*, *Saccharomycodes* (spoilage genus), *Yarrowia*, *Erysiphe*, and *Metschnikowia* (the latter is an "innocent" and "fermenting" genus; Morrison-Whittle and Goddard, 2018; **Table 2**). As fungal populations, bacterial populations in the vineyard and must have been shown to be highly similar (Bokulich et al., 2014; Portillo Mdel and Mas, 2016). Bacterial populations in the musts are dominated by Bacillales, Enterobacteriales, Lactobacillales, Pseudomonadales, and Rhodospirillales, with a higher proportion of LAB than what observed on vine leaves (Bokulich et al., 2014; Portillo Mdel and Mas, 2016; **Table 2**).

Noticeably, the clear geographical diversification of microbial populations observed in musts weakens during fermentation (Morrison-Whittle and Goddard, 2018), probably because of a collapse of microbial diversity as *Saccharomyces* yeasts displace other species (Goddard, 2008). Nevertheless, as the fermentation process proceeds, must populations have been shown to increasingly resemble those found on vine barks, possibly due to the high frequency of *Saccharomyces* spp. yeasts found in both fermentation and bark samples (Morrison-Whittle and Goddard, 2018). *Saccharomyces cerevisiae* is almost always the species dominating the fermentation, but other yeast species (*Candida* spp. and *Hanseniaspora* spp.) have been shown to be present at high frequencies, especially during the early phases of the process (Portillo Mdel and Mas, 2016; Stefanini et al., 2016). In some occasions *Candida* spp and *Hanseniaspora* spp have also been shown to be able to dominate the fermentation (David et al., 2014; Stefanini et al., 2016).

A great step forward in our understanding of bacterial populations composition during alcoholic fermentation was achieved thanks to the application of metagenomic approaches. In fact, studies carried out before the advent of metagenomics suggested some bacterial species could not persist during alcoholic fermentation due to their sensitivity to alcohol: according to culture-based studies, the abundance of AAB was considered to decrease from 10^6-10^7 colony forming units (CFU)/ml in must to 10^2-10^3 CFU/ml at the end of alcoholic fermentation (Du Toit and Pretorius, 2002). Contrarily, the use of

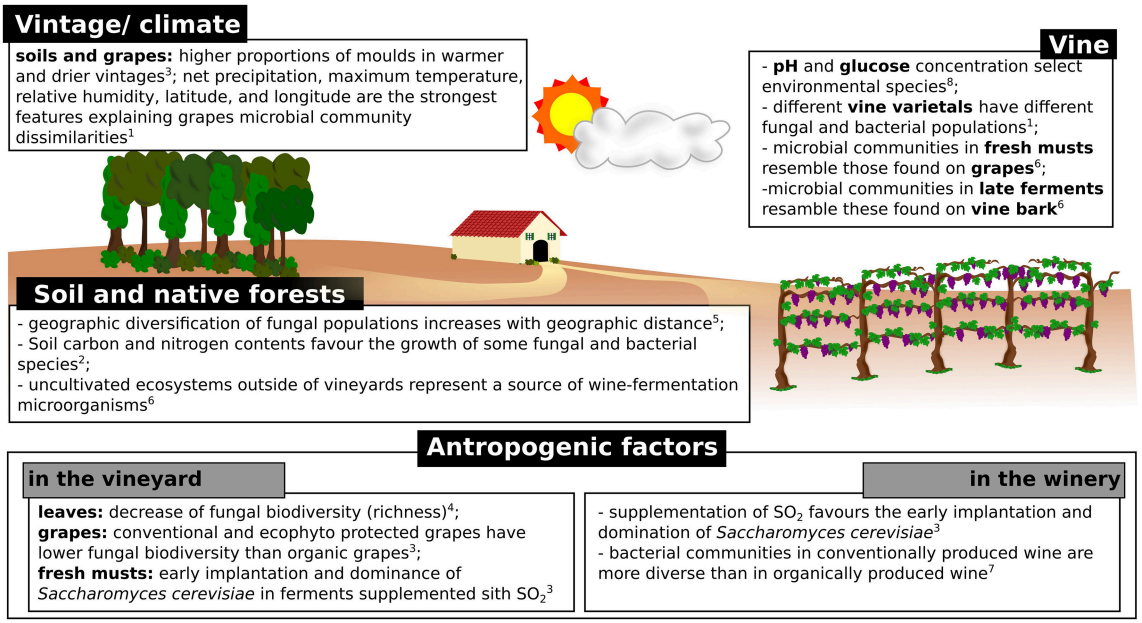

FIGURE 2 | Schematic representation of the factors known to influence the composition of microbial populations involved in wine fermentations. [1](Bokulich et al., 2014); [2](Burns et al., 2015); [3](Grangeteau et al., 2017); [4](Pinto et al., 2014); [5](Miura et al., 2017); [6](Morrison-Whittle and Goddard, 2018); [7](Piao et al., 2015); [8](Stefanini et al., 2016).

culture-independent methods reported that the high abundances of AAB, in particular of *Gluconobacter* (Acteobacteraceae), remained elevated throughout fermentation (Andorrà et al., 2010; Bokulich et al., 2012; Portillo Mdel and Mas, 2016). Nevertheless, the abundances of AAB detected by means of metagenomic approaches were shown to decrease with fermentation, possibly also because of the increase of LAB abundances (Portillo Mdel and Mas, 2016). In general, the inoculation of the yeast *S. cerevisiae* strains, a practice currently used to support and control the fermentation, has been shown to largely impact on the composition of bacterial populations, and to reduce the biodiversity, inducing a reduction of acetic acid bacteria (Bokulich et al., 2012).

As previously described for the vineyard, also the winery environment is a source of microorganisms involved in must fermentation (Bokulich et al., 2013; Belda et al., 2017). Under normal cleaning conditions, large populations of fungi and bacteria are found on winery surfaces prior to harvest (**Table 2**) (Bokulich et al., 2013). Such persistent microorganisms encompass fermenting, spoiling and innocent fungi and bacteria: *Pseudomonas*, *Enterobacteriaceae*, *Bacillus*, *S. cerevisiae, Cryptococcus* spp., *Aureobasidium pullulans*, and *Aspergillus* spp. (Bokulich et al., 2013; **Table 2**). Hence, these microorganisms will potentially contribute to the fermentation process of the following vintage.

Environmental Factors Influencing Microbial Populations

Among the known environmental factors known to influence the microbial populations found in various vineyard specimens, the vintage is probably the most relevant (**Figure 2**). The number and type of taxa identified in grape samples are associated with vintage characteristics, including factors such as temperature and rainfall (Bokulich et al., 2014; Grangeteau et al., 2017). Grangeteau and collaborators showed that the total number of fungal species and the proportion of molds were greater in warmer and drier vintages compared to cold vintages with heavy precipitations (Grangeteau et al., 2017). In addition, the *Botryotinia*, *Cladosporium*, and *Phoma* genera were found only in warm and dry vintages, while the *Monilinia* genus was found in vintages with lower temperatures and greater precipitations (Grangeteau et al., 2017). Similarly, Bokulich and collaborators showed the vintage effect on microbial populations present in must samples (Bokulich et al., 2014; **Figure 2**), with maximum temperature and relative humidity being among the strongest features explaining microbial community dissimilarities across grape microbial community patterns (Bokulich et al., 2014).

Other factors shaping the composition of wine fermentation-related microbial populations are the physical characteristics of the vineyard. Burns and collaborators showed that high abundances of Alphaproteobacteria and Actinobacteria families were found in vineyard soils having high contents of carbon or nitrogen (Burns et al., 2015). Contrarily, Sphingomonadaceae, Comamonadaceae, Pseudomonadaceae, Xanthomonadaceae, Micrococcaceae, Nocardiaceae, Flavobacteriaceae, Bacillaceae, and Paenibacillaceae were more abundant in soils showing low amounts of carbon or nitrogen sources (Burns et al., 2015). Unfortunately, the analyzed vineyards, located in the Napa Valley (California), showed several characteristics correlated with each other (i.e., elevation was positively correlated with

latitude, slope, and average annual precipitation), hence probably preventing the identification of further associations (Burns et al., 2015). However, the topological characteristics of the vineyard have been shown to greatly influence the composition of wine-related microbial populations (Portillo Mdel et al., 2016). In fact, *Pseudomonas* (an innocent genus), Haemophilus, Oxalobacteraceae, *Sphingomonas*, have been shown to be constantly present in grape samples from vineyards exposed to East, while *Staphylococcus* (innocent genus), *Streptococcus*, Micrococcaceae, Enhydrobacter, and Aeromonadaceae have been shown to be typical of flat vineyards (Portillo Mdel et al., 2016).

Notably, it has been shown that the vine cultivar influences the composition of fungal and bacterial populations (Bokulich et al., 2014). Bokulich and collaborators showed that *Capnodiales*, *Protobacteria*, and *Penicillium* were more abundant in Chardonnay grapes, Cabernet Sauvignon grapes were enriched in β-*Proteobacteria*, *Bacteroidetes*, *Clostridia*, *Dothideomycetes*, *Agaricomycetes*, *Tremellomycetes*, *Microbotryomycetes*, and *Saccharomycetaceae*; and *Firmicutes*, *Gluconobacter*, *Eurotiomycetes* (*Aspergillus*), *Leotiomycetes*, and *Saccharomycetes* were more abundant in Zinfandel (Bokulich et al., 2014).

In addition, must chemical-physical factors have been shown to play a relevant role in selecting microbial populations. For example, acidic musts (low pH) show high amounts of the environmental species *Pichia membranifaciens*, whereas *Wickerhamomyces anomalus*, *Pichia bialowiezense*, *Guehomyces* spp., *Cladosporium* spp., *Torulaspora delbrueckii*, and *Nakazawaea holstii* showed a preference for an environment characterized by a low ethanol concentration, high glucose concentration, and mildly acidic pH, as is usually the case for must in the early stages of fermentation (Stefanini et al., 2016). However, the composition of microbial populations is highly dynamic during the conversion of must into wine, and several studies have been done to dissect the dynamics of microbial populations. In general, the richness of both bacterial and fungal populations decreases during the process (Pinto et al., 2015). The fungal population, which is dominated by environmental species in musts, shows an initial growth of non-*Saccharomyces* (i.e., *Hanseniaspora*, *Metschnikowia*, *Pichia*, and *Torulaspora*). Later, the number of species is reduced, and a few yeast species are abundant in spontaneous fermentations: *S. cerevisiae, Candida zemplinina*, *Hanseniaspora* spp., *Metschnikowia* spp., and *Lachancea* spp. (Pinto et al., 2015; Stefanini et al., 2016).

Anthropogenic Factors Influencing Microbial Populations

Aiming to the optimization of the product, winemakers intervene in several stages of the process, from the vineyard up to the winery. The most common human interventions encompass the decision of using protective treatments in the vineyard, inoculating musts with either selected microorganisms or enriched environmental populations (*pied de cuve*), or adding chemicals to the must to eradicate spoiling microorganisms. Several different farming approaches are nowadays adopted in the vineyard, among which the most extreme are the conventional, based on the application of chemical fungicides and biofertilizers, and the biodynamic and organic approaches, avoiding the use of pesticides and herbicides. Some efforts have been made to evaluate the effects of these approaches on microbial communities associated with wine production. The fungal richness in grapes was found to be higher in conventional and ecophyto (same compounds used in conventional protection, at a lower dosage) than in organic (treated with only pyrethrin, copper, and sulfur) vineyards (Grangeteau et al., 2017). Basidiomycota (especially *Cryptococcus*) were mainly found in organic vineyards, as well as *Fusarium* and *Mucor*, whereas the fermenting genera *Saccharomyces*, *Metschnikowia*, and *Hanseniaspora* are mainly associated with the conventional method (Grangeteau et al., 2017). The effects of the farming approaches were also observed in microbial populations found in the must. In fact, the fungal biodiversity was found to be higher in musts from biodynamic vineyards (treated with sulfur, copper oxide, organic fungicides) than in conventional (chemical fungicides and biofertilizers are applied) and integrated (application of biofertilizers, mycorrhizae, combination of systemic and surface protectants for pest control) vineyards (Bagheri et al., 2015).

Conventional farming approaches make use of repetitive and various chemical treatments in the vineyard, which have been shown to influence both fungal and bacterial communities present on vine leaves (Pinto et al., 2014) and grapes (Setati et al., 2015). Chemical treatments affect the microbial biodiversity, especially reducing the relative abundances of *Aureobasidium* spp., *Cryptovalsa*, *Bulleromyces*, *Diaporthe*, and increasing the relative abundances of *Alternaria*, *Claviceps*, *Guignardia*, *Lewia*, *Puccinia*, *Sporormiella*, *Stemphylium*, and *Ustilago* on leaves (Pinto et al., 2014). When different combinations of chemicals were sequentially applied in the vineyard, each treatment was shown to affect the whole fungal community (Pinto et al., 2014). After treatments with chemicals encompassing the active element sulfur, a noticeable reduction was observed for the abundances on vine leaves of the genera *Aureobasidium*, *Rhodotorula,* and *Candida* (Pinto et al., 2014). In addition, the abundance of *Aureobasidium* was also affected by treatments supplemented with folpet, an agricultural fungicide used for the control of downy and powdery mildew and gray mold infections (Pinto et al., 2014). Concerning bacteria, chemical treatments have been shown to decrease the relative abundances of Enterobacteriaceae, Pseudomonadaceae, Comamonadaceae and Xanthomonadaceae families (Pinto et al., 2014; **Figure 2**).

Aiming to the control of spoilage microorganisms, winemakers have adopted in the winery a series of protocols including the control of temperature, the inoculation of *S. cerevisiae* strains, and the supplementation of musts with chemicals (i.e., SO_2). The inoculation of *S. cerevisiae*, a technique adopted since the mid-late nineteenth century (Muller-Thurgau, 1896), is aimed at exploiting the vigorous fermentative capacity of this species to obtain a very efficient ethanol production and impose the inoculated strain over the rest of the microbiota, potentially able to spoil the wine (Piskur et al., 2006). The inoculation of *S. cerevisiae* reduces the biodiversity of microbial populations, and in particular of acetic acid bacteria, possibly

by increasing the fermentation rate and the must temperature consequently (Bokulich et al., 2012). Currently, winemakers are interested in using non-*Saccharomyces* yeasts during alcoholic fermentation to increase wine complexity and differentiation (Lleixà et al., 2016). To meet this requirement, companies have started to study and commercialize *Torulaspora delbrueckii* and *M. pulcherrima* (Jolly et al., 2014). In addition, researchers have started to investigate the possibility to exploit one of the non-*Saccharomyces* genera most abundant in grape must, *Hanseniaspora*, and *H. vinae* has so far shown the most promising potential as fermentation starter (Lleixà et al., 2016). Interestingly, the inoculated *H. vinae* strains were shown to persist at high frequencies in musts only during the initial days of fermentation, and, despite being overturned by natural *S. cerevisiae* strains, were able to modify the organoleptic properties of wine (further details in section Effects of Microbial Populations on the Quality of Wine Fermentation; Lleixà et al., 2016).

Grangeteau and collaborators showed that the human intervention during the fermentation process (on musts) can modify the composition of microbial populations with a reduced impact than the human activities in the vineyard (Grangeteau et al., 2017). Indeed, the type of protection applied in the vineyard (conventional, ecophyto or organic) was shown to have the major effect on the dynamics of fungal populations during the fermentation (Grangeteau et al., 2017). However, also the supplementation of musts with SO_2 had an effect, favoring the early implantation and domination of the genus *Saccharomyces* (Grangeteau et al., 2017). Similarly, bacterial communities were shown to be affected by the supplementation of SO_2 (Bokulich et al., 2015; Piao et al., 2015). Bokulich and collaborators showed a dose-dependent effect of SO_2, with 25 mg/l SO_2 being the minimal concentration required to stabilize the bacterial population, also resulting in the control of *Gluconobacter* and LAB (Bokulich et al., 2015). However, the same study also revealed that the inoculation of *S. cerevisiae* had the same effect of SO_2 on bacterial populations and that the effect was not additive with the supplementation of SO_2 (Bokulich et al., 2015). A similar result was reported by Piao and collaborators, revealing higher abundances of the spoiling *Gluconobacter oxydans* and, in a minor extent, *Acetobacter*, in organic fermentations (not supplemented with SO_2), compared to conventional fermentations (supplemented with 55.8 mg/L SO_2; Piao et al., 2015).

Effects of Microbial Populations on the Quality of Wine Fermentation

As described in previous sections, a wealth of studies based on metagenomic approaches have investigated microbial populations associated with wine production, not only to describe them, but also to identify factors affecting their compositions. Contrarily, only a few studies have explored the associations between microbial communities and wine organoleptic characteristics (Bokulich et al., 2016; Lleixà et al., 2016; Stefanini et al., 2017b). It is worth mentioning that most of current studies on the associations between microbial communities and organoleptically relevant compounds were aimed at identifying correlations, without claiming causation. In other words, the identification of positive or negative correlations does not mean that the microbe produces (positive correlation) or is killed/controlled by (negative correlation) the compound. Rather, correlations could be potential markers to predict wine metabolite composition (Bokulich et al., 2016). Further studies should be done to assess the potential role of microorganisms in flavor production (Bokulich et al., 2016).

The geographical differentiation observed for microbial populations was also observed for wine metabolites (Knight et al., 2015; Bokulich et al., 2016). This observation encouraged Bokulich and collaborators to search for correlations between microbial (fungal and bacterial) genera abundances and metabolite amounts (Bokulich et al., 2016). Noticeably, associations were identified between Leuconostocaceae (with *O. oeni* as the best sequence hit) and a metabolite tentatively assigned as methyl benzoate, phenylacetate, or p-anisaldehyde, between *Hanseniaspora uvarum* and a metabolite tentatively identified as acetophenone, phenylacetaldehyde, or 3-methyl benzaldehyde, and between *Pichia guilliermondii* and a two metabolites identified as octanoic acid and $C_6H_{10}O_2$ (either acid, ester, or lactone) (Bokulich et al., 2016). Noticeably, several of the compounds identified as being associated to microbial species are known to have scents lending wine either pleasant or unpleasant characteristic, e.g., methyl benzoate has pungent, heavy, floral odor with fruity undertones; p-anisaldehyde has an intensely sweet floral odor; phenylacetaldehyde has a rose-like scent; octanoic acid has an unpleasant odor [information obtained from PubChem (Kim et al., 2016) and "the good scent company" website, http://www.thegoodscentscompany.com/]. Other correlations have been identified among fungal genera and volatile compounds in withering *V. vinifera* L. cv. Corvina grapes and musts of Amarone, a dry wine produced exclusively in the Italian region of Valpolicella (Verona) (Stefanini et al., 2017b). The fungal genus *Phoma*, found at high frequencies in withering Corvina grapes, showed a positive correlation with (3E)-3-hexenoic acid. The *Diplodia* genus, highly abundant in musts, was found to be positively correlated with 1-pentanol (amyl alcohol, having a balsamic, fusel, oil, sweet, vanilla flavor) and 2,6-dimethoxy phenol (syringol, having a bacon, balsamic, phenol, powdery, smoke, woody flavor). Contrarily, other genera highly abundant in musts showed negative correlations with volatile compounds known to have a relevant impact on wine aroma. The genus *Candida* showed a negative correlation with *p*-formilphenol, having an almond, balsam, sweet, woody flavor, and dichloromethane, having a sweet smell. The *Cytospora* genus showed a negative correlation with paraldehyde (aromatic and sweet smell), and tetradecane (alkane, mild, waxy smell). The genus *Metschnikowia* was found to have negative correlations with (3E)-3-hexenoic acid (acid, cheesy, fruity, grass, sweaty flavor), isoamyl acetate (banana and pear), dibutyl phthalate (faint smell), paraldehyde (sweet and aromatic smell), p-formaldehyde (almond, balsam, sweet, woody smell), triethylene glycol (odorless, but potentially acting as disinfectant), and dichloromethane (sweet smell). Both *Cytospora* and *Metschnikowia* showed negative correlations

with caprylic acid (cheesy, rancid smell) and octadecane (alkane smell).

As previously stated (section Anthropogenic Factors Influencing Microbial Populations), not-*Saccharomyces* strains are being studied as potential starters (aka strains inoculated in musts to promote alcoholic fermentation) to increase wine complexity and differentiation. The inoculation of *Hanseniaspora vinae* was shown to modify the organoleptic characteristics of wine, despite the inoculated strain was rapidly replaced by natural *S. cerevisiae* strains present in the must (Lleixà et al., 2016). In particular, the amounts of N-acetyl tyamine and 1H-indole-3-ethanol acetate ester, usually not found in wine fermentations, were found only in musts inoculated with *H. vinae*, and phenethyl acetate, conferring floral, fruity and honey-like aromas to wine, was 50 times more abundant in wines fermented with *H. vinae* (Lleixà et al., 2016). Noticeably, Lleixà and collaborators also reported that wine-tasters selected and easily distinguished wines fermented with *H. vinae*, indicating that the early presence of this species can greatly modify the characteristics of the wine (Lleixà et al., 2016).

FUTURE CHALLENGES FOR METAGENOMIC APPROACHES: THE SUB-SPECIES LEVEL

Amplicon-based approaches allow us to obtain a general picture of the microbiota but have a taxonomic resolution that, in the best situations, assigns individuals at the species level (Stefanini et al., 2016). Although this might be sufficient to describe and compare populations at the large scale, in some situations a higher resolution is necessary. For instance, *S. cerevisiae* isolated from different geographical locations have shown different genetic and phenotypic characteristics, thus suggesting the existence of geographically-specific lineages of this yeast (Yarza et al., 2014). Hence, the disclosure of microbial populations at the strain level is of great interest to better understand the distribution and diffusion of microorganisms from the vineyard to the winery and among vineyards.

Aiming to identifying different strains of a given species, a few culture-independent procedures have been developed. Among these procedures, MetaMLST (Zolfo et al., 2017) and *SID* (Stefanini et al., 2017a) are based on approaches used to identify isolates by means of genetic markers, MLST (multilocus sequences typing) and microsatellites sequencing, respectively. MetaMLST allows the identification of strains by comparing whole-genome metagenomic sequences with databases of species-specific loci (Zhang et al., 2016). Contrarily, *SID* is based on the use of microsatellites, non-coding DNA sequences composed by small repeated units (2–6 bp) which are repeated a variable number of times in different individuals (Legras et al., 2005). Hence, *SID* identifies the combination of microsatellite profiles of strains from a reference dataset most likely composing the microsatellite profile obtained on a complex sample (e.g., microbial DNA extracted from must, grapes; Stefanini et al., 2017a). MetaMLST and *SID* enable the identification of different strains according to the similarity of the sample profile to the profiles present in reference databases. The use of MetaMLST to wine fermentation is currently limited due to the availability of MLST databases enriched in bacterial and fungal species of clinical interest (Zolfo et al., 2017), thus making this approach not suitable for wine-related samples. On the contrary, microsatellite sequencing has been widely used to type microorganisms in fermentation, but most of such studies were limited to the *S. cerevisiae* species (Legras et al., 2005, 2007; Ezov et al., 2006; Richards et al., 2009).

Recently, another tool has been proposed by the Segata group, StrainPhlAn (Truong et al., 2017). StrainPhlAn is based on reconstructing consensus sequence variants within species-specific marker genes identified for MetaPhlAn2 and building a phylogenetic tree on the consensus sequences to identify different strains (Truong et al., 2015). The species-specific markers (∼1 million markers from >7,500 species) (Truong et al., 2017) used in MetaPhlAn analyses have been identified by comparing the genomes available from the Integrated Microbial Genomes system, encompassing publicly available bacterial, archaeal, eukaryotic, and phage genomes, as well as engineered, environmental and host-associated microbiome samples (Truong et al., 2015). Hence, since it is not biased toward clinically-relevant microbes, this approach holds a great potential in supporting the identification of microbial strains present in wine-related metagenomes.

CONCLUSIONS

Metagenomic approaches are largely contributing to the dissection of the so-called "microbial terroir," microbial communities typical of the geographical area of wine production. Thanks to these approaches, the rapid and exhaustive characterization of microbial populations present in various specimens associated with vineyards, wineries, and fermentation is nowadays possible. In addition to evaluating the existence of a microbial terroir, new studies allowed the identification of several environmental and human-related factors influencing the composition of microbial populations, and hence potentially affecting their fermentative performances. And yet, despite the great contribution made by these studies, the microbial spreading and persistence from the vineyard to the winery are still far from being completely dissected. Further studies, exploring a wider variance of vine varietals, comparing different procedures (adopted in the vineyard and in the winery) and different environments, will increase our knowledge of this complicated process. Probably one of the most complex achievement is the separation of topological variables characterizing the vineyard and environmental variables characterizing a "vintage." A proper comparison of microbial populations in environments varying by only one or few variables will help in this goal. In addition, the observation of clear geographic diversification of fungal populations, and weaker diversification of bacterial communities may indicate the need for understanding the role of vectors in moving microbes across areas. Indeed, while bacterial and fungal spores can blow in the wind and be transported

among distant geographic locations, not-airborne yeasts require animals to be vectored among distant (by birds) or close (by insects) locations (Francesca et al., 2012; Stefanini et al., 2012). A complete survey of the microbiota of these vectors will help in completely understanding the fluxes of microorganisms relevant for wine fermentation. The complete understanding of all the factors influencing the composition of microbial populations and their passage from the vineyard to fermenting musts will help winemakers by disclosing the association between variables and outcomes, thus allowing the adoption of the most appropriate techniques according to environmental changes.

AUTHOR CONTRIBUTIONS

IS wrote the first draft of the review. DC proofed the drafts. IS and DC finalized the review.

FUNDING

This work was supported by EU Framework Programme 7 Collaborative Project [242220]-JPI ENPADASI. While writing this review, IS was supported by a fellowship from the Wellcome Warwick Quantitative Biomedicine Programme (Institutional Strategic Support Fund: 105627/Z/14/Z).

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

frontiers in Microbiology

REVIEW
published: 22 February 2018
doi: 10.3389/fmicb.2018.00288

Copy Number Variation in Fungi and Its Implications for Wine Yeast Genetic Diversity and Adaptation

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In recent years, copy number (CN) variation has emerged as a new and significant source of genetic polymorphisms contributing to the phenotypic diversity of populations. CN variants are defined as genetic loci that, due to duplication and deletion, vary in their number of copies across individuals in a population. CN variants range in size from 50 base pairs to whole chromosomes, can influence gene activity, and are associated with a wide range of phenotypes in diverse organisms, including the budding yeast *Saccharomyces cerevisiae*. In this review, we introduce CN variation, discuss the genetic and molecular mechanisms implicated in its generation, how they can contribute to genetic and phenotypic diversity in fungal populations, and consider how CN variants may influence wine yeast adaptation in fermentation-related processes. In particular, we focus on reviewing recent work investigating the contribution of changes in CN of fermentation-related genes in yeast wine strains and offer notable illustrations of such changes, including the high levels of CN variation among the *CUP* genes, which confer resistance to copper, a metal with fungicidal properties, and the preferential deletion and duplication of the *MAL1* and *MAL3* loci, respectively, which are responsible for metabolizing maltose and sucrose. Based on the available data, we propose that CN variation is a substantial dimension of yeast genetic diversity that occurs largely independent of single nucleotide polymorphisms. As such, CN variation harbors considerable potential for understanding and manipulating yeast strains in the wine fermentation environment and beyond.

Keywords: structural variation, alcohol fermentation, sugar metabolism, gene duplication, gene loss, population genomics

OPEN ACCESS

Edited by:
Aline Lonvaud,
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Reviewed by:
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Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: *12 December 2017*
Accepted: *07 February 2018*
Published: *22 February 2018*

Citation:
Steenwyk JL and Rokas A (2018) Copy Number Variation in Fungi and Its Implications for Wine Yeast Genetic Diversity and Adaptation. Front. Microbiol. 9:288. doi: 10.3389/fmicb.2018.00288

INTRODUCTION

Genetic variation in natural populations is shaped by diverse biological processes, such as genetic drift and natural selection (Chakravarti, 1999), and is, in part, responsible for phenotypic variation. For example, arginine auxotrophy in the baker's yeast *Saccharomyces cerevisiae* is a Mendelian inherited trait due to polymorphisms in the *ARG4* locus (Brauer et al., 2006), whereas variation in *S. cerevisiae* colony morphology is a complex trait driven by variants in several different genes (Taylor et al., 2016). The aforementioned yeast phenotypes are all caused by SNPs or small insertions and deletions, which are by far the most well characterized types of genetic variation

Abbreviations: BIR, break-induced recombination; CN, copy number; HR, homologous recombination; NHR, non-homologous repair; SNPs, single nucleotide polymorphisms.

not only in yeast, but in any kind of organism (Sachidanandam et al., 2001; McNally et al., 2009; Schacherer et al., 2009). In recent years, however, several studies in diverse organisms have revealed that genomes also harbor an abundance of structural variation, which too contributes to populations' genetic and phenotypic diversity (Stranger et al., 2007; Zhang et al., 2009).

Variation in the structure of chromosomes, or structural variation, encompasses a wide array of mutations including insertions, inversions, translocations, and CN variants (i.e., duplications and deletions) (Feuk et al., 2006) and, in humans, accounts for an estimated average of 74% of the nucleotide differences between two genomes (Rahim et al., 2008). The major influence of several types of structural variation, such as large-scale inversions, translocations, and insertions, on phenotype is better understood because many such variants can be microscopically examined and lead to classic human genetic disorders, such as Down's syndrome (Youings et al., 2004; Rausch et al., 2012; Gu et al., 2016). In contrast, many CN variants are submicroscopic and eschewed attention until the advent of whole genome sequencing technologies (Feuk et al., 2006).

Copy number variants are defined as duplications or deletions that range from 50 base pairs to whole chromosomes (**Figure 1**) and can significantly influence phenotypic diversity (Lieber, 2008; Riethman, 2009; Zhang et al., 2009; Arlt et al., 2014). For example, in humans, the CN of the salivary amylase gene, *AMY1*, is higher in populations with high-starch diets and correlated with salivary protein abundance thereby improving digestion of starchy foods (Perry et al., 2007). Levels of CN variation have been examined in diverse organisms across the tree of life, including animals (e.g., Humans; *Homo sapiens*: Sudmant et al., 2015, House mouse; *Mus musculus*: Pezer et al., 2015), plants (e.g., soybean; *Glycine max*: Cook et al., 2012, maize; *Zea mays*: Swanson-Wagner et al., 2010) and fungi (e.g., *Cryptococcus neoformans*: Hu et al., 2011, *Brettanomyces bruxellensis*: Curtin et al., 2012, *Batrachochytrium dendrobatidis*: Farrer et al., 2013, *Zymoseptoria tritici:* Hartmann and Croll, 2017). Additionally, CN variants spanning genes can be a major platform for functional divergence of gene duplicates (e.g., through subfunctionalization or the partitioning of a set of ancestral functions across duplicates), including the evolution of new functions (neofunctionalization) (Lynch and Conery, 2000; Soria et al., 2014; Reams and Roth, 2015). For example, duplicated phospholipase genes that have undergone neofunctionalization are responsible for the evolution and diversification of snake venom and snake species (Lynch, 2007), whereas clusters of tandemly duplicated genes are associated with phenotypic diversity in many traits and organisms (Ortiz and Rokas, 2017).

Saccharomyces cerevisiae has been an important model for genetics, genomics, and evolution (Goffeau et al., 1996; Botstein et al., 1997; Winzeler et al., 1999). Much of what we know about the evolutionary history of *S. cerevisiae* stems from investigating genome-wide patterns of SNPs among globally distributed strains. Examination of genome-wide patterns of SNP variation has yielded valuable insights into yeast function in the wine fermentation environment. For example, 13 SNPs in *ABZ1,* a gene associated with nitrogen biosynthetic pathways, have been shown to modify the rate of fermentation and nitrogen utilization during fermentation (Ambroset et al., 2011).

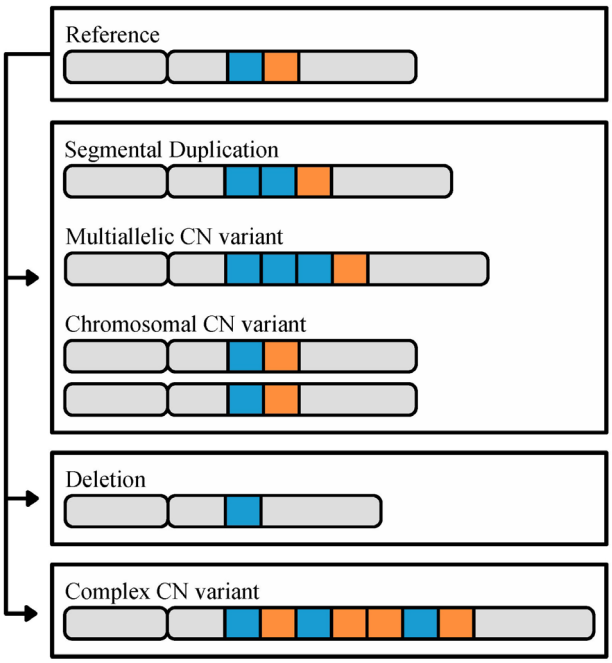


FIGURE 1 | The different types of CN variation. CN variants range in size (50 base pairs or greater) to whole chromosomes, and are identified through comparison to a reference genome. In this cartoon, a reference chromosome containing two highlighted loci, in blue and orange, is shown on top. The second chromosome illustrates an example of a segmental duplication CN, in which there are two copies of the blue locus. The third chromosome illustrates an example of a multiallelic CN variant, where the duplicated locus contains 3 or more copies. The fourth pair of chromosomes illustrates a CN variant associated with the duplication of an entire chromosome. Finally, the last two chromosomes illustrate deletion and complex CN variants, respectively; deletion CN variants are associated with loci that are not present relative to the reference, and complex CN variants refer to a combination of duplications, deletions, insertions, and/or inversions relative to the reference. In some organisms, such as budding yeast (Dunn et al., 2012; Bergstrom et al., 2014) and humans (Riethman, 2009), CNVs tend to biased in their genomic location toward subtelomeres.

Interrogations of genome-wide patterns of SNPs have also shown that industrial lineages – including those of beer, bread, cacao, sake, and wine – often mirror human history (Schacherer et al., 2009; Sicard and Legras, 2011; Cromie et al., 2013; Gallone et al., 2016; Gonçalves et al., 2016), suggesting that human activity has greatly influenced *S. cerevisiae* genome evolution (Yue et al., 2017). Furthermore, SNP-based studies have repeatedly found that wine strains of *S. cerevisiae* exhibit low levels of genetic diversity (Liti et al., 2009; Schacherer et al., 2009; Sicard and Legras, 2011; Cromie et al., 2013; Borneman et al., 2016), consistent with a historical population bottleneck event that reduced wine yeast genetic variation. The low SNP diversity among wine yeast strains has led some to suggest that wine strain development may benefit from the introduction of genetic variation from yeasts outside the wine lineage (Borneman et al., 2016). However, recent studies examining CN variation among wine associated strains of *S. cerevisiae* have identified considerable genetic diversity (Gallone et al., 2016; Gonçalves

et al., 2016; Steenwyk and Rokas, 2017), suggesting that standing CN variation in wine strains may be industrially relevant.

In the present review, we begin by surveying the molecular mechanisms that lead to CN variant formation, we next discuss the contribution of CN variation to the genetic and phenotypic diversity in fungal populations, and close by examining the CN variation in wine yeasts and the likely phenotypic impact of CN variants in the wine fermentation environment.

COPY NUMBER VARIATION AND THE MOLECULAR MECHANISMS THAT GENERATE IT

Copy number variants, a class of structural variants, are duplicated or deleted loci that range from 50 base pairs (bp) to whole chromosomes in length (**Figure 1**) and have a mutation rate 100–1,000 times greater than SNPs (Zhang et al., 2009; Arlt et al., 2014; Sener, 2014). CN variable loci can in turn be broken down into three subclasses (**Figure 1**) (Estivill and Armengol, 2007). The first subclass encompasses variants that originate via duplications; in the genome, these can appear as either identical or nearly identical copies, or multi-allelic CN variants (Bailey and Eichler, 2006; Usher and McCarroll, 2015). The extreme version of this subclass are chromosomal CN variants that correspond to duplications of entire chromosomes. The second subclass encompasses CN variants that originate via deletion leading to the loss of the sequence of a locus in the genome. The third subclass includes complex CN variants where a locus exhibits a combination of duplication, deletion, insertion, and inversion events (Usher and McCarroll, 2015).

Copy number variants are commonly generated from aberrant DNA repair via three mechanisms: HR, NHR, and environmental stimulation (**Figure 2**) (Hastings et al., 2009b; Hull et al., 2017). HR is a universal process associated with DNA repair and requires high sequence similarity across 60–300 bps (Hua et al., 1997; Petukhova et al., 1998). HR is initiated by double-strand breaks caused by ionizing radiation, reactive oxygen species, and mechanical stress on chromosomes such as those associated with collapsed or broken replication forks (Khanna and Jackson, 2001; Aylon and Kupiec, 2004; Hastings et al., 2009b). Improper repair by HR can result in duplication, deletion, or inversion of genetic material (Reams and Roth, 2015). Non-allelic HR (also known as ectopic recombination), defined as recombination between two different loci of the same or different chromosomes that share sequence similarity and are $\geq$300 base pairs in length, is among the most well-studied examples of improper repair (Kupiec and Petes, 1988; Prado et al., 2003). Most evidence of non-allelic HR resulting in CN variation is directly associated with low copy repeats or transposable elements (Xu and Boeke, 1987; Hurles, 2005). For example, a duplication and deletion may result during unequal crossing over of homologous sequences (**Figure 2A**) (Carvalho and Lupski, 2016). Improper HR may also occur at collapsed or broken replication forks by BIR (**Figure 2B**). BIR requires 3′ strand invasion at the allelic site of stalled replication to properly restart DNA synthesis (**Figure 2B-i**) (Llorente et al., 2008), however, template switching, the non-allelic pairing of homologous sequences, in the backward (**Figure 2B-ii**) or forward (**Figure 2B-iii**) direction can result in a duplication or deletion, respectively (Morrow et al., 1997; Smith et al., 2007). Although HR occurs with high fidelity, errors in the process, which are thought to increase in frequency during mitosis and meiosis, can generate CN variants (Hastings et al., 2009b).

In contrast to HR, NHR utilizes microhomologies (typically defined as ∼65% or more sequence similarity of short sequences up to ten bases long) or does not require homology altogether, and can too lead to CN variant formation (Daley et al., 2005; McVey and Lee, 2008). NHR can occur by two mechanisms: non-replicative and replicative (Hastings et al., 2009b). Non-replicative mechanisms include non-homologous end joining and microhomology-mediated end-joining (Lieber, 2008; McVey and Lee, 2008). Non-homologous end-joining refers to the direct ligation of sequences in a double-strand break (Daley et al., 2005). Prior to ligation, there may be a loss of genetic material or the addition of free DNA (e.g., from transposable elements or mitochondrial DNA) (Yu and Gabriel, 2003). Microhomology-mediated end joining is similar to non-homologous end-joining but occurs more frequently, requires different enzymes, and leverages homologies 1–10 base pairs in length to ensure more efficient annealing (Yu et al., 2004; Lieber, 2008). Non-homologous end-joining and microhomology-mediated non-homologous end-joining are primarily associated with small insertions and deletions and therefore are not likely to be a major driver of CN variation (Yu and Gabriel, 2003; Gu et al., 2008). Replicative mechanisms of CN variant formation include replication slippage, fork stalling, and microhomology BIR. Replication slippage occurs along repetitive stretches of DNA resulting in the duplication or deletion of sequence between repetitive regions (Hastings et al., 2009b). Fork stalling is thought to cause large CNVs of 20 kb average length through template switching between distal replication forks rather than within a replication fork (Slack et al., 2006). However, fork stalling without distal template switching can also be highly mutagenic and induce CN variants (Paul et al., 2013; Hull et al., 2017). Lastly, microhomology-mediated break-induced replication occurs when the 3′ end of a collapsed fork anneals with any single-stranded template that it shares microhomology with to reinitiate DNA synthesis (**Figure 2B**) (Hastings et al., 2009b). Annealing can occur in the backward (**Figure 2B-ii**) or forward (**Figure 2B-iii**) direction of the allelic site causing a duplication or deletion, respectively, and is thought to be the primary cause of low copy repeats (Hastings et al., 2009a).

The third mechanism is associated with an epigenetic mark that can stimulate the formation of CN variants. Histone acetylation, specifically H3K56ac, is, in part, environmentally driven (Turner, 2009), associated with highly transcribed loci, and can promote CN variant formation through repeated fork stalling or template switching (**Figure 2C**) (Hull et al., 2017). For example, it has been shown that exposure to environmental copper stimulates the generation of CN variation in *CUP1,* a gene that is associated with copper resistance when duplicated (Fogel and Welch, 1982), thereby increasing the likelihood of favorable alleles that exhibit increased copper resistance (Hull et al., 2017). Similarly, environmental formaldehyde exposure was shown to

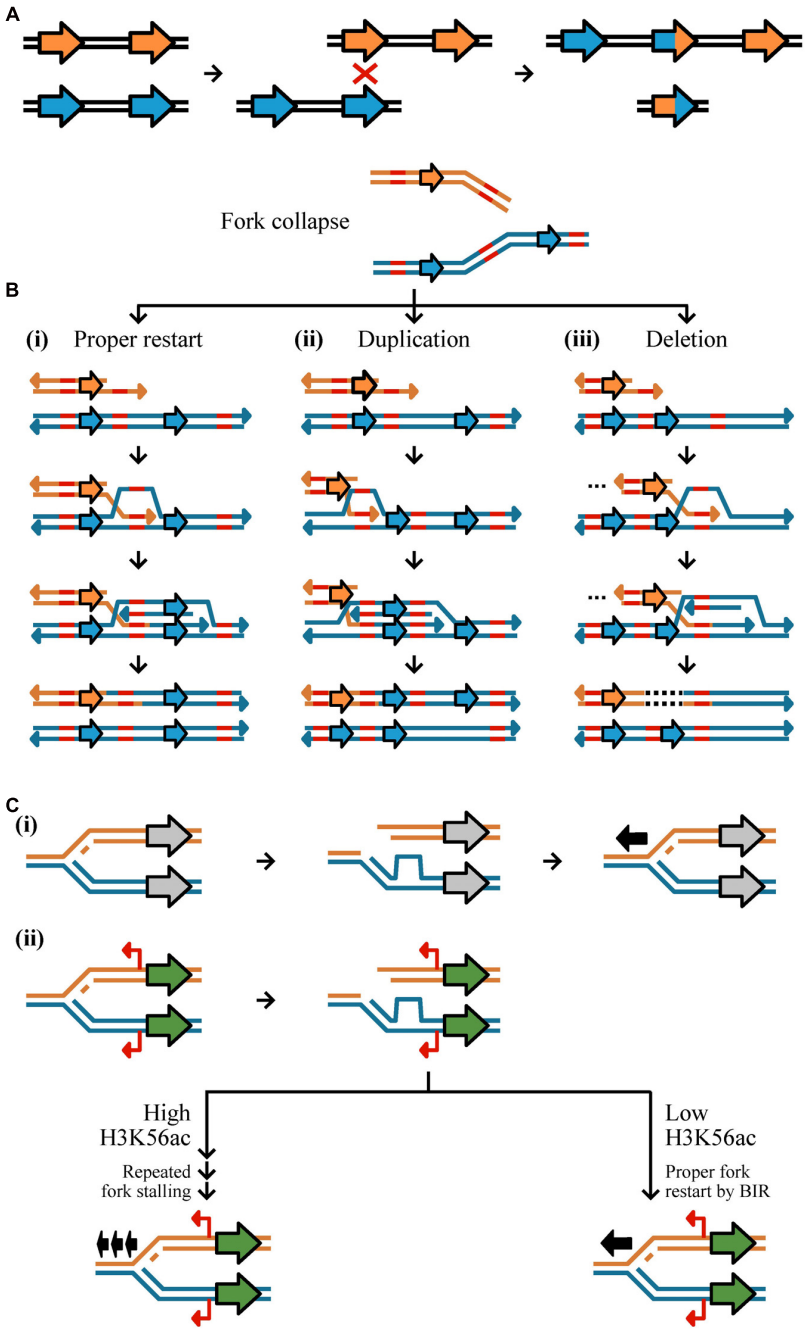


FIGURE 2 | Mechanisms of CN variant formation. CN variants typically occur as a result of aberrant replication via homologous recombination, non-homology based mechanisms, and environmentally stimulated processes. **(A)** Unequal crossing over during recombination may result in duplication and deletion. Here, two equal strands of DNA with two genes (represented by the orange or blue arrows) have undergone unequal crossing over due to the misalignment of a homologous sequence. This results in one DNA strand having three genes and the other one gene. **(B,C)** A major driver of CN variant formation is aberrant DNA replication. (**B**, top) Double strand breaks at replication forks or collapsed forks are often repaired via Break-induced replication (BIR). (i) Proper BIR starts with strand invasion of a homologous or microhomologous sequence (shown in red) to allow for proper fork restart. (ii) If template switching occurs in the backward direction, a segment of DNA will have been replicated twice resulting in a duplication; (iii) in contrast, template switching in the forward direction results in a deletion represented by a dashed line in the DNA sequence. Erroneous BIR may be mediated by microhomologies as well. **(C)** CN variants may be stimulated near genes that are highly expressed due to an increased chance of fork stalling. (i) If a replication fork breaks down near a gene that is not expressed (gray) and restarts once (represented by one black arrow), no mutation will occur. (ii) If a replication fork breaks down near a gene that is expressed (green) with cryptic unstable transcripts (red) then there may be two outcomes dependent on the degree of the H3K56ac acetylation mark. If there are low levels of H3K56ac, it is more likely that there will be proper fork restart by BIR (represented by one black arrow). If there are high levels of H3K56ac, it is more likely that there will be repeated fork stalling (represented by three black arrows) (see Figure 8 from Hull et al., 2017).

stimulate CN variation (Hull et al., 2017) of the *SFA1* gene, which confers formaldehyde resistance at higher CNs (Wehner et al., 1993). Altogether, these experiments provide insight to how perturbations of an environmental parameter may stimulate CN variation at a locus associated with adaptation in the new environment (Hull et al., 2017).

COPY NUMBER VARIATION AS A SOURCE OF PHENOTYPIC DIVERSITY

Copy number variants can have multiple effects on gene activity, such as changing gene dosage (i.e., gene CN; **Figure 3**) and interrupting coding sequences (Itsara et al., 2009; Sener, 2014). These effects can be substantial; for example, 17.7% of gene expression variation in human populations can be attributed to CN variants (Stranger et al., 2007). Furthermore, changes in human gene expression attributed to CN variants have little overlap with changes in gene expression caused by SNPs, suggesting the two types of variation independently affect gene expression (Stranger et al., 2007). Additionally, gene CN tends to correlate with levels of both gene expression and protein abundance (Perry et al., 2007; Stranger et al., 2007; Henrichsen et al., 2009). For example, changes in gene expression and therefore protein abundance caused by chromosomal CN variation in human chromosome 21 are thought to contribute to Down syndrome (Kahlem et al., 2004; Aivazidis et al., 2017).

COPY NUMBER VARIATION AS A SOURCE OF GENETIC AND PHENOTYPIC DIVERSITY IN FUNGAL POPULATIONS

Copy number variant loci contribute to population genetic and phenotypic diversity (**Box 1**), such as virulence (Hu et al., 2011; Farrer et al., 2013), in diverse fungal species, including the baker's yeast *Saccharomyces cerevisiae* (ASCOMYCOTA, Saccharomycetes) (Strope et al., 2015; Gallone et al., 2016; Gonçalves et al., 2016; Steenwyk and Rokas, 2017), *Saccharomyces paradoxus* (ASCOMYCOTA, Saccharomycetes) (Bergstrom et al., 2014), the fission yeast *Schizosaccharomyces pombe* (ASCOMYCOTA, Schizosaccharomycetes) (Jeffares et al., 2017), the wheat pathogen *Zymoseptoria tritici* (ASCOMYCOTA, Dothideomycetes) (Hartmann and Croll, 2017), the human fungal pathogens *Cryptococcus deuterogattii* (BASIDIOMYCOTA, Tremellomycetes) (previously known as *Cryptococcus gattii* VGII; Steenwyk et al., 2016) and *C. neoformans* (Hu et al., 2011), and the amphibian pathogen *Batrachochytrium dendrobatidis* (CHYTRIDIOMYCOTA, Chytridiomycetes) (Farrer et al., 2013).

Importantly, the degree of CN variation (which can be represented by CN variable base pairs per kilobase) in fungal populations is not always correlated to the degree of SNP variation (which can be represented by SNPs per kilobase) (**Figure 4A**). For example, there is no correlation between CN variable base pairs per kilobase and SNPs per kilobase among

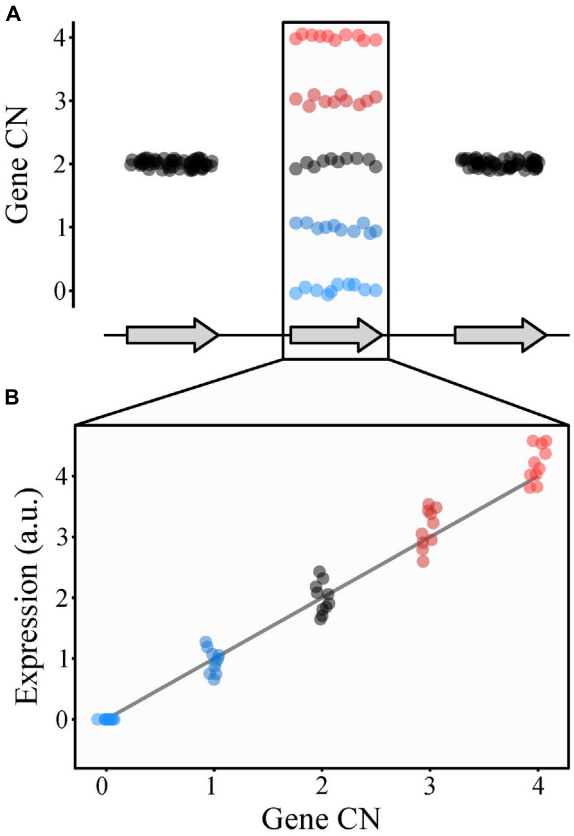
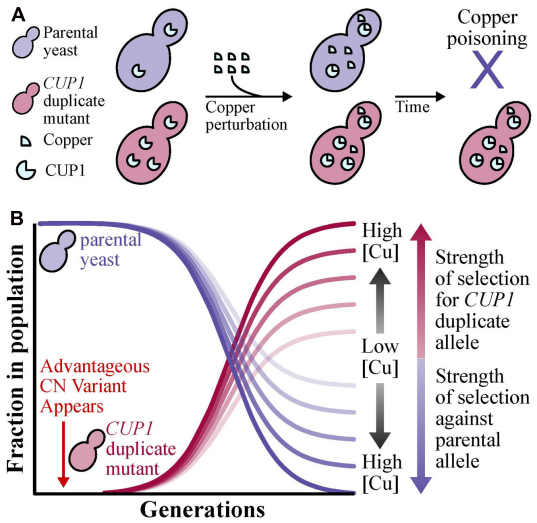


FIGURE 3 | Copy number variation can alter gene expression. **(A)** Consider a gene whose CN ranges from 0 to 4 (blue to black to red) among individuals (represented by dots) in a population (middle gene). **(B)** Generally, CN and gene expression (represented as arbitrary units or a.u.) correlate with one another such that individuals with lower CN values will have lower levels of gene expression of that gene while those with higher CN values will have higher levels of gene expression.

S. cerevisiae wine strains (Steenwyk and Rokas, 2017) and a population of *Cryptococcus deuterogattii* (Steenwyk et al., 2016). Interestingly, both populations harbor low levels of SNP diversity; for *S. cerevisiae* wine strains this is due to a single domestication-associated bottleneck event (Liti et al., 2009; Schacherer et al., 2009; Sicard and Legras, 2011; Cromie et al., 2013), whereas for *C. deuterogattii* this is because the samples stem from three clonally evolved subpopulations from the Pacific Northwest, United States (Engelthaler et al., 2014). In contrast, a significant correlation is observed between CN variable base pairs per kilobase and SNPs per kilobase among individuals in a globally distributed population of *S. pombe* (Jeffares et al., 2015).

The proportion of the genome exhibiting CN and SNP variation also varies across *S. cerevisiae, S. pombe,* and *C. deuterogattii* populations. For example, CN variable base pairs per kilobase are significantly different between the three populations (**Figure 4B**), with the fraction of CN variable base pairs per kilobase being greatest in *S. cerevisiae* wine strains, followed by *C. deuterogattii,* and then *S. pombe*. Notably, wine strains of *S. cerevisiae* exhibit higher levels of CN variation



BOX 1 | Standard population genetic principles of shifts in allele frequencies (Felsenstein, 1976; Moritz, 1994) can be applied to CN variants. To illustrate the case, we provide an example using the *CUP1* locus, where high CN provides protection against copper poisoning (Fogel and Welch, 1982), of how the allele frequency of a CN variant can increase through its phenotypic effect. Suppose that in a yeast population exposed to copper that all individuals do not harbor CN variation at the *CUP1* locus. Through a mutational event, a beneficial *CUP1* allele that contains two or more copies of the locus may appear in the population. **(A)** Yeast with two or more copies of *CUP1*, which in turn lead to higher *CUP1* protein levels, will be better and more efficient at copper sequesteration unlike the parental allele and therefore avoiding copper poisoning (Fogel and Welch, 1982). **(B)** Assuming a large population size and strong positive selection, changes in allele frequency will occur in the population due to changes in yeast survivability and ability to propagate. More specifically, the frequency of the beneficial allele (i.e., *CUP1* duplications) will increase depending on the strength of selection, which increases as the concentration of environmental copper increases, and the parental allele will decrease.

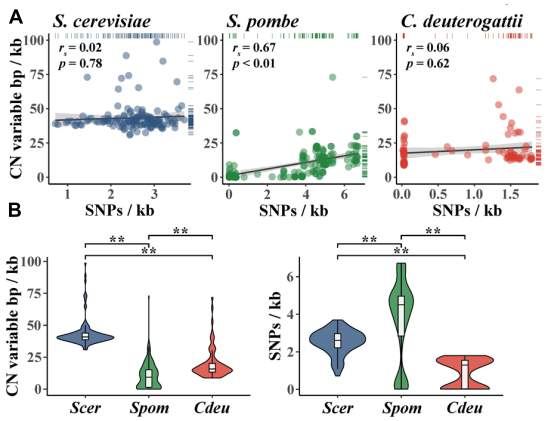


FIGURE 4 | Comparison of genomic content affected by CN variants and SNPs in three fungal species. **(A)** SNPs per kb is not significantly correlated with CN variable base pairs per kb in *S. cerevisiae* wine strains (blue; $r_S = 0.02$; $p = 0.78$; Spearman rank correlation) and *C. deuterogattii* (red; $r_S = 0.06$; $p = 0.62$; Spearman rank correlation); the reverse is true in *S. pombe* (green; $r_S = 0.67$; $p < 0.01$; Spearman rank correlation). (**B**, left) CN variable base pairs per kb in wine strains of *S. cerevisiae* is greater than *C. deuterogattii* and *S. pombe* ($p < 0.01$; Kruskal–Wallis and $p < 0.01$ for all Dunn's test pairwise comparisons with Benjamini–Hochberg multi-test correction). (**B**, right) SNPs per kb is low among *S. cerevisiae* wine strains (*Scer*) compared to *S. pombe* (*Spom*) but greater than a clonally expanded population of *C. deuterogattii* (*Cdeu*) ($p < 0.01$; Kruskal–Wallis and $p < 0.01$ for all Dunn's test pairwise comparisons with Benjamini–Hochberg multi-test correction). CN variants from Jeffares et al. (2015, 2017) (*Spom*); Steenwyk et al. (2016) (*Cdeu*); Steenwyk and Rokas (2017) (*Scer*) were all greater than 100 base pairs and smaller than whole chromosomes. Accordingly, CN variants represented here do not include whole chromosomes (i.e., aneuploidy). **Indicates a *p*-value < 0.01.

than sake strains but lower than beer strains (Gallone et al., 2016). In contrast, there are fewer SNPs per kilobase in the *S. cerevisiae* population compared to *S. pombe* but more compared to *C. deuterogattii* (**Figure 4B**). Additionally, several different *S. cerevisiae* lineages (e.g., wine, sake, etc.) have more CN variation but less SNP variation than the sister species, *S. paradoxus,* further highlighting the importance of CN variation to *S. cerevisiae* genome evolution (Bergstrom et al., 2014). Interestingly, *S. cerevisiae* CN variants are not evenly distributed across the genome, but tend to occur most frequently within subtelomeric regions (Dunn et al., 2012; Bergstrom et al., 2014). For example, across 132 wine yeast strains, 46 and 67% of the most CN diverse loci and genes, respectively, are observed in the subtelomeric regions (Steenwyk and Rokas, 2017).

How CN variants influence gene expression and phenotype in fungi is not well known. Examination of the contribution of CN variants to gene expression and phenotypic variation in *S. pombe* shows that partial aneuploidies (i.e., large CN variants) influence both local and global gene expression (Chikashige et al., 2007); in addition, CN variants are positively correlated with gene expression changes ($r_s = 0.71$; $p = 0.01$; Spearman rank correlation; reported in Jeffares et al., 2017). Genome-wide association analyses of numerous phenotypes in *S. pombe* showed that structural variants accounted for 11% of phenotypic variation (CN variants accounted for 7% of that variation and rearrangements for 4%; Jeffares et al., 2017). The phenotypes significantly influenced by CN variants included growth rate, growth in various free amino acids (e.g., tryptophan, isoleucine), growth in the presence of various stressors (e.g., hydrogen peroxide, ultraviolet radiation, minimal media), and sugar utilization in winemaking (Jeffares et al., 2017). However, how much of the phenotypic impact of CN variants is due to genetic drift or adaptation remains largely unknown. Functional analyses of single genes have provided some insight for adaptive CN variants. For example, in *S. cerevisiae,* CN variants have been shown to influence ecologically-relevant phenotypes; *CUP1* duplications have been repeatedly associated with resistance to copper (Fogel and Welch, 1982; Strope et al., 2015) and duplications in the *MAL* loci, which facilitate the utilization of maltose, the main carbon source during beer fermentation and present in sake fermentations, are frequently observed among beer and sake yeast strains, (Vidgren et al., 2005; Gallone et al., 2016; Gonçalves et al., 2016).

Although more studies are needed, these findings argue that CN variation may be a substantial contributor to the total genetic

and phenotypic variation of fungal populations. Additionally, the variation in the correlation between CN and SNP variation across fungal populations (**Figure 4**) suggests that levels of SNP variation are not always a good proxy for levels of CN variation.

COPY NUMBER VARIATION AND ITS IMPACT ON WINE YEAST ADAPTATION IN FERMENTATION-RELATED PROCESSES

During the wine making process, *S. cerevisiae* yeasts are barraged with numerous stressors such as high acidity, ethanol, osmolarity, sulfites, and low levels of oxygen and nutrient availability (Marsit and Dequin, 2015). Not surprisingly, *S. cerevisiae* strains isolated from wine making environments tend to be more robust to acid, copper, and sulfite stressors than yeasts isolated from beer and sake environments (Gallone et al., 2016). These biological differences are, at least partially, explained by variants, including CN variants, found at different frequencies or uniquely in wine yeasts. Although it is not known whether most of these CN variant differences are driven by natural selection or genetic drift, CN variation in several cases is associated with ecologically-relevant genes and traits. Below, we discuss what is known about the CN profile of genes from *S. cerevisiae* wine yeast strains associated with these stressors that may reflect diversity in stress tolerance or metabolic capacity and efficiency (**Figure 5**).

CN Variable Genes Related to Stress

Many of the CN variable genes that have been identified among wine strains of *S. cerevisiae* (Ibáñez et al., 2014; Gallone et al., 2016; Gonçalves et al., 2016; Steenwyk and Rokas, 2017) are associated with fermentation processes (**Table 1**), which supports the hypothesis that CN variation plays a significant role in microbial domestication (Gibbons and Rinker, 2015). For example, *CUP1* is commonly duplicated among wine yeast strains, but not among yeasts in the closely related natural oak lineage (Almeida et al., 2015; Strope et al., 2015). Duplications in *CUP1* have been shown to confer copper resistance (Warringer et al., 2011) and their occurrence in wine yeast strains may have been driven by the human use of copper as a fungicide to combat powdery mildews in vineyards since the 1800's (Fay et al., 2004; Almeida et al., 2015).

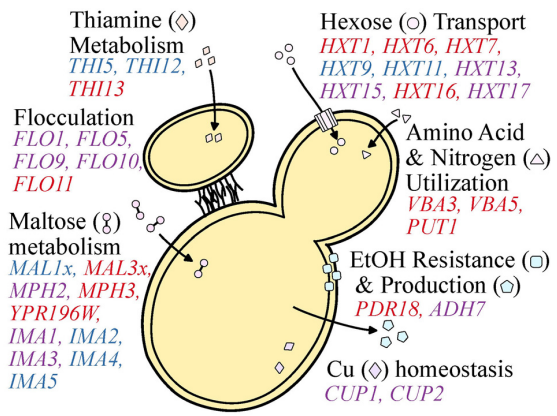


FIGURE 5 | Copy number variable genes that affect functions important to wine making. Functional categories (e.g., Cu and Fe homeostasis, maltose metabolism, etc.) are shown in black font. Genes of interest are shown proximal to the category described and are colored blue, red, or purple to represent a gene observed to be primarily deleted, duplicated, or both across populations and studies investigating *S. cerevisiae* wine strains. Genes found to be both duplicated and deleted present an opportunity for oenologists to capitalize on standing genetic diversity to select for particular flavor profiles or yeast performance.

Wine yeasts have also evolved strategies that favor survival in the wine fermentation environment, such as flocculation. This aggregation of yeast cells is associated with escape from hypoxic conditions, as it promotes floating and reaching the air-liquid interface where oxidative metabolism is possible (Martínez et al., 1997; Fidalgo et al., 2006). Flocculation is also favorable for oenologists as it facilitates yeast removal in post-processing (Soares, 2011) and is associated with the production of flavor enhancing ester-containing compounds (Pretorius, 2000). Flocculation is controlled by the *FLO* family of genes (Fidalgo et al., 2006; Govender et al., 2008). Examination of patterns of CN variation in *FLO* gene family members shows frequent duplications in *FLO11* as well as numerous duplications and deletions in *FLO1*, *FLO5*, *FLO9*, and *FLO10* (Gallone et al., 2016; Steenwyk and Rokas, 2017). Additionally, multiple independent studies have reported the GO terms CELL AGGREGATION (GO:0098743) and AGGREGATION OF UNICELLULAR ORGANISMS (GO:0098630) to be significantly enriched among CN variable genes in wine yeasts (Gallone et al., 2016; Steenwyk and Rokas, 2017). Interestingly, the same GO terms are only enriched among deleted genes in the beer and Asia/sake lineages (Gallone et al., 2016) suggesting these genes may be particularly important for wine yeasts. In fact, this has been demonstrated for "flor" or "sherry" yeasts, where partial duplications in the Serine/Threonine-rich hydrophobic region of *FLO11* are associated with the adaptive phenotype of floating to the air-liquid interface to access oxygen (Fidalgo et al., 2006). Furthermore, the same partial duplications have also been observed in the more general wine lineage (Steenwyk and Rokas, 2017), suggesting that the benefits associated with this phenotype may not be unique to "flor" yeasts.

Copy number variation is also observed in genes related to stuck (incomplete) or sluggish (delayed) fermentations. Stuck fermentations are caused by a multitude of factors including nitrogen availability, nutrient transport, and decreased resistance to starvation (Salmon, 1989; Thomsson et al., 2005). Two genes associated with decrease resistance to starvation, *ADH7* and *AAD3*, are sometimes duplicated or deleted among wine yeast strains (Steenwyk and Rokas, 2017). Diverse CN profiles of *ADH7*, an alcohol dehydrogenase that reduces acetaldehyde to ethanol during glucose fermentation, and *AAD3*, an aryl-alcohol dehydrogenase whose null mutant displays greater starvation sensitivity (Walker et al., 2014), suggest variable degrees of starvation sensitivity and therefore fermentation performance. Additionally, wine yeasts are enriched for duplication in *PDR18* (Gallone et al., 2016), a transporter that aids in resistance to

TABLE 1 | Genes associated with fermentation-related processes that exhibit CN variation among *Saccharomyces cerevisiae* wine strains.

Process (organized alphabetically)	Gene	Primarily duplicated, deleted, or both	References (organized by publication date)
Amino acid and nitrogen utilization	*VBA3, VBA5, PUT1*	Duplicated	Ibáñez et al., 2014; Gallone et al., 2016
Cu and Fe homeostasis	*CUP1, CUP2*	Both	Fay et al., 2004; Warringer et al., 2011; Almeida et al., 2015; Steenwyk and Rokas, 2017
	FIT2, FIT3, FRE3	Duplicated	Gallone et al., 2016
Ethanol resistance and production	*PDR18*		Gallone et al., 2016
	ADH7	Both	Steenwyk and Rokas, 2017
Flocculation	*FLO11*	Duplicated	Steenwyk and Rokas, 2017
	FLO1, FLO5, FLO9, FLO10	Both	Gallone et al., 2016; Steenwyk and Rokas, 2017
Hexose transport	*HXT1, HXT4, HXT6, HXT7, HXT16*	Duplicated	Gallone et al., 2016; Steenwyk and Rokas, 2017
	HXT9, HXT11	Deleted	Gallone et al., 2016; Steenwyk and Rokas, 2017
	HXT13, HXT15, HXT17	Both	Gallone et al., 2016; Steenwyk and Rokas, 2017
Maltose metabolism	*MAL3x, MPH3, YPR196W*	Duplicated	Gallone et al., 2016; Gonçalves et al., 2016; Steenwyk and Rokas, 2017
	MAL1x, IMA2, IMA4, IMA5	Deleted	Gallone et al., 2016; Gonçalves et al., 2016; Steenwyk and Rokas, 2017
	MPH2, IMA1, IMA3	Both	Gallone et al., 2016; Steenwyk and Rokas, 2017
Thiamine metabolism	*THI13*	Duplicated	Steenwyk and Rokas, 2017
	THI5, THI12	Deleted	Steenwyk and Rokas, 2017

ethanol stress, one of the traits that differentiates wine from other industrial strains. Another gene associated with decreased resistance to starvation that also exhibits CN variation is *IMA1* (Steenwyk and Rokas, 2017), a major isomaltase with glucosidase activity (Teste et al., 2010).

CN Variable Genes Related to Metabolism

Nutrient availability and acquisition is a major driving factor of wine fermentation outcome. Among the most important nutrients dictating the pace and success of wine fermentation is sugar availability (Marsit and Dequin, 2015). The most abundant fermentable hexose sugars in the wine environment include glucose and fructose (Marques et al., 2015), whose transport is largely carried out by genes from the hexose transporter (*HXT*) family (Boles and Hollenberg, 1997). A reproducible evolutionary outcome of yeasts exposed to glucose-limited environments, which are reflective of late wine fermentation, is duplication in the high-affinity hexose transporters, such as *HXT6* and *HXT7* (Brown et al., 1998; Dunham et al., 2002; Gresham et al., 2008, 2010), suggesting that changes in transporter CN are adaptive. Interestingly, GO terms such as HEXOSE TRANSMEMBRANE TRANSPORT (GO:0035428), GLUCOSE IMPORT (GO:0046323), and MONOSACCHARAIDE TRANSPORT (GO:0015749) are significantly enriched among duplicated CN variable genes in the wine lineage primarily due to duplications repeatedly observed in the *HXT* gene family among wine yeast strains (Dunn et al., 2012; Gallone et al., 2016; Steenwyk and Rokas, 2017). More specifically, *HXT13*, *HXT15*, and *HXT17* exhibit CN variation among wine strains, *HXT1, HXT6, HXT7,* and *HXT16* are more commonly duplicated, and *HXT9* and *HXT11* are more commonly deleted (Gallone et al., 2016; Steenwyk and Rokas, 2017).

Similarly striking patterns of CN variation are observed for genes associated with maltose metabolism (Gallone et al., 2016; Gonçalves et al., 2016; Steenwyk and Rokas, 2017). The two *MAL* loci in the reference genome of *S. cerevisiae* S288C, *MAL1,* and *MAL3*, that contain three genes which encode for a permease (*MALx1*), a maltase (*MALx2*), and a *trans*-activator (*MALx3*) (Michels et al., 1992; Naumov et al., 1994). The *MAL* loci are primarily associated with the metabolism of maltose (Michels et al., 1992), an abundant sugar during beer fermentation, and are commonly duplicated among beer yeast strains (Gallone et al., 2016; Gonçalves et al., 2016), however, this locus would be expected to be primarily deleted among wine yeasts as maltose is in relatively low abundance compared to other sugars during wine fermentation. As expected, MALTOSE METABOLIC PROCESS (GO:0000023) is among the significantly enriched GO terms across deleted genes in the wine yeast strains (Gallone et al., 2016) due to the deletion of the *MAL1* locus (Gallone et al., 2016; Gonçalves et al., 2016; Steenwyk and Rokas, 2017). In contrast, the *MAL3* locus is primarily duplicated among wine yeast strains (Gonçalves et al., 2016; Steenwyk and Rokas, 2017). Interestingly,

part of the *MAL3* locus, *MAL32,* has been demonstrated to be important for growth on turanose, maltotriose, and sucrose (Brown et al., 2010), which are present in the wine environment, albeit in small quantities (Victoria Moreno-Arribas and Carmen, 2013), suggesting potential function on secondary substrates or perhaps another function.

Equally important as sugar availability in determining fermentation outcome is nitrogen acquisition (Marsit and Dequin, 2015). Genes associated with amino acid and nitrogen utilization are commonly duplicated among wine yeast strains. Notable examples of such duplications are the amino acid permeases, *VBA3* and *VBA5* (Gallone et al., 2016), and *PUT1*, a gene that aids in the recycling or utilization of proline (Ibáñez et al., 2014).

Copy number variation is also observed in genes of the *THI* family, which are all involved in biosynthesis of hydroxymethylpyrimidine, a thiamine, or vitamin B_1, precursor (Rodríguez-Navarro et al., 2002; Wightman and Meacock, 2003; Li et al., 2010), another important determinant of wine fermentation outcome. Several *THI* gene family members are CN variable; *THI5* and *THI12* are typically deleted, while *THI13* is commonly duplicated (Steenwyk and Rokas, 2017). Expression of *THI5* is commonly repressed or absent in wine strains, as it is associated with an undesirable rotten-egg smell and taste in wine (Bartra et al., 2010; Brion et al., 2014). Interestingly, *THI5* is deleted in greater than 90% of examined wine strains (Steenwyk and Rokas, 2017) but is duplicated in several other strains of *S. cerevisiae*, as well as in its sister species *S. paradoxus* and the hybrid species *S. pastorianus* (Wightman and Meacock, 2003).

CONCLUSION AND PERSPECTIVES

An emerging body of work suggests that CN variation is an important, largely underappreciated, dimension of fungal genome biology and evolution (Hu et al., 2011; Farrer et al., 2013; Gallone et al., 2016; Gonçalves et al., 2016; Steenwyk et al., 2016; Hartmann and Croll, 2017; Steenwyk and Rokas, 2017). Not surprisingly, numerous questions remain unresolved. For example, we have detailed numerous mechanisms that lead to the generation of CN variation but the relative contribution of each remains unclear. Additionally, both the genomic organization and genetic architecture of CN variants remain largely unknown. For example, are duplicated copies typically found in the same genomic neighborhood or are they dispersed? Similarly, what percentage of phenotypic differences among fungal strains is explained by CN variation?

The same can be said about the role of CN variation in yeast adaptation to the wine fermentation environment. We still lack computational methods for distinguishing the footprint of natural selection and genetic drift on CN variation. Comparison of genome-wide patterns of CN variation among yeast populations responsible for the fermentation of different wines (e.g., white and red), coupled with functional studies, would provide insight to how human activity has shaped the genome of yeasts associated with particular types of wine. Additionally, most sequenced wine strains originate from Italy, Australia, or France. Genome sequencing of yeasts from underrepresented regions (e.g., Africa and the Americas) may provide further insight to CN variable loci unique to each region and the global diversity of wine yeast genomes.

Another major set of questions are associated with examining the impact of CN variable loci at the different stages of wine fermentation. Insights on how CN variable loci modify gene expression, protein abundance and in turn fermentation behavior and end-product would be immensely valuable. A complementary, perhaps more straightforward, approach would be focused on examining the phenotypic impact of single-gene or gene family CN variants, such as the ones discussed in previous sections (e.g., genes belonging to the *ADH*, *HXT, MAL*, and *VBA* families; **Table 1**) on fermentation outcome; this approach would also aid distinguishing adaptive and neutral CN variants. Such studies may provide an important bridge between scientist, oenologist, and wine-maker to enhance fermentation efficiency and consistency between batches or in the design of new wine flavor profiles.

Although this review focused solely on the contribution of *S. cerevisiae* CN variation, it is important to keep in mind that several other yeasts are also part of the wine fermentation environment. Members of many other wine yeast genera (e.g., *Hanseniaspora, Saccharomycodes,* and *Torulaspora*) are known to modify properties wine fermentation end product (Ciani and Maccarelli, 1998). Furthermore, recent sequencing projects have made several non-conventional wine yeast genomes publically available such as several *Hanseniaspora* species (Sternes et al., 2016; Seixas et al., 2017), *Starmerella bacillaris* (Lemos Junior et al., 2017), *Lachancea lanzarotensis* (Sarilar et al., 2015), and *Brettanomyces bruxellensis,* which has already been demonstrated to harbor CN variants (Curtin et al., 2012). In-depth sequencing of populations from these yeast species and others associated with wine will provide insight to niche specialization within the wine environment as well as greatly enhance our understanding of CN variation and its role in the ecology and evolution of fungal populations.

AUTHOR CONTRIBUTIONS

JS and AR chose the topic of the review and identified the areas that it would cover and the figures that it would contain. JS wrote the first draft of the manuscript and designed the figures. AR provided several rounds of extensive feedback on both the manuscript and the figures.

FUNDING

JS was supported by the Graduate Program in Biological Sciences at Vanderbilt University. Research in AR's laboratory was supported by the National Science Foundation (DEB-1442113), the Burroughs Wellcome Fund, and the March of Dimes Prematurity Research Center Ohio Collaborative.

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

frontiers in Microbiology

ORIGINAL RESEARCH
published: 07 May 2018
doi: 10.3389/fmicb.2018.00910

Co-evolution as Tool for Diversifying Flavor and Aroma Profiles of Wines

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The products of microbial metabolism form an integral part of human industry and have been shaped by evolutionary processes, accidentally and deliberately, for thousands of years. In the production of wine, a great many flavor and aroma compounds are produced by yeast species and are the targets of research for commercial breeding programs. Here we demonstrate how co-evolution with multiple species can generate novel interactions through serial co-culture in grape juice. We find that after ∼65 generations, co-evolved strains and strains evolved independently show significantly different growth aspects and exhibit significantly different metabolite profiles. We show significant impact of co-evolution of *Candida glabrata* and *Pichia kudriavzevii* on the production of metabolites that affect the flavor and aroma of experimental wines. While co-evolved strains do exhibit novel interactions that affect the reproductive success of interacting species, we found no evidence of cross-feeding behavior. Our findings yield promising avenues for developing commercial yeast strains by using co-evolution to diversify the metabolic output of target species without relying on genetic modification or breeding technologies. Such approaches open up exciting new possibilities for harnessing microbial co-evolution in areas of agriculture and food related research generally.

Keywords: wine yeast, co-evolution, metabolite analysis, microbial interactions, co-culture

OPEN ACCESS

Edited by:
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United States

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

Received: *25 December 2017*
Accepted: *19 April 2018*
Published: *07 May 2018*

Citation:
Morrison-Whittle P, Lee SA, Fedrizzi B and Goddard MR (2018) Co-evolution as Tool for Diversifying Flavor and Aroma Profiles of Wines. Front. Microbiol. 9:910. doi: 10.3389/fmicb.2018.00910

1. INTRODUCTION

For thousands of years humans have benefited from the products of microbial metabolism as they form the basis of all fermented foods and beverages (Blandino et al., 2003; Hutkins, 2007). In wine production, yeast species metabolize sugars and other compounds in grape juice and convert these into alcohol and a vast array of flavor and aroma compounds (Pretorius, 2000; Ciani et al., 2010). It is beneficial to have some control over the balance of desirable metabolites in the final wine, as this underpins the quality and value of finished wines. A large fraction of wine metabolites are produced by a variety of yeasts found naturally associated with grapes and their ferments, and yeast metabolism has been the subject of intensive research for many years (Pretorius, 2000; Ciani et al., 2010; Knight et al., 2015). Traditionally, harnessing desirable yeast metabolites has been achieved through breeding programs or by genetic modification of the main fermentative species: *Saccharomyces cerevisiae*. Over the last few years however, researchers have begun to explore the possibilities of altering the balance of flavor and aroma metabolites by inoculating more than one species of yeast into commercial ferments (Anfang et al., 2009; Ciani et al., 2010). Here we take this a step further and demonstrate the ability to harness microbial interactions using co-evolution as a means of diversifying and altering the metabolism of yeast species.

Microbial metabolism is influenced by a number of factors and classically, is understood to be largely a product how a microbe's genome interacts with its physical and chemical environment. Just as a microbe's genome will be subject to evolutionary change across multiple generations, so too will the manifestation of genome evolution on metabolism. A number of studies have demonstrated that microbial metabolism can significantly shift over a number of generations when grown consistently in controlled conditions (e.g., Fong et al., 2005; Gresham et al., 2008; Behe, 2010; Padfield et al., 2016). In addition to these adaptive shifts in metabolism in response to novel environments, the presence of other species may also alter the metabolism and evolutionary trajectories of bacterial species (Lawrence et al., 2012; Barraclough, 2015).

Species interactions have a profound effect on the evolution and ecological dynamics of biological species (Cadotte et al., 2008; Harmon et al., 2009; Bassar et al., 2010; Poltak and Cooper, 2011). These interactions may be broadly categorized as: antagonistic (competition, predation, ammensalism, and parasitism); neutral (such as commensalism); or mutualistic (such as cross-feeding)—as reviewed in West et al. (2007). The origin of these interactions through co-evolution has important consequences for overall metabolic regulation/flux (West et al., 2007). Lawrence et al. (2012) demonstrated the use of co-culture with serial transfers as a means of inducing co-evolution between bacterial species. In doing so, they demonstrated that bacteria independently and co-evolved showed significantly different reproductive success when subsequently co-cultured, that was consistent with evolved mutualistic cross-feeding behavior in co-evolved lines. Furthermore, the authors showed that these novel interactions were associated with significantly different patterns of metabolic regulation in co-evolved species.

In this study, we apply the experimental approach of Lawrence et al. (2012) to evaluate the evolution of novel microbial interactions between microbial eukaryotes: the grape and wine ferment associated yeasts *Candida glabrata* and *Pichia kudriavzevii*. We go on to quantify the impact of co-evolution on the production of 38 commercially important flavor and aroma compounds produced during experimental ferments with *S. cerevisiae*.

2. METHODS

2.1. Selection of Fungal Species

Initially 96 vineyard derived non-*Saccharomyces* isolates from our culture collection were grown in commercially harvested Sauvignon Blanc juice deriving from Marlborough, New Zealand. The SO_2 concentration of this juice, hereafter referred to as "juice A", was adjusted to 20 mg/L. Each non-*Saccharomyces* isolate was added to 200 μL of juice and incubated for 24 h. Isolates that grew readily (as measured by optical density) were then grown on yeast-extract peptone dextrose (YPD) agar to determine colony morphology. For ease of identification in co-cultures, isolates of different colony morphology were paired. All combinations of isolate pairs were then co-inoculated separately into juice A and incubated for either 24, 48, or 72 h. Ultimately, the final isolate pairs selected from all co-culture combinations were those that: (1) grew quickly in juice A over a 24-h period; and (2) grew at similar rates, yielding approximately equal numbers of colonies after spread-plating co-culture aliquots on YPD agar. Of these plated isolates, two colony phenotypes predominated, and were *Candida glabrata* and *Pichia kudriavzevii*. The identity of these isolates was confirmed by sequence homology of PCR amplicons at the D1/D2 26S rDNA locus using NL1 and NL4 primers (Kurtzman and Robnett, 2003; Romanelli et al., 2010).

2.2. Serial Transfers

Two experimental groups were initiated: "independently evolved" and "co-evolved". All independently evolved and co-evolved isolates of *C. glabrata* and *P. kudriavzevii* derived from a single colony of each species, and the ancestral isolates were stored in suspended animation at −80°C. The generation of all experimental strains are shown in **Figure 1**. Independently evolved lines were prepared by suspending *C. glabrata* and *P. kudriavzevii* in distilled water at equivalent optical densities, and 50 μL inoculated into seven wells (biological replicates) of 96-deepwell plates containing 200 μL of juice A for each. Distilled water was added to 200 μL of juice A in one well to act as a negative control for each plate.

Co-evolved lines were prepared by thorough vortex-mixing a 50:50 mix of the two species (See **Figure 1**). From this combined suspension, 50 μL was inoculated into 200 μL of juice A in seven wells of a 96-deepwell plate, and distilled water added to appropriate wells to act as a negative control. The two plates containing independently evolved lines and the single plate containing co-evolved lines were incubated at 28°C for 24 h. After 24 h, the contents of each well in each plate were mixed by pipetting, and 50 μL of each culture was transferred to 200 μL of fresh juice A, and incubated at 28°C for 24 h. This transfer procedure to fresh juice was repeated a total of 30 times to continuously grow independently evolved and co-evolved lines for ∼65 generations. Independently evolved and co-evolved isolates of *C. glabrata* and *P. kudriavzevii* were recovered after serial transfer by spread plating on YPD, from which single colonies were isolated and stored in15% (v/v) glycerol at −80°C (See **Figure 1**).

2.3. Growth Media

While the independently evolved and co-evolved lines of *C. glabrata* and *P. kudriavzevii* were evolved in juice A, downstream growth and metabolite assays were also carried out in a second juice B. Juice B was prepared as a blend from a number of other Sauvignon Blanc juice stocks donated by various commercial wineries. Conducting all analyses in two juices allowed us to test whether any significant differences between independently evolved and co-evolved lines were specific to the environment (juice) in which they evolved, or whether any evolved interactions were also expressed in different environments (juice chemistries). For both juices, 10 L of frozen juice was thawed prior to inoculation and sterilized at room temperature overnight using dimethyl dicarbonate (DMDC) in 25 L carboys. Each juice was mixed thoroughly and 200 mL was dispensed into sterilized 250 mL flasks with one-way airlocks 24 h prior to inoculation with *C. glabrata* and *P. kudriavzevii* strains.

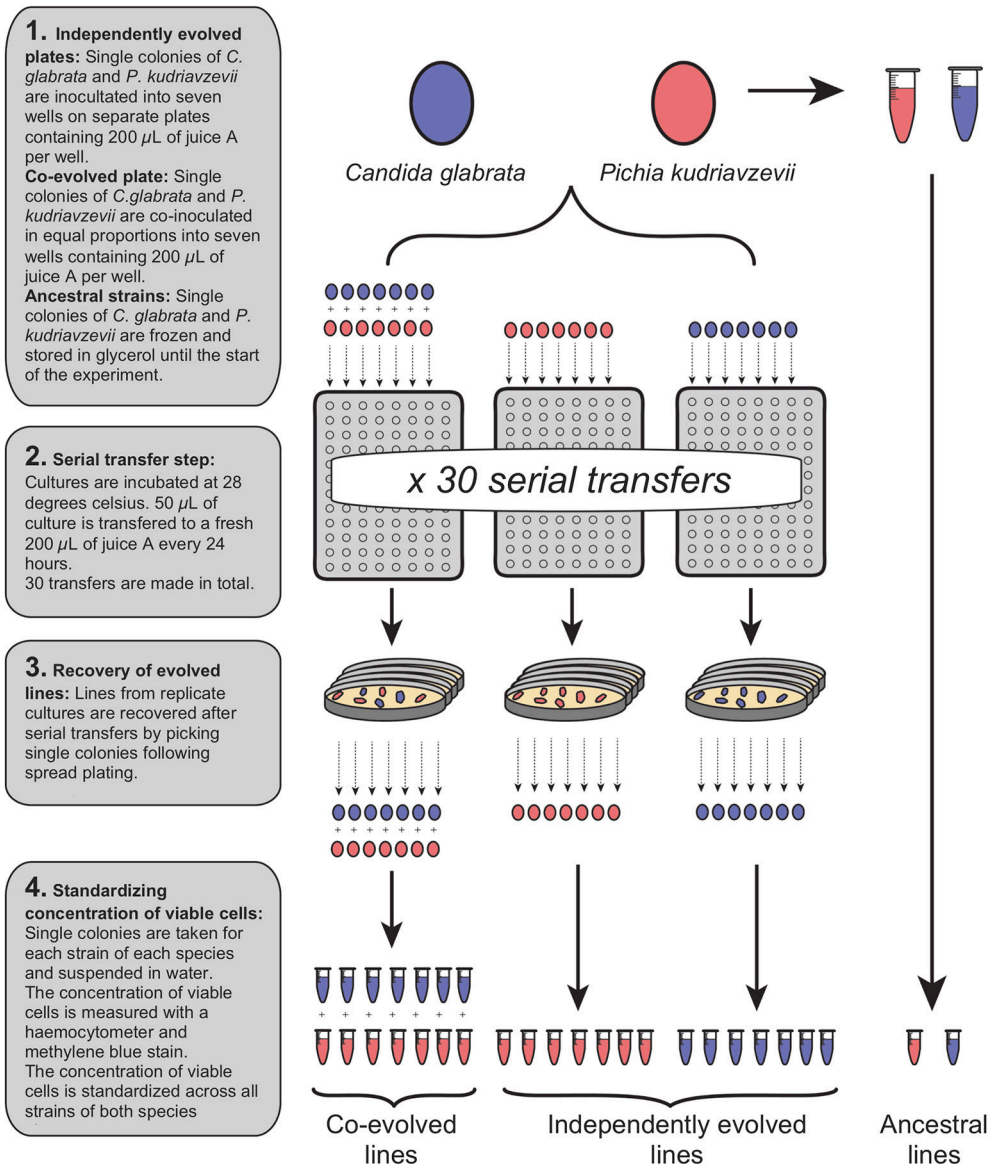

FIGURE 1 | Generation of independently evolved and co-evolved yeast lines from a single ancestral colony by serial transfer in fresh juice.

2.4. Flask Ferments

Prior to inoculation of juices A and B, *C. glabrata* and *P. kudriavzevii* were recovered from glycerol storage by growth in YPD, and each sample was then transferred to 50 mL Falcon tubes and pelleted at 3,000 g for 5 min. The resulting pellets were re-suspended in 10 mL of distilled water and transferred to fresh 15 mL Falcon tubes. The concentration of viable cells was enumerated using a haemocytometer with methylene blue staining solution. The concentration of viable cells was standardized to the sample with the lowest cell concentration. *C. glabrata* and *P. kudriavzevii* co-evolved strains were re-paired with their respective partner. Independently evolved strains of *C. glabrata* and *P. kudriavzevii* were paired arbitrarily. Flasks were co-inoculated by inoculating 1 mL of both *C. glabrata* and *P. kudriavzevii*, resulting in a final concentration of 2.52×10^5 cells mL^{-1} for both species (see **Figure 2**). All inoculated juice was then incubated for 50 h at 28°C after which all were inoculated with the same VL3 commercial strain of *S. cerevisiae* to a final concentration of 2.38×10^3 cells mL^{-1} to emulate a commercial situation and ferment to dryness. Flasks were incubated for 15 days at 28°C. After fermentation, cells were pelleted by centrifugation at 3,000 g for 5 min after which the supernatant was decanted and stored at −20°C for downstream juice metabolite analysis.

2.5. Bioscreen C™ Growth Assays

Relative fitness was estimated by maximum growth rate (V_{max}), lagtime, and cell-densities at 12, 24, and 48 h using Bioscreen C™

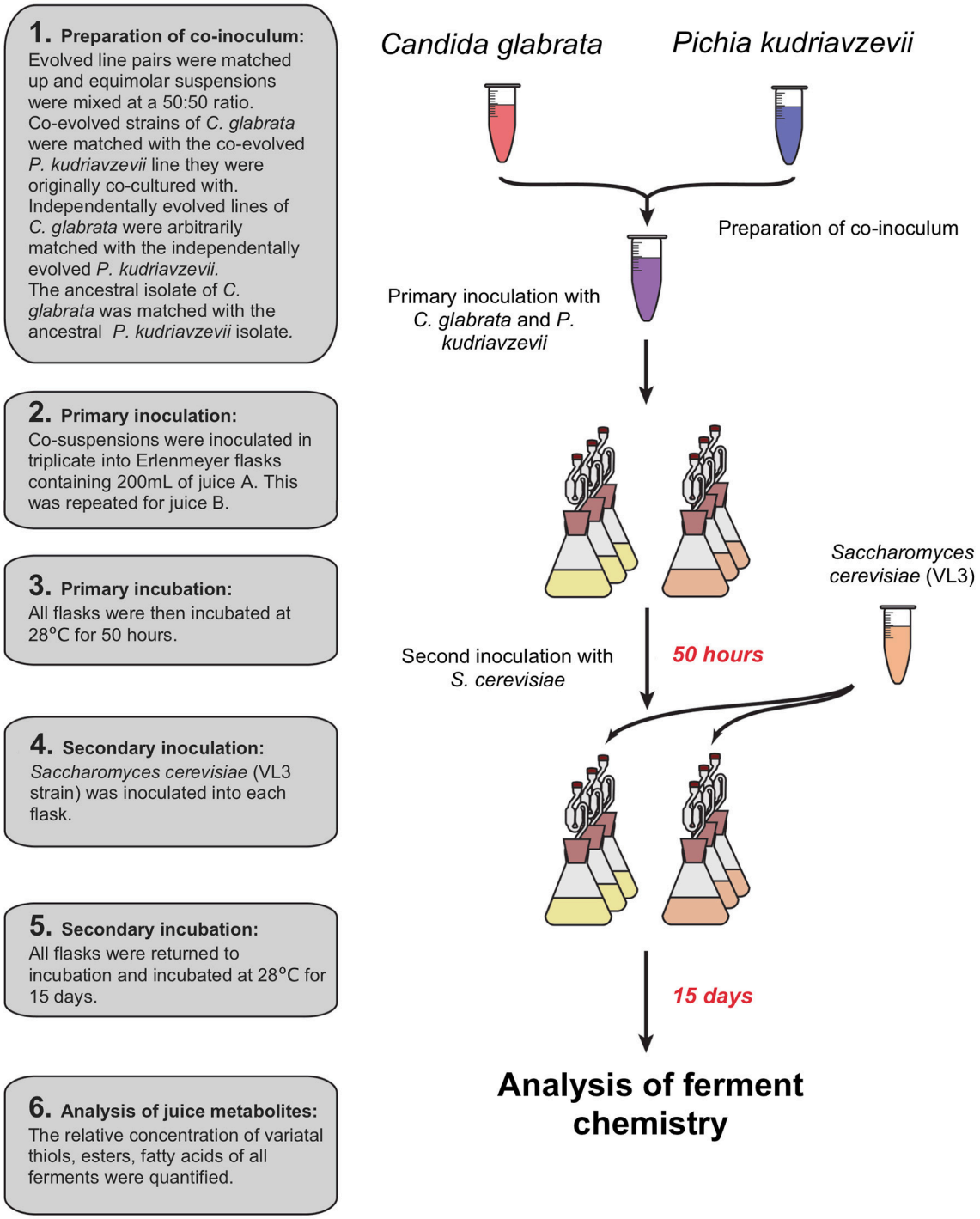

FIGURE 2 | Sequential inoculation of experimental ferments: primary inoculation of *C. glabrata* and *P. kudriavzevii* pairs then a secondary inoculation of *S. cerevisiae* after 50 h.

spectrophotometer/incubator. Maximum growth rate is defined as the maximum change in optical absorbance (wideband filter 420–580 nm) over a sliding 10 hr window; lagtime was defined as the time until a culture reached V_{max}, and cell density was approximated by optical absorbance. Each strain was grown from frozen glycerol stocks in liquid YPD for 24 h prior to analysis. The concentration of viable cells was enumerated using methylene blue stain and all samples were standardized to the sample of the lowest concentration of viable cells. 15 μL of each strain suspension was added to separate 100-well Bioscreen plates

(300 μL capacity) containing 185 μL of juice A and B separately. Each strain was inoculated into five technical replicates per treatment, producing a final concentration of 2.9×10^3 viable cells per well.

2.6. Metabolite Analysis of Co-inoculated Ferments

To evaluate metabolic output, the relative concentrations of two varietal thiols, fifteen esters, six higher alcohols, four C6 compounds, six terpenes, and five fatty acids of all ferments were quantified following the method described in Knight et al. (2015). Varietal thiols (3MH, 3MHA) were quantified using an ethyl propiolate derivatization and analyzed on an Agilent 6890N gas chromatograph (Santa Clara, CA, USA) equipped with a 7683B automatic liquid sampler, a G2614A autosampler and a 593 mass selective detector as outlined in Herbst-Johnstone et al. (2013b). Esters, alcohols, C6 compounds, terpenes, and fatty acids were quantified simultaneously using a HS-SPME/GC-MS method outlined in Herbst-Johnstone et al. (2013a). Raw data was transformed with GCMSD Translator and peak integration was performed using MS Quantitative Analysis, both part of the Agilent MassHunter Workstation Software (Version B.04.00, Agilent Technologies).

2.7. Species Identification/Contamination Controls

DNA from single colonies was extracted using Zymo Soil DNA extraction kits (Irvine, CA, USA), and species identity confirmed through Sanger sequencing of the D1/D2 region of the 26S rDNA using NL1 and NL4 fungal primers (Kurtzman and Robnett, 2003). One isolate recovered from the co-evolved *C. glabrata* serial-transfer plate could not be amplified using fungal primers and appeared to be a bacterial contaminant. All ferment and Bioscreen samples that contained this contaminant were excluded from all analysis.

2.8. Statistical Analysis

To test whether independently evolved and co-evolved lines had significantly different growth rates and cell-densities, separate one-way full factorial ANOVAs were conducted for each juice, testing: maximum growth rate (V_{max}); lagtime; and cell densities at 12, 24, and 48 h after inoculation. To test whether the metabolic profiles of independently evolved and co-evolved lines significantly differed from each other, we implemented two-way full factorial permutational multivariate ANOVA (permanova) of Jaccard dissimilarities between metabolite profiles. Separate tests were conducted for: esters, fatty acids, terpenes, C6 compounds, and all metabolites combined.

3. RESULTS

3.1. Relative Fitness of Evolved *C. glabrata* and *P. kudriavzevii*

The evolution of interactions between microbes may be manifest in differential reproductive success (or fitness). Antagonistic interactions are predicted to lower the net reproductive

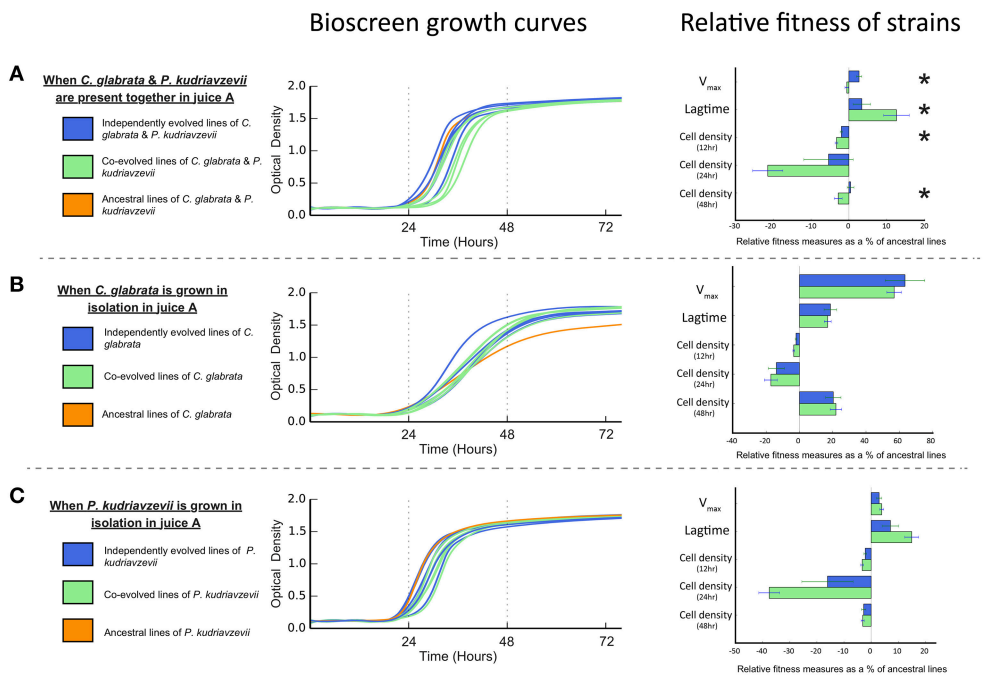

FIGURE 3 | Bioscreen growth curves and relative fitness measures in juice A of co-evolved, independently evolved, and ancestral lines of **(A)** *C. glabrata* and *P. kudriavzevii* when grown together. **(B)** *C. glabrata* when grown in isolation. **(C)** *P. kudriavzevii* when grown in isolation. Relative fitness measures—V_{max}, lagtime, and cell densities—are expressed as the proportional difference between evolved lines and the ancestral line. Significant differences between co-evolved and independently evolved strains are denoted by "∗".

success when in co-culture (Lawrence et al., 2012). Conversely, mutualistic interactions, such as cross-feeding, are predicted to increase the net reproductive co-culture success (Lawrence et al., 2012). Bioscreen analyses show co-cultures of co-evolved *C. glabrata* and *P. kudriavzevii* pairings have significantly lower net V_{max} than co-cultures of individually evolved strains [$F_{(1, 9)} = 13.266$, $P = 0.005382$; see **Figure 3A**]. Moreover, the lagtime of co-evolved co-cultures was significantly greater than individually evolved co-cultures [$F_{(1, 9)} = 5.2517$, $P = 0.04765$], which reached peak growth rate roughly 2 h sooner. This difference in net growth rate between co-evolved and individually evolved strain pairs resulted in co-cultures having lower cell densities after 12 h [$F_{(1, 9)} = 7.2366$, $P = 0.02479$] and 48 h [$F_{(1, 9)} = 5.7289$, $P = 0.04032$], but this difference was not significant at 24 h [$F_{(1, 9)} = 3.9378$, $P = 0.0785$].

Lawrence et al. (2012) found that cross-feeding behavior between co-evolved bacterial species represented an adaptive trade-off, as co-evolved strains had significantly lower fitness than independently evolved equivalents when grown in isolation. To test whether such an adaptive trade-off was apparent in these microbial eukaryotes, we measured the growth rates and cell densities of both individually evolved and co-evolved *C. glabrata* and *P. kudriavzevii* when grown in isolation. Co-evolved strains of *C. glabrata* were just as fit as individually evolved lines (as shown in **Figure 3B**) when grown in isolation, as there was no significant difference in V_{max} or lagtime [V_{max}: $F_{(1, 9)} = 0.2244$, $P = 0.647$; lagtime: $F_{(1,9)} = 0.125$, $P = 0.7318$. We found no significant difference between the cell densities of co-evolved and individually evolved *C. glabrata* at 12, 24, and 48 h [12 h: $F_{(1, 9)} = 3.5403$, $P = 0.09258$; 24 h: $F_{(1, 9)} = 0.2687$, $P = 0.6167$; 48 h: $F_{(1, 9)} = 0.0712$, $P = 0.7956$]. Co-evolved *P. kudriavzevii* were equally as fit as individually evolved lines (see **Figure 3C**), with no significantly difference in V_{max} [$F_{(1, 9)} = 0.5513$, $P = 0.4767$] or lagtime [$F_{(1, 9)} = 3.6597$, $P = 0.08803$]. As was observed with *C. glabrata*, co-evolved and individually evolved *P. kudriavzevii* did not have significantly different cell-densities at 12, 24, or 48 h [12 h: $F_{(1, 9)} = 2.2205$, $P = 0.1704$; 24 h: $F_{(1, 9)} = 3.808$, $P = 0.08278$; 48 h: $F_{(1, 9)} = 0.1226$, $P = 0.7343$]. The fact that cell densities are equivalent between individually and co-evolved lines for each species shows that these populations have expanded to the same extents at each cycle. Thus, both treatments (individually and co-evolved) experienced approximately the same number of generations across the experiment for *C. glabrata* and *P. kudriavzevii*.

3.2. The Relative Fitness of Evolved Strains Across Juices

Co-evolved lines displayed decreased fitness compared to individually evolved lines for some fitness components when subsequently co-cultured in a different juice (see Figures S1, S5, S6). Co-evolved lines had significantly longer lagtimes than individually evolved lines [$F_{(1, 9)} = 11.729$, $P = 0.007571$], but they did not have significantly different V_{max} [$F_{(1, 9)} = 3.1321$, $P = 0.1105$] in juice B. Co-evolved lines showed significantly lower cell densities than individually evolved lines at 12 h [V_{max}: $F_{(1,9)} = 9.3651$, $P = 0.01357$], but not at 24 or 48-h [24 h: $F_{(1, 9)} = 0.7227$, $P = 0.4173$; 48 h: $F_{(1, 9)} = 4.5186$, $P = 0.06246$] in juice B.

There was no significant difference in growth rate or cell density (see Figure S1B) between co-evolved and individually evolved lines of *C. glabrata* in juice B. However, co-evolved *P. kudriavzevii* were less fit than individually evolved in juice B for some fitness components (see Figure S1C): co-evolved lines showed no significant difference in V_{max} or lagtime [V_{max}: $F_{(1,9)} = 0.903$, $P = 0.3668$; lagtime: $F_{(1, 9)} = 0.7421$, $P = 0.4113$], but did show significantly lower cell densities than individually evolved lines in Juice B at 12 h [$F_{(1, 9)} = 19.428$, $P = 0.001701$], but not at 24 and 48 h [24 h: $F_{(1, 9)} = 1.1411$, $P = 0.3132$; 48 h: $F_{(1, 9)} = 1.1933$, $P = 0.303$].

3.3. The Evolution of Metabolite Profiles

We quantified the relative abundance of 38 metabolites in wine fermented by the variously treated *C. glabrata* and *P. kudriavzevii* lines, along with VL3. Both juice-type and evolution status significantly affected the overall metabolite profiles as indicated by two-way permanova analysis of Jaccard dissimilarities (**Table 1**), but there was no significant interaction between these ($R^2 = 0.0128$, $P = 0.6279$). This difference persists when broken down into major metabolic groups, but with varying levels of significance: esters (Juice: $R^2 = 0.2210$, $P = 0.0121$; strain status: $R^2 = 0.1508$, $P = 0.0334$); C6 compounds (Juice: $R^2 = 0.7839$, $P < 0.0001$; strain status: $R^2 = 0.0375$, $P = 0.0309$); and terpenes (Juice: $R^2 = 0.5273$, $P < 0.0001$; strain status: $R^2 = 0.0882$, $P = 0.0203$)—see Table S1. Of all classes, co-evolution had the greatest effect on ester profiles, where co-evolution induced approximately two-thirds the magnitude of the effect of juice in determining changes of esters profiles (Esters—effect of juice: $R^2 = 0.221$; effect of evolution status: $R^2 = 0.151$).

Overall, the effect of environment (juice) explains three times the variation than whether isolates were co-evolved or individually evolved, but the effect of evolution status is significant, and these are displayed in multidimensional scaling plots (see **Figure 4** and Figures S2–S4). Evolution status significantly impacted the metabolite profiles overall, and analyses of individual metabolite concentrations indicate a number of compounds which drive this difference, particularly

TABLE 1 | Results of Permutation ANOVA of Jaccard dissimilarities between overall metabolite profiles using independently evolved and co-evolved lines across two juices (9,999 permutations).

Effect	*df*	*SS*	*MS*	F_{pseudo}	R^2	*P*
Juice	1	0.62054	0.62054	14.4233	0.38460	0.0005
Culture status	1	0.19789	0.19789	4.5995	0.12265	0.0222
Interaction	1	0.02063	0.02063	0.4795	0.01279	0.6279
Residuals	18	0.77442	0.04302		0.47997	
Total	21	1.61347			1.00000	

df, degrees of freedom; SS, sum-of-squares; MS, mean sum-of-squares; F_{pseudo}, pseudo F-statistic; R^2, R-squared value; P, p-value.

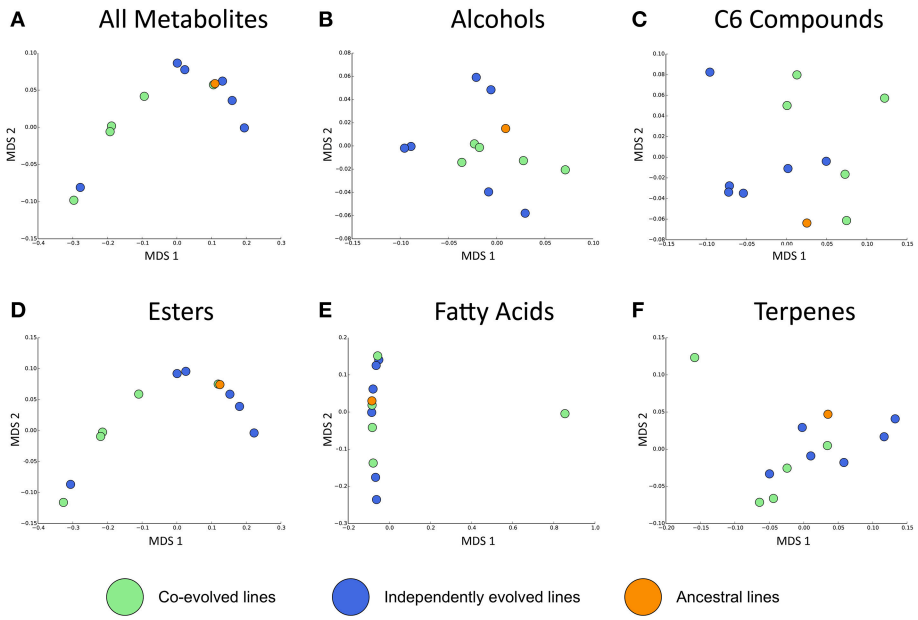

FIGURE 4 | Jaccard dissimilarities of metabolite profiles—averaged across technical replicates—for **(A)** All metabolites, **(B)** Higher alcohols, **(C)** C6 compounds, **(D)** Esters, **(E)** Fatty acids, **(F)** Terpenes in Juice A.

increases in trans-2-hexenal and decanoic acid, and decreases in 3 MH and ethyl phenylacetate. The relative abundances of each compound in derived lines compared to ancestral lines is shown in (**Figure 5** and Figure S7).

4. DISCUSSION

We found that after ∼65 generations of co-culture in Sauvignon Blanc juice, *C. glabrata* or *P. kudriavzevii* appear to have co-evolved, and that this co-evolution has significantly shifted the balance and composition of many of the flavor and aroma compounds we quantified. This study demonstrates the use of co-evolution as a means of diversifying the metabolic products of commercially important microbes. To the best of our knowledge, this is the first time that the evolution of microbial interactions in the lab has been shown to significantly modify the metabolite profiles of experimental wine ferments.

Contrary to the findings of Lawrence et al. (2012) we did not find evidence of the evolution of cross-feeding indicating the evolution of mutualistic interactions. Instead we see co-evolved strains of *C. glabrata* and *P. kudriavzevii* display lower V_{max} and cell densities than independently evolved strains. The reduced fitness of the co-evolved strains when grown together is consistent with antagonistic interactions between species that appear absent in independently evolved equivalents. When interactions between microbes are antagonistic, chemical energy available for reproduction is reduced by the metabolic costs of stress responses elicited by other microbes or on producing metabolites that reduces the reproductive success of other microbes.

One important consideration of utilizing co-evolution to alter microbial metabolism is generation time. As the number of generations increases, so does the likelihood that the phenotype of different evolutionary lines will diverge from one another. It is possible that the apparently antagonistic interaction between co-evolved *C. glabrata* and *P. kudriavzevii* may not represent a stable evolutionary state, and may intensify or change entirely given more generation time. A number of studies of experimental co-cultures have reported that the nature of microbial interactions do change over time (Poltak and Cooper, 2011; Andrade-Domínguez et al., 2014); some become increasingly mutualistic, others increasingly antagonistic. This phenotypic variation through time further increases the pool of yeast phenotypes from which strains can be selected and bred from as transitional phenotypes can be archived in glycerol storage.

It is important to note that this experimental design does not resolve whether the up-regulation or down-regulation of any one compound is a result of adaptation to other members of co-culture. Metabolic traits may not be adaptive in themselves but may covary with traits that are through gene linkage (Gould and Lewontin, 1979). Furthermore, our experimental design does not allow us to determine what species is driving the abundance of any one metabolite. For example, it is unclear whether *C. glabrata* or *P. kudriavzevii* directly affect the concentration of sensory compounds (by producing or metabolizing them) or whether they affect them indirectly by altering the metabolism of one or more co-fermenting partners. What this study does show is that the co-evolution of yeast strains naturally present on fruits and their ferments may be employed to manipulate the products of commercial fermentation.

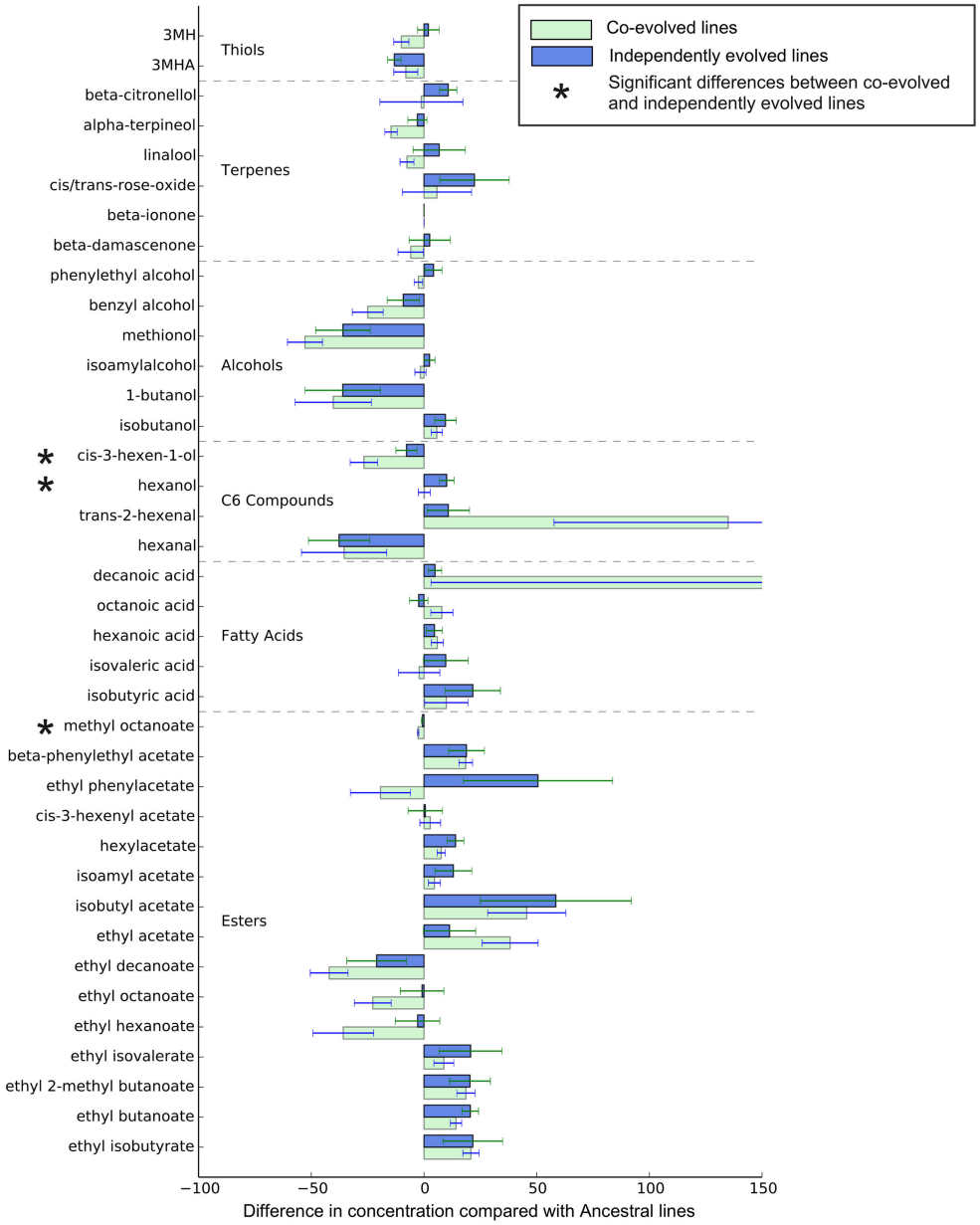

FIGURE 5 | Relative metabolite concentrations of co-evolved and independently evolved lines in Juice A—the juice they were evolved in. The concentrations of metabolites are the proportional difference in concentration compared to the ancestral line.

Another key consideration for this study is species number. In this study we report on interactions between two species, but it should be noted that the complexity and nature of microbial interactions can differ dramatically depending on the numbers present in co-culture (Barraclough, 2015; Fiegna et al., 2015). Lawrence et al. (2012) used 4 bacterial species in a simulated community and detected evidence of mutualistic co-evolution. Fiegna et al. (2015) found in experimentally assembled biofilm communities that species interactions evolved to be less negative over time, particularly in diverse communities. It seems reasonable to suggest that the nature and impact of microbial interactions on metabolite profiles may vary depending on the number and types of yeast species used. This complexity greatly enhances the potential for commercial researchers to generate a vast number of possible phenotypes—and subsequently, flavor and aroma profiles—by co-evolving a small number of yeasts in different combinations.

It should also be noted that antagonistic, neutral, or mutualistic microbial interactions do not predict whether the interaction is commercially valuable. The value of any microbial interaction in changing the metabolite profiles of any commercially valuable microbe depends on what metabolite

profile is considered desirable. Inducing co-evolution between wine yeasts merely represents a tool for diversifying the metabolite output of prospective yeast species. By diversifying the possible phenotype of yeast species, one can increase the pool from which strains can be selected, bred from, or used directly.

Furthermore, while this study infers that serial co-culture significantly alters microbial metabolism as the result of evolutionary change, we did not quantify any sequence changes in the genomes after serial co-culture beyond Sanger sequencing of a single locus. Here we demonstrate that serial co-culture significantly altered microbial metabolism and that this metabolic variation was heritable and persisted in subsequent generations after the co-culture step. However, as we did not quantify and genetic change, we cannot exclude the possibility that the changes in microbial metabolism are a consequence of epigenetic changes and not changes in genomic sequences, but we can conclude these changes are heritable. We would argue that if natural selection for microbial interaction is driving the formation and maintenance of these genetic and/or epigenetic changes, then the genetic changes in the genome would be predicted given enough generations.

Co-evolution is a powerful mechanism with which researchers can diversify or differentiate the metabolic activity of scientifically and/or commercially important organisms. Interactions between yeasts in commercial ferments, whether coincidental or derived from co-evolution, undoubtedly play a role in shaping the sensory properties of many commercial wines, especially those produced by spontaneous fermentation of harvested grape juice. Fermentative foods represent a powerful model for dissecting processes of microbial community formation (Wolfe and Dutton, 2015). Here we demonstrate the potential for utilizing both biotic and abiotic pressures to diversify the metabolic activity of commercially valuable yeast species. This study provides a tentative insight into the commercial value of microbial co-evolution; the practical applications of controlling wine sensory properties are vast, and elucidating the many mechanisms of evolution opens up exciting new areas of agriculture and food related research generally.

AUTHOR CONTRIBUTIONS

PM-W conceived and design the overall experimental design, trialed and selected non-*Saccharomyces* yeast isolates, carried out the continuous co-culture design through serial transfers of co-culture to fresh media, extracted DNA from all yeast isolates carried out BioscreenTM analysis of growth rates and cell densities, prepared juices A and B for experimental fermentation, carried out experimental fermentation of juices in 200 ml flasks, prepared resultant wine samples for downstream metabolite analysis, statistically analyzed all data, prepared primary manuscript, and proof-read and prepared final manuscript. SL confirmed the species identity of experimental strains through Sanger sequencing of D1/D2 26S locus amplified by PCR using NL1 and NL4 primers. Soon also quantified the concentrations of variatal thiols, esters, terpenes, C6 compounds, higher alcohols and fatty acids present in wine samples derived from experimental fermentation. BF provided expertise with volatile measurements. MG assisted in the experimental design, and assisted in the preparation of the final manuscript.

FUNDING

This work was funded by grants to MG from the New Zealand Ministry Innovation and Employment, Plant and Food Research Ltd., and New Zealand Winegrowers.

ACKNOWLEDGMENTS

We thank Pernod Ricard Winemakers New Zealand who kindly donated the grape juice for this experiment, and Alexandria Leonard for her assistance in editing the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.00910/full#supplementary-material

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

frontiers in Microbiology

ORIGINAL RESEARCH
published: 11 April 2018
doi: 10.3389/fmicb.2018.00670

Aromatic Amino Acid-Derived Compounds Induce Morphological Changes and Modulate the Cell Growth of Wine Yeast Species

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Yeasts secrete a large diversity of compounds during alcoholic fermentation, which affect growth rates and developmental processes, like filamentous growth. Several compounds are produced during aromatic amino acid metabolism, including aromatic alcohols, serotonin, melatonin, and tryptamine. We evaluated the effects of these compounds on growth parameters in 16 different wine yeasts, including non-*Saccharomyces* wine strains, for which the effects of these compounds have not been well-defined. Serotonin, tryptamine, and tryptophol negatively influenced yeast growth, whereas phenylethanol and tyrosol specifically affected non-*Saccharomyces* strains. The effects of the aromatic alcohols were observed at concentrations commonly found in wines, suggesting a possible role in microbial interaction during wine fermentation. Additionally, we demonstrated that aromatic alcohols and ethanol are able to affect invasive and pseudohyphal growth in a manner dependent on nutrient availability. Some of these compounds showed strain-specific effects. These findings add to the understanding of the fermentation process and illustrate the diversity of metabolic communication that may occur among related species during metabolic processes.

Keywords: aromatic alcohols, serotonin, tryptamine, quorum sensing, pseudohyphal growth, non-*Saccharomyces*, invasive growth

OPEN ACCESS

Edited by:
Aline Lonvaud, Université de Bordeaux, France

Reviewed by:
Warren Albertin, Institut des Sciences de la Vigne et du Vin (ISVV), France
Hervé Alexandre, Université de Bourgogne, France

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

Received: *22 December 2017*
Accepted: *21 March 2018*
Published: *11 April 2018*

Citation:
González B, Vázquez J, Cullen PJ, Mas A, Beltran G and Torija M-J (2018) Aromatic Amino Acid-Derived Compounds Induce Morphological Changes and Modulate the Cell Growth of Wine Yeast Species. Front. Microbiol. 9:670. doi: 10.3389/fmicb.2018.00670

INTRODUCTION

Wine is produced by alcoholic fermentation, in which grape sugars are metabolized into ethanol by yeast. During grape ripening, the surfaces of berries are primarily colonized by non-*Saccharomyces* yeast, such as *Hanseniaspora*, *Starmerella* (sym *Candida*), *Hansenula*, or *Metschnikowia*. Microorganisms belonging to the *Saccharomyces* genus are present in low abundance and are difficult to detect in initial must (Ribéreau-Gayon et al., 2006). For this reason, during spontaneous fermentation, non-*Saccharomyces* yeasts are responsible for initiating alcoholic fermentation and are then out-competed by *S. cerevisiae* throughout fermentation (Heard and Fleet, 1988; Fleet, 2003; Ribéreau-Gayon et al., 2006). Traditionally, the low ethanol tolerance and competitiveness of non-*Saccharomyces* yeasts compared to *Saccharomyces* species (Ribéreau-Gayon et al., 2006) has resulted in a lack of interest in these yeast species for many years. However, recently, the importance of non-*Saccharomyces* strains in alcoholic fermentation has

become appreciated, particularly in terms of their contribution to wine aroma, during the early steps of fermentation. Indeed, these species have been reported to impact, sometimes positively, winemaking via the production of high amounts of aromatic compounds, such as aromatic alcohols, ethyl esters, and acetate esters (Romano et al., 2003; García et al., 2010; Jolly et al., 2014; Belda et al., 2017). Furthermore, these strains appear to be present throughout much of the fermentation process, although this finding has been neglected because such strains are difficult to culture (Millet and Lonvaud-Funel, 2000; Wang et al., 2015a, 2016).

Saccharomyces cerevisiae is a unicellular fungi that reproduce asexually by budding and is able to undergo filamentous growth to scavenge for nutrients (Wendland and Philippsen, 2001; Verstrepen and Klis, 2006; Cullen and Sprague, 2012). Filamentous growth includes morphological changes that involve the global reorganization of cellular processes to produce a new cell type. Cells alter their budding pattern, becoming more elongated and remaining attached to each other through the formation of pseudohyphae. Moreover, under certain conditions, yeast cells penetrate surfaces through a process known as invasive growth (Roberts and Fink, 1994). Although much of the genetic characterization of this response has been performed in *S. cerevisiae* strains on the Σ1278b background (Gimeno et al., 1992; Cullen and Sprague, 2000), the response has also been studied in many strains and genera (Gimeno and Fink, 1994; San-blas et al., 1997; Lo and Dranginis, 1998). For example, the human pathogen *Candida albicans* (Hornby et al., 2001; Chen et al., 2004; Biswas et al., 2007; Kruppa, 2009) undergoes pseudohyphal and hyphal growth (pathogenic form), which confers the ability to infect human tissues (Lo et al., 1997; Leberer et al., 2001; Rocha et al., 2001). Filamentous growth in yeasts has been reported to occur in response to cell density and several molecules, such as aromatic alcohols and ethanol, have been identified as stimuli that induce these morphological changes (Gimeno et al., 1992; Dickinson, 1996; Lorenz et al., 2000; González et al., 2017). Indeed, aromatic alcohols, tyrosol (TyrOH), tryptophol (TrpOH), and phenylethanol (PheOH), which are derived from the amino acids tyrosine, tryptophan, and phenylalanine, respectively, have been suggested to act as quorum sensing molecules (QSMs) in yeasts, regulating cell density and evoking morphogenetic transitions (Chen et al., 2004; Chen and Fink, 2006). Moreover, nitrogen limitation results in the increased production of aromatic alcohols, leading to elevated filamentous growth in *S. cerevisiae*. In this species, PheOH and TrpOH act as inducers of morphogenesis, while TyrOH has no detectable effects (Chen and Fink, 2006). However, in *C. albicans*, these alcohols exhibit the opposite behavior: TyrOH promotes pseudohyphal growth, whereas PheOH and TrpOH inhibit it. The finding that different aromatic alcohols exert different responses on morphogenesis depending on the yeast species implicates these molecules as inducers of species-specific effects (Chen and Fink, 2006). In a recent study, González et al. (2017) showed that ethanol specifically induced filamentous growth under nitrogen-limiting conditions, whereas aromatic alcohols did not. Thus, environmental conditions impact the efficacy of these compounds. Non-*Saccharomyces* yeasts, such as *Hanseniaspora uvarum*, *Pichia kudriavzevii*, and *Pichia fabianii*, undergo filamentous growth under nutrient-limited conditions (nitrogen or carbon) or in the presence of other stress factors (Pu et al., 2014; van Rijswijck et al., 2015), but the roles of these alcohols have not been extensively explored.

During alcoholic fermentation, yeast synthesizes compounds that, depending on the concentration, can be inhibitory to their own growth or the growth of other yeast species. A primary example is ethanol, which is a potent inhibitory compound for growth. Other metabolites, such as short-to-medium-chain fatty acids (e.g., acetic, hexanoic, octanoic, and decanoic acids) and yeast killer toxins, also inhibit growth and even induce the death of certain yeast species, including strains of *S. cerevisiae* (Pérez et al., 2001). Recently, interactions between species were shown to be impacted by the secretion of compounds by yeast during alcoholic fermentation (Ciani and Comitini, 2015; Wang et al., 2015b; Albergaria and Arneborg, 2016). To our knowledge, there have been no studies investigating the effects of aromatic alcohols or other QSMs synthesized during alcoholic fermentation on the growth and vitality of wine yeasts. Moreover, the effects of aromatic alcohols on the filamentous growth of non-*Saccharomyces* wine yeast species have not been explored. The investigation of these areas might help to unravel the possible roles of QSMs in the interactions between yeasts during alcoholic fermentation. Moreover, direct microbial interactions (i.e., through physical contact) are reportedly involved in the growth inhibition of non-*Saccharomyces* yeast, although such mechanisms are dependent on cell density, when cultures are competing for space (Nissen et al., 2003, 2004; Pérez-Nevado et al., 2006; Renault et al., 2013).

Additionally, through tryptophan metabolism, yeasts also produce other metabolites that are related to indoles, such as serotonin, melatonin, or tryptamine. Serotonin and melatonin are of special relevance for their bioactivity in higher organisms, including humans. Rodriguez-Naranjo et al. (2012) demonstrated that melatonin is produced during alcoholic fermentation by yeast, and different strains and species synthesize this compound at different concentrations. The role of melatonin in yeasts is still unclear, although a recent paper showed that the compound demonstrated possible antioxidant activity in response to oxidative damage by hydrogen peroxide in *S. cerevisiae* (Vázquez et al., 2017). On the other hand, tryptamine has also been detected in red wines at mg/L concentrations after malolactic fermentation (Wang et al., 2014). Serotonin appears to exert antifungal activity against *Candida* and *Aspergillus* spp. *in vitro* (Lass-Flörl et al., 2002, 2003).

Thus, the objective of this study was to evaluate the effects of different compounds derived from aromatic amino acid metabolism and produced during alcoholic fermentation on the growth and physiology of different wine yeast species. We first described an analysis of the growth parameters of different yeast strains and species in the presence of increasing concentrations of specific compounds of interest. Then, the effects of aromatic alcohols and ethanol, which are well-known morphogenesis inducers in *S. cerevisiae*, were examined for their impact on the filamentous growth of different non-*Saccharomyces* wine species.

MATERIALS AND METHODS

Strains and Growth Media

Eight strains from *Saccharomyces* species and two strains from four species of non-*Saccharomyces* yeast were used in the study. The *S. cerevisiae* strains included the laboratory strain Σ1278b, the wine strains SB (Marullo et al., 2007), QA23, T73, P5, and P24 (Lallemand, Canada), the animal nutrition strain Sc20 and the hybrid *S. kudriavzevii/S. cerevisiae* Vin7 (Oenobrands SAS, France) (Borneman et al., 2012). The non-*Saccharomyces* yeasts were *Starmerella bacillaris* (sym. *Candida zemplinina*) (Cz4-CECT13129, Cz11), *H. uvarum* (Hu4-CECT13130, Hu11), *Metschnikowia pulcherrima* (Mpp-CECT 13131, FLAVIA), and *Torulaspora delbrueckii* (Tdp-CECT 13135, BIODIVA). FLAVIA and BIODIVA are commercial strains (Lallemand, Canada) whereas the other non-*Saccharomyces* strains were isolated from grapes/wine media (Padilla et al., 2016). Yeasts were typically grown on YPD [2% (w/v) peptone, 1% (w/v) yeast extract, 2% (w/v) glucose, and 2% (w/v) agar] at 28°C.

Effects on Yeast Growth

Yeasts were pre-cultured for 48 h on minimal medium [(MM) 1x Yeast Nitrogen Base (YNB) without (w/o) amino acids (aa) or ammonium, 2% (w/v) glucose, and 10 mM $(NH_4)_2SO_4$ (280 mgN/L)] at 28°C and then inoculated into each medium, adjusting the initial optical density (OD_{600nm}) to 0.2. To evaluate the effects of nitrogen concentration, yeasts were grown on MM and on low nitrogen medium [(LNM) 1x YNB w/o aa or ammonium, 2% (w/v) glucose, and 1 mM $(NH_4)_2SO_4$ (28 mgN/L)]. Media were supplemented with increasing concentrations of melatonin (Mel), tryptamine (Trpm), serotonin (Ser), tyrosol (TyrOH), phenylethanol (PheOH), and tryptophol (TrpOH), ranging from 50 to 1000 mg/L. All assays were performed using a POLARstar Omega microplate reader (BMG LABTECH, Germany) and were performed in triplicate at 28°C for 48 h. Microplate wells were filled with 250 μL of inoculated media. A control well-containing medium without inoculum was used to determine the background signal. Measurements were taken every 30 min after pre-shaking the microplate for 30 s at 500 rpm. For each growth curve, the variables generation time (GT) and maximal growth (OD max) were calculated according to Warringer and Blomberg (2003). Briefly, for the GT determination, a slope was calculated between every second consecutive measurement for the whole growth curve (OD values were previously $\log_{10}$ transformed). Of the seven highest slopes, the highest two were discarded, and the mean for the following five was defined as maximum division rate. The GT was obtained dividing the $\log_{10}$ 2 by the maximum division rate. The lag phase was calculated using the program GrowthRates (Hall et al., 2014).

Statistical Data Processing

All experiments were performed in triplicate. The data was subjected to one-way analysis of variance (ANOVA), and Tukey's *post hoc* test (XLSTAT Software) was used to evaluate significant differences between the control condition (no addition) and the addition of each compound. The results were considered statistically significant at $p < 0.05$. For each compound, relative values were calculated using the condition in the absence of added compound (0 mg/L) as a control [(condition-control)/control]. To better understand the interactions between the calculated parameters and their effects on yeast growth, principal component analysis (PCA) was performed using XLSTAT Software at a concentration of 1000 mg/L for each compound and under both nitrogen conditions (MM and LNM) for all strains tested.

Filamentous Growth Assays

Yeast Strains, Media, and Growth Conditions

For the filamentous growth assay, two strains of each non-*Saccharomyces* species were tested, using the strain QA23 (*S. cerevisiae*) as a control (González et al., 2017). Yeasts were grown on minimal medium [MM – 1x YNB w/o aa or ammonium, 2% (w/v) glucose, and 10 mM $(NH_4)_2SO_4$] with agitation (120 rpm) for 16 h at 28°C before seeding on plates for filamentation analysis. To evaluate invasive and pseudohyphal growth, three different media were used, with variations in glucose and nitrogen concentrations: SAD – synthetic medium [1x YNB w/o aa or ammonium, 2% (w/v) glucose, and 37 mM $(NH_4)_2SO_4$ and 2% (w/v) agar], SALG – synthetic medium with low glucose [1x YNB w/o aa or ammonium, 0.5% (w/v) glucose, and 37 mM $(NH_4)_2SO_4$ and 2% (w/v) agar] (González et al., 2017), and SLAD – synthetic low-ammonium dextrose medium [SLAD – 1x YNB w/o aa or ammonium, 2% (w/v) glucose, and 50 μM $(NH_4)_2SO_4$ and 2% (w/v) agar]. To test the effects of aromatic alcohols, the above media were supplemented with 500 μM of TyrOH (6,90 mg/L), TrpOH (8,06 mg/L) or PheOH (6,10 mg/L) or 2% (v/v) ethanol. Those concentrations were chosen according our previous studies (González et al., 2017).

Invasive and Pseudohyphal Growth Assays

Cells pre-grown in MM for 16 h were harvested by centrifugation, washed once in sterile water, and adjusted to an OD_{600nm} of 2.0. Subsequently, 10 μl of cells were spotted in triplicate on semisolid agar media. Plates were incubated at 30°C for 3, 5, and 7 days depending on the experiment. Invasive growth was determined in a plate washing assay (Roberts and Fink, 1994). Colonies were photographed before and after the plates were washed in a stream of water, after which the colonies were rubbed from the surface with a gloved finger. ImageJ software[1] was used to quantitative invasive growth in the plate-washing assay. The background intensity was determined for each spot and subtracted from the densitometry of the invasive area. Densitometry analysis was performed on invasive patches over multiple days. The data was subjected to one-way ANOVA and Tukey's *post hoc* test (XLSTAT Software) was used to evaluate significant differences on invasion intensity between media. The results were considered statistically significant at $p < 0.05$. The examination of pseudohyphae was determined as described by Gimeno et al. (1992). Before washing the plates, the colony periphery was observed and photographed each day under microscopy (Raman FT-IR).

[1] http://rsb.info.nih.gov/ij/

RESULTS

Effects of the Presence of Aromatic Amino Acid-Derived Compounds on Yeast Growth

To evaluate the effects of amino acid-derived compounds on yeast growth, five strains of *S. cerevisiae* and one strain of each non-*Saccharomyces* species were grown in the presence of 1000 mg/L of Mel, Ser, Trpm, TyrOH, PheOH, or TrpOH. As these molecules are derived from nitrogen metabolism, and QSMs are produced during nutrient limitation, we tested their effects under two different nitrogen conditions: 1 and 10 mM $(NH_4)_2SO_4$ (**Figure 1**). As an example, the growth curves obtained with *S. cerevisiae* QA23 (**Figure 1A**) and *S. bacillaris* Cz4 (**Figure 1B**) in the presence of 1000 mg/L of the different compounds and 10 mM $(NH_4)_2SO_4$ are shown. In the QA23 strain, Ser completely inhibited cell growth. In addition to this dramatic phenotype, other subtle phenotypes were observed. TrpOH caused a reduction in growth rate and maximal growth, and Trpm increased the lag phase. The other compounds tested did not significantly affect the growth profile. In comparison, the growth of strain Cz4 was reduced by TrpOH and Trpm, but not by the other compounds. Therefore, different compounds cause the growth inhibition of different species.

The relative values of OD max (**Figure 1C**) and GT (**Figure 1D**) were calculated for each compound, using the condition without addition as a control (absolute values can be found in Supplementary Table S1). Overall, the addition of these compounds (with the exception of Mel) exerted negative impacts on the maximal growth obtained for most of the tested strains (**Figure 1C**). Ser decreased the OD max in all yeast species, particularly under low nitrogen conditions, while Trpm and aromatic alcohols had a major impact in non-*Saccharomyces* strains under both nitrogen conditions. On the other hand, Ser caused growth reduction in all strains, increasing their GT (**Figure 1D**). In general, this increase was significant for *Saccharomyces* strains under both nitrogen conditions but only under low nitrogen conditions for most non-*Saccharomyces* strains. Increases in GT were also observed when the medium was supplemented with TrpOH in all the non-S*accharomyces* strains under both nitrogen conditions. The other two aromatic alcohols, PheOH and TyrOH, exerted no effects in *Saccharomyces* strains, and at 1 mM, among non-*Saccharomyces* strains, only the Tdp strain was affected by PheOH, and *S. bacillaris* by TyrOH. In general, the relative OD max or GT presented a similar trend under both nitrogen conditions; the most relevant differences consisted of greater effects from Ser in the non-*Saccharomyces* strains under low nitrogen concentration. The effects of these compounds were impacted by exogenous nitrogen levels, although in a strain-dependent manner. The impact of ethanol on yeast growth was also analyzed, but no significant differences were observed at 1000 mg/L for any of the yeast species studied (data not shown). Based on these results, at high nitrogen concentration *Saccharomyces* and non-*Saccharomyces* strains clustered into two different groups in a PCA (Supplementary Figure S1A), primarily attributable to the higher reduction in the OD max on non-*Saccharomyces* strains due to the presence of aromatic alcohols and Trpm. Under low nitrogen conditions (Supplementary Figure S1B), all strains of *Saccharomyces* were included in the same cluster, but non-*Saccharomyces* strains were plotted into two different groups because *T. delbrueckii* clustered separately from the other non-*Saccharomyces* species, because of their higher GT in PheOH.

Effects of the Concentrations of Aromatic Amino Acid-Derived Compounds on Yeast Growth

According to our previous results, the effects of certain aromatic amino acid-derived compounds were slightly greater under low nitrogen conditions than under high nitrogen conditions. For this reason, we investigated how the increasing concentrations of these compounds (from 50 to 1000 mg/L) affect the growth of a larger collection of wine yeast in nitrogen-limiting conditions (absolute values of GT and maximal growth obtained for each strain and condition can be found in Supplementary Tables S2, S3).

When different concentrations of the metabolites were tested, we observed again that Ser (**Figure 2** and Supplementary Figure S2), TrpOH (**Figure 3** and Supplementary Figure S2), and Trpm (**Figure 4** and Supplementary Figure S2) exerted higher impacts on the cell growth of yeast strains and in some cases in the lag phase. On the other hand, TyrOH and PheOH only affected to the growth of non-*Saccharomyces* strains (Supplementary Figure S3), even at low concentrations (50 mg/L) in the case of PheOH.

The effects of different Ser concentrations on the *S. cerevisiae* Sc20 strain are shown as an example (**Figure 2A**). Clear inhibition of yeast growth was observed at concentrations of Ser above 500 mg/L, increasing GT and decreasing the OD max. Interestingly, GT and OD max values obtained in the presence of Ser were strongly correlated (R^2 0.8204), indicating that this compound influenced both growth parameters for most strains (**Figure 2B**). As shown in **Figure 3C**, all wine strains were affected by the presence of Ser in the medium, and the increase in GT was directly proportional to the Ser concentration, illustrating a dose-dependent effect. Instead, the laboratory strain Σ1278b was barely affected by this compound. Most *S. cerevisiae* strains showed growth inhibition starting from 250 mg/L, primarily in Vin7, T73, P5, and P24. Conversely, the strains of *S. bacillaris*, *H. uvarum*, and *M. pulcherrima* appeared to be more tolerant to this compound. On the other hand, *T. delbrueckii* presented a specific profile, as growth was only affected above 750 mg/L, but they exhibited the highest growth inhibition at 1000 mg/L. The effects of Ser on the relative OD max of the strains showed a profile similar to GT (Supplementary Figure S2).

For most strains, the addition of TrpOH caused a decrease in growth in a dose-dependent manner (see **Figure 3A** and Supplementary Figure S2). The presence of TrpOH had a greater impact on GT than on maximal growth (**Figure 3B**), particularly in non-*Saccharomyces* strains (**Figure 3C**). Among them, the most tolerant strain was *M. pulcherrima* Mpp, which was only slightly affected at high doses of TrpOH. Conversely, the other *M. pulcherrima* strain, FLAVIA, was one of the most

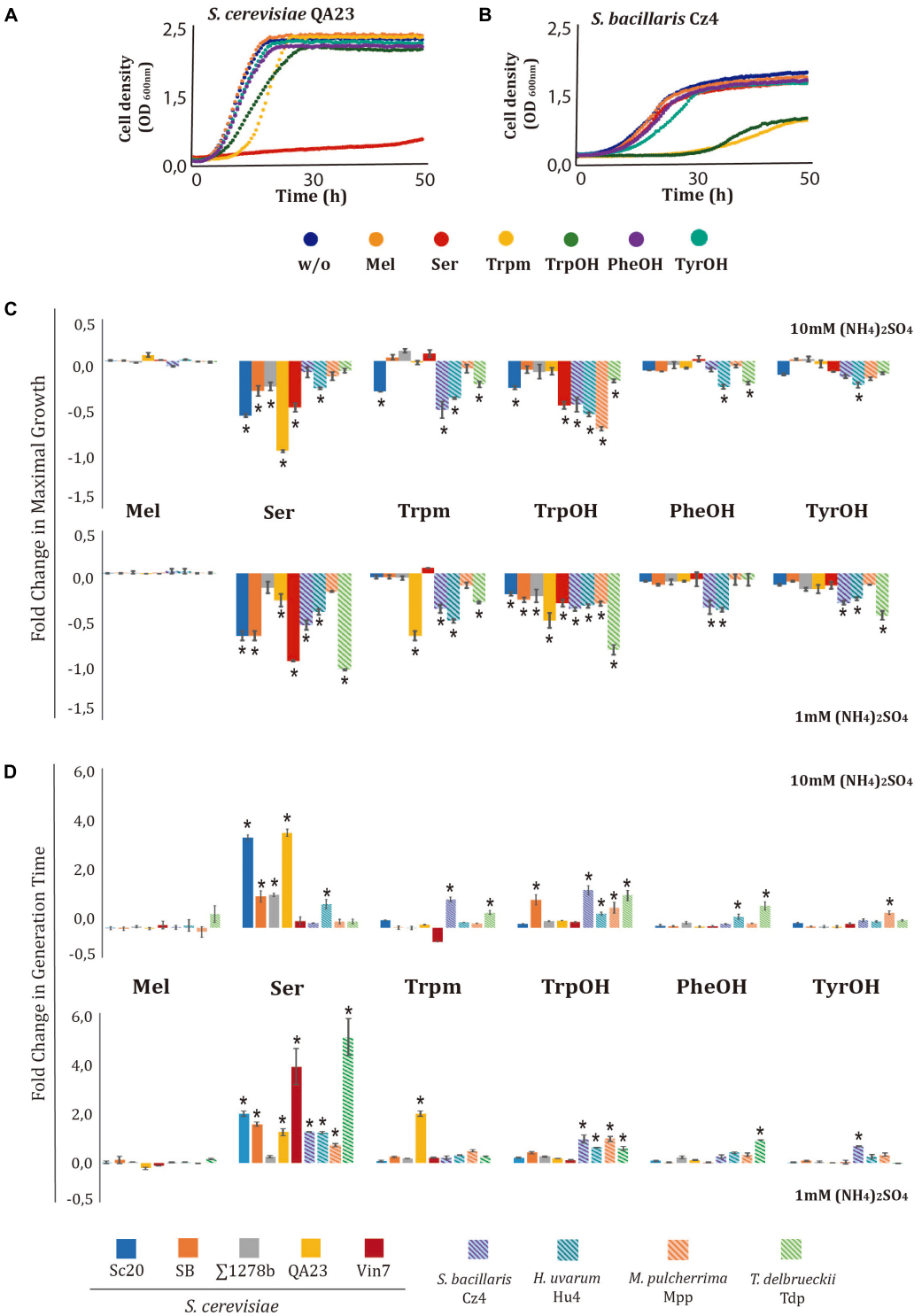

FIGURE 1 | Effects of aromatic amino acid-derived compounds on the growth of wine yeast species at different nitrogen concentrations. The effects of Mel, Ser, Trpm, TrpOH, PheOH, and TyrOH on the growth of four strains of *S. cerevisiae* and four of non-*Saccharomyces* were determined. Yeast were grown for 48 h at 28°C in minimal medium with two different nitrogen concentrations [10 mM or 1 mM $(NH_4)_2SO_4$] and supplemented with 1000 mg/L of each compound. Non-supplemented cultures were used as controls. Experiments were carried out in triplicate. Growth curves of *S. cerevisiae* QA23 **(A)** and *S. bacillaris* Cz4 **(B)**, with the different compounds added at 10 mM $(NH_4)_2SO_4$ medium are shown. For each nitrogen condition and compound, maximal growth **(C)**, and generation time **(D)** was calculated. The fold-change for each growth parameter was determined in relation to its control condition. Statistical analysis was performed using Tukey's test by comparing the effects of each compound in the different strains; asterisk denotes a p-value < 0.05.

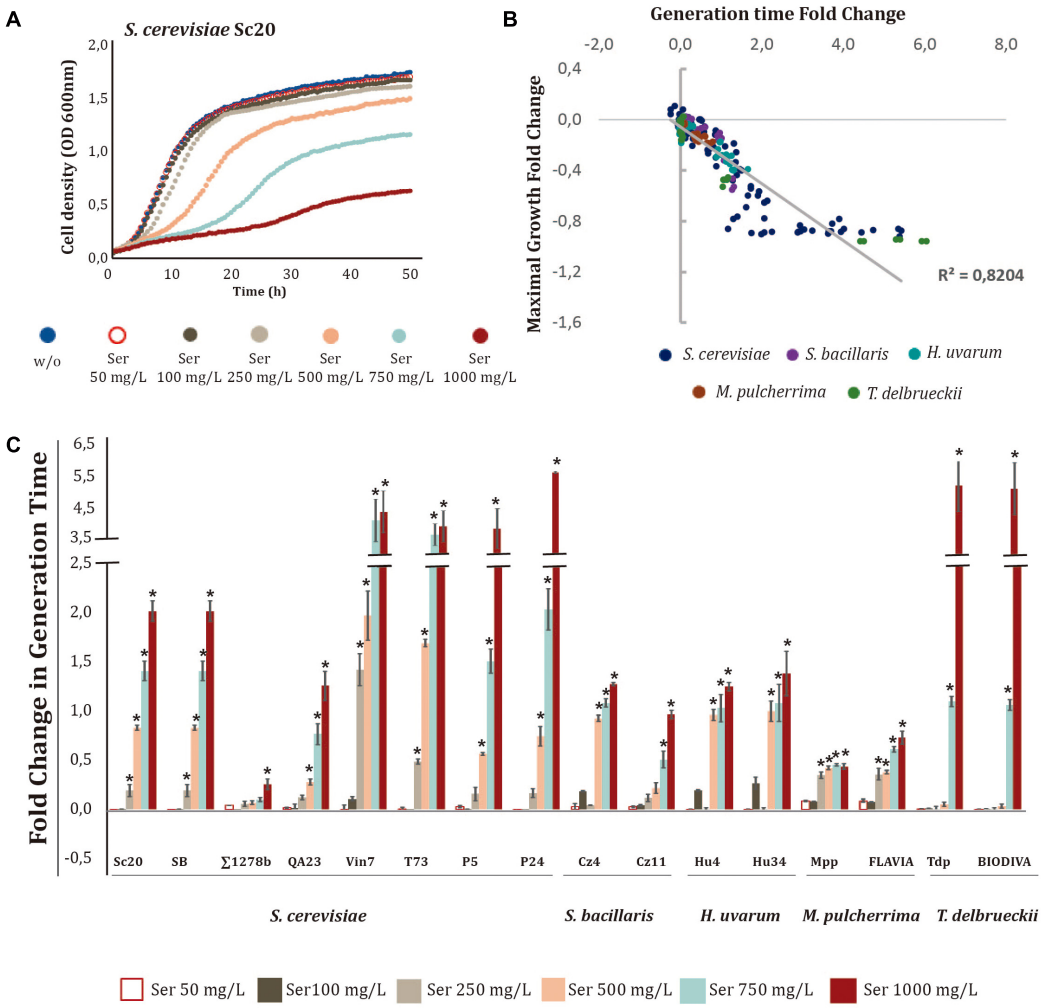

FIGURE 2 | Effects of increasing serotonin (Ser) concentrations on yeast growth. Ser was added to minimal medium [1 mM $(NH_4)_2SO_4$] at increasing concentrations (50, 100, 250, 500, 750, and 1000 mg/L). **(A)** Growth curves obtained with *S. cerevisiae* Sc20. **(B)** Correlation between the generation time and maximal growth fold-changes obtained with different yeast species. **(C)** Generation time fold-change for each strain at different Ser concentrations. Statistical analysis was performed, using Tukey's test and comparing the effects of Ser concentrations in each strain; asterisk denotes a p-value < 0.05. The fold-change for each growth parameter was determined in relation to the control (no-supplemented condition, w/o).

heavily affected, indicating that sensitivity to TrpOH is strain-dependent. In general, the *S. cerevisiae* strains were less affected by TrpOH.

Trpm influenced differently the growth of yeast strains, resulting in increases in the lag phase or in the GT, decreases in the OD max, and even no inhibitory effects at all (see two examples in **Figure 4A**). Thus, within the same species, we observed different responses to the presence of Trpm. For example, among *S. cerevisiae* strains, Vin7 only showed an increase during the lag phase, and there were no significant effects on the other growth parameters; QA23 primarily increased its GT and decreased the OD max, while the other *S. cerevisiae* strains were barely affected by Trpm (**Figures 4B,C** and Supplementary Figure S2). On the other hand, non-*Saccharomyces* strains were more affected by the presence of this biogenic amine, even at low concentrations, modifying all the growth parameters. Interestingly, in *M. pulcherrima* strains, the effects of Trpm on the OD max and GT were not dose-dependent, demonstrating similar inhibition from 100 to 1000 mg/L (**Figure 4** and Supplementary Figure S2).

Effects of Culture Medium Composition on Filamentous Growth in Non-*Saccharomyces* Species

The aromatic alcohols and ethanol have been described as molecules signaling morphological changes in different yeasts, primarily in *S. cerevisiae* and *C. albicans*; therefore, we analyzed their effects on the non-*Saccharomyces* strains. We first studied invasive growth on rich (SAD) and nutrient-limiting [glucose (SALG) and nitrogen (SLAD)] media for all strains using

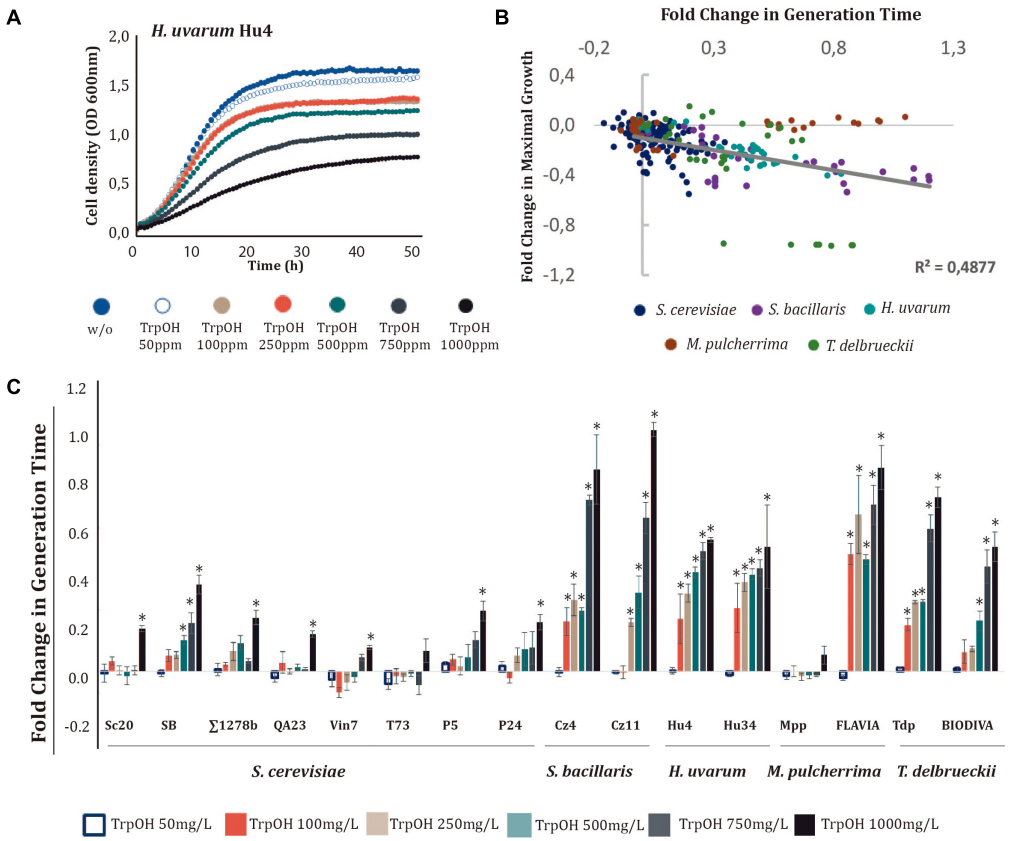

FIGURE 3 | Effects of increasing tryptophol (TrpOH) concentrations on yeast growth. TrpOH was added to minimal medium [1 mM $(NH_4)_2SO_4$] at increasing concentrations (50, 100, 250, 500, 750, and 1000 mg/L). **(A)** Growth curves obtained with *H. uvarum* Hu4. **(B)** Correlation between the generation time and maximal growth fold-changes obtained with different yeast species. **(C)** Generation time fold-change for each strain at different TrpOH concentrations. Statistical analysis was performed using the Tukey's test and comparing the effects of TrpOH concentrations in each strain; asterisk denotes a p-value < 0.05. The fold-change for each growth parameter was determined in relation to the control (no-supplemented condition, w/o).

S. cerevisiae QA23 as a control (**Figure 5**). Interestingly, all strains exhibited a certain degree of invasive growth. Moreover, media limited for glucose or nitrogen resulted in enhanced invasive growth for most of them. Specifically, on SLAD plates, most strains showed invasive growth that was significantly higher than on SAD, with the exception of *H. uvarum* strains. *M. pulcherrima* and *T. delbrueckii* strains were the most invasive non-*Saccharomyces* yeasts in the absence of nitrogen. Carbon source limitation (SALG) had a similar effect as nitrogen; most strains presented significant invasive growth compared to rich media, with the exception of the two *T. delbrueckii* strains.

We also determined the ability of these yeasts to form pseudohyphae by analyzing the morphology of their colonies on SAD, SLAD, and SALG media. **Figure 6** shows the morphology of the colony peripheries at day 7. *H. uvarum* strains exerted the highest pseudohyphal phenotype, mainly in limitation of nitrogen (SLAD), similarly to the control strain. Surprisingly, these *H. uvarum* strains were also able to produce pseudohyphae on rich media. *M. pulcherrima* and *S. bacillaris* strains formed few filaments only in SLAD medium, and none of the tested strains underwent pseudohyphae in SALG medium. Thus, the lack of glucose was not a limiting factor to trigger this aspect of the filamentous growth response in non-*Saccharomyces* yeast.

Effects of Alcohols on Filamentous Growth in Non-*Saccharomyces* Species

The effects of alcohols on invasive growth were assayed on SAD, SALG, and SLAD plates, both with and without supplementation with different alcohols. In general, the effects of alcohols varied depending on the medium and the species (**Figure 7A**). On SAD medium (**Figure 7B**), TrpOH and PheOH promoted invasive growth in the *S. cerevisiae* strain. Among non-*Saccharomyces* species, PheOH only stimulated invasive growth in *H. uvarum*, while ethanol and TrpOH only in *T. delbrueckii*. Furthermore, no significant effects were observed in *S. bacillaris* or in *M. pulcherrima*. On SALG plates (**Figure 7C**), aromatic alcohols significantly decreased the invasive growth of the commercial QA23 strain. Among non-*Saccharomyces* strains, TrpOH and PheOH significantly promoted invasive

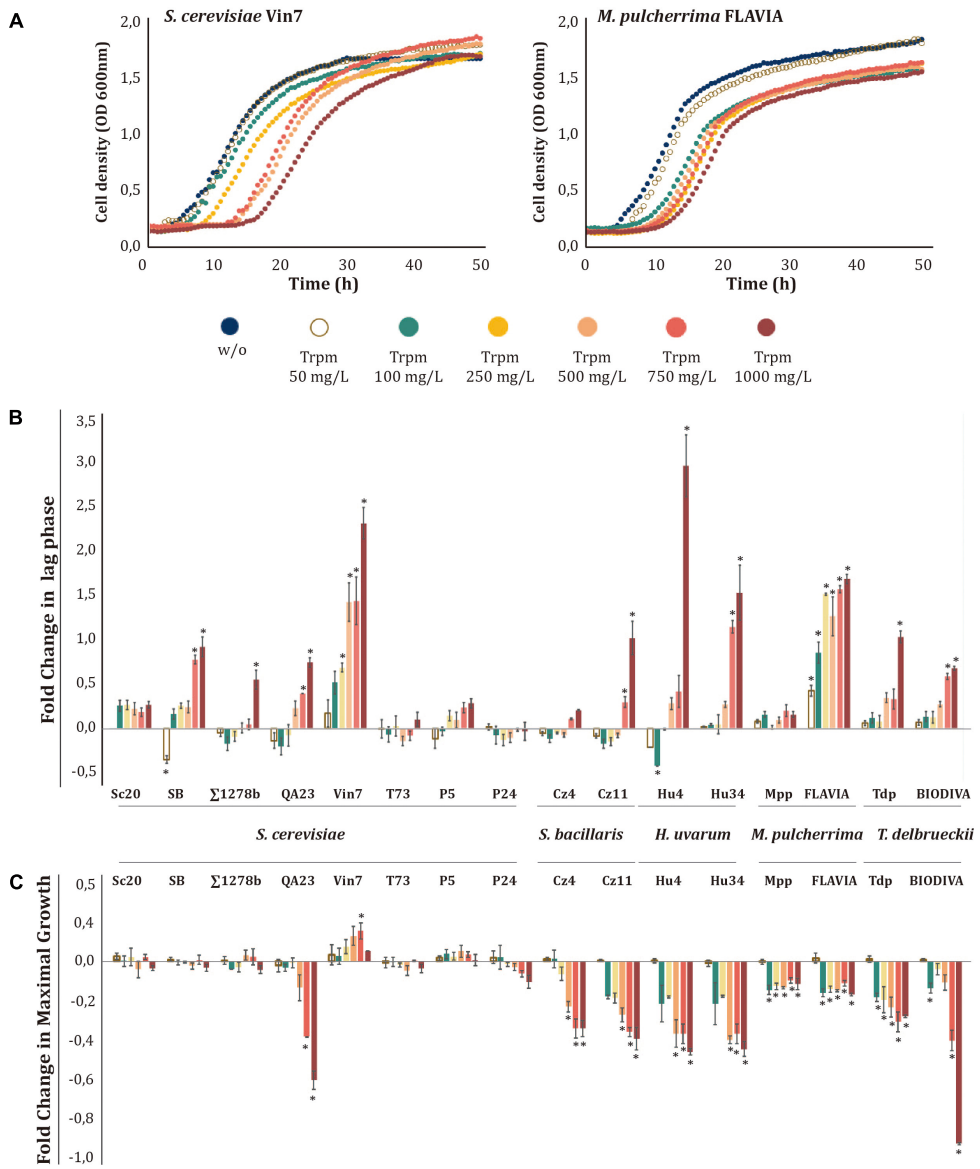

FIGURE 4 | Effects of increasing tryptamine (Trpm) concentrations on yeast growth. Trpm was added to minimal medium [1 mM $(NH_4)_2SO_4$] at increasing concentrations (50, 100, 250, 500, 750, and 1000 mg/L). **(A)** Growth curves obtained with *S. cerevisiae* Vin7 and *M. pulcherrima* FLAVIA. **(B)** Lag phase fold-change for each strain at different Trpm concentrations. **(C)** Maximal growth fold-change for each strain at different Trpm concentrations. Statistical analysis was performed comparing the effects of Trpm concentrations in each strain, using Tukey's test statistical method; asterisk denotes a p-value < 0.05. The fold-change for each growth parameter was determined in relation to the control (no-supplemented condition, w/o).

growth on *H. uvarum* Hu4 and *T. delbrueckii* Tdp, respectively. Ethanol appeared to strengthen invasive growth in *S. bacillaris*, *M. pulcherrima*, and *T. delbrueckii*, while TyrOH presented similar effects in the two strains of *M. pulcherrima* and in the commercial *T. delbrueckii* BIODIVA strain. On SLAD plates (**Figure 7D**), ethanol induced invasive growth in the QA23 strain, as well as in both strains of *S. bacillaris* and *T. delbrueckii*. *H. uvarum* and *S. bacillaris* increased their invasive growth in the presence of PheOH. On the other hand, TyrOH significantly reduced the invasive growth of *M. pulcherrima* strains.

To study the effects of alcohols in pseudohyphal growth, we focused on SLAD medium (**Figure 8**). Ethanol and PheOH stimulated pseudohyphal formation in *S. cerevisiae*. However, the addition of alcohols to agar plates resulted in a reduction in filamentation in both strains of *H. uvarum*. Similar to *S. cerevisiae*, ethanol changed growth patterns to a more filamentous form in *S. bacillaris*, but the aromatic alcohols tested did not affect pseudohyphae development. TyrOH considerably increased filament formation in *M. pulcherrima*. Moreover, the two strains of *T. delbrueckii* tested did not form pseudohyphae when starved for nitrogen in the presence of any alcohol tested.

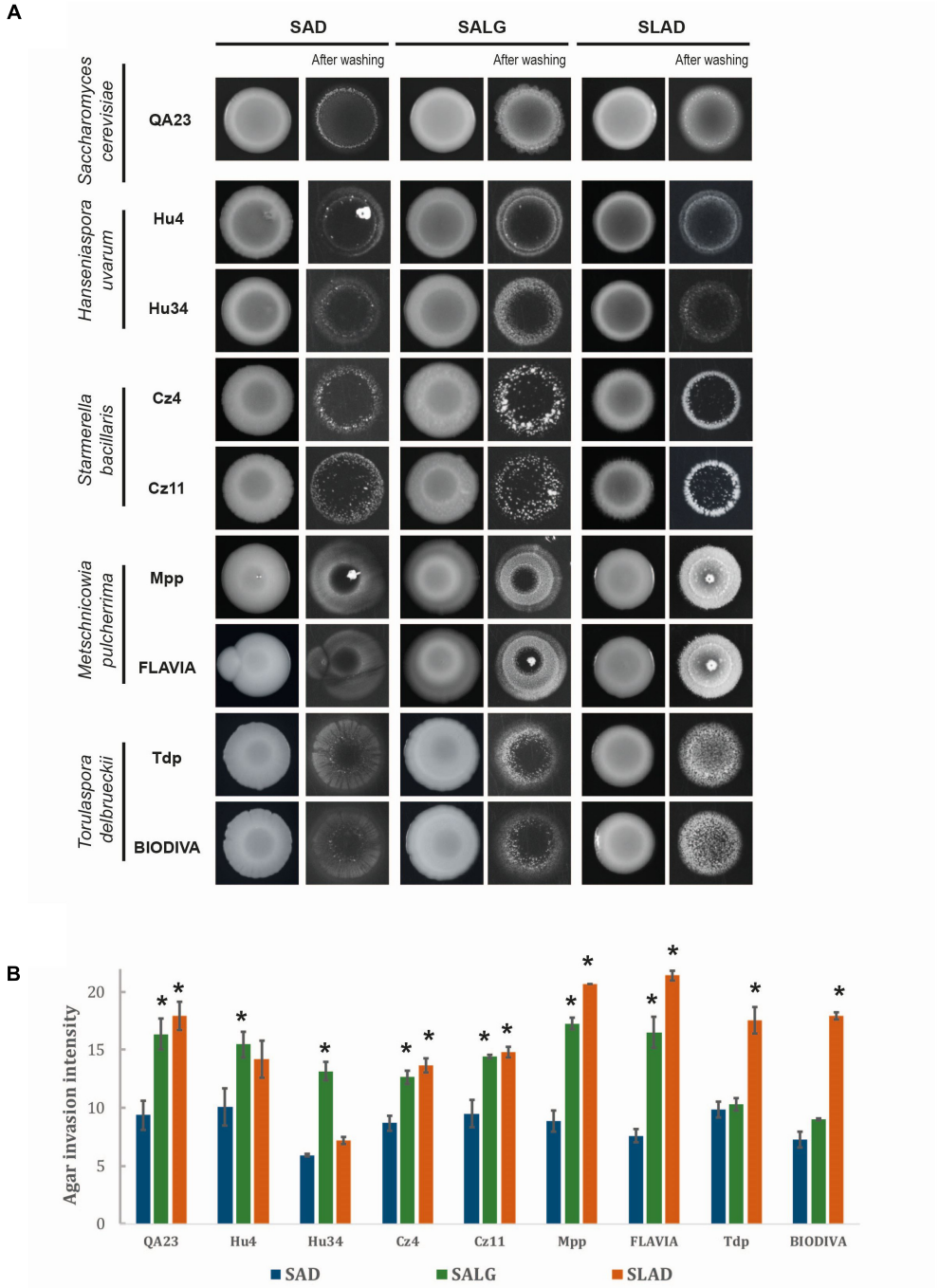

FIGURE 5 | Invasive growth phenotypes of different wine yeast species. **(A)** In a plate washing assay (PWA), equal concentrations of cells were spread on media with different nutrient contents and incubated for 5 days at 28°C. **(B)** Quantification of invasive growth was performed after washing the plate via densitometry analysis. Cells were spotted in triplicate, and the average values are shown. Statistical analysis was carried out by comparing each strain with respect to rich media (SAD), using Tukey's test statistical method; asterisk denotes a p-value < 0.05.

DISCUSSION

No organisms exist in isolation, all species share common environments and compete for nutrients. Interactions between organisms are commonplace and may be diverse. Although there are many examples of cooperation and symbiotic relationships among organisms, many interactions are combative, with one species profiting from another's detriment. An excellent example of this is seen on rotting fruit, where yeast and other microorganisms compete for sugar food sources.

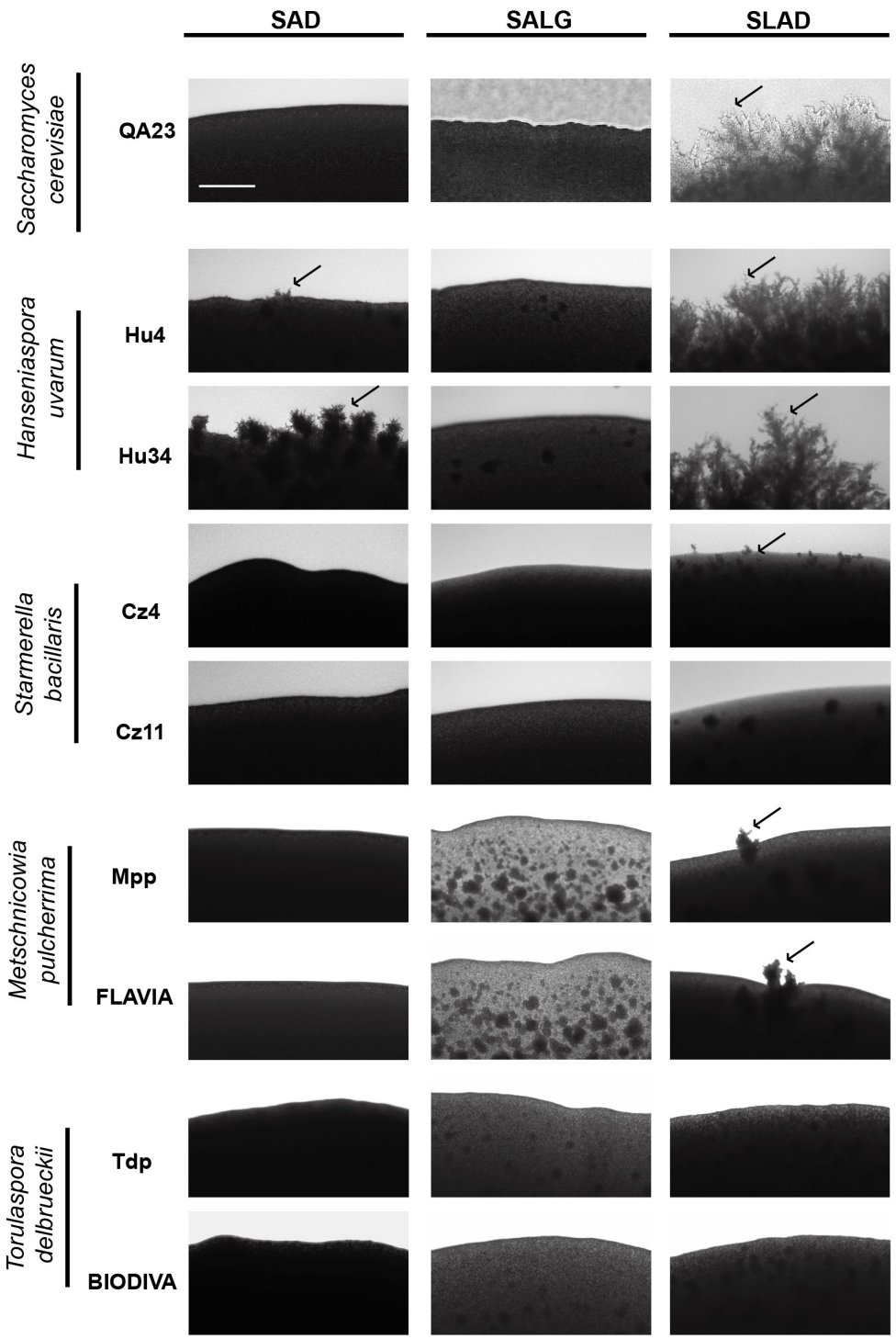

FIGURE 6 | Pseudohyphal growth phenotypes of different wine yeast species. Cells were spotted on rich medium (SAD) and nutrient limitation media (SALG and SLAD). Colony peripheries were photographed after incubation for 5 days at 28°C. Scale bar is 50 μm. Arrows mark examples of pseudohyphae.

Non-*Saccharomyces* yeasts are predominant in grape must, even during the first stages of spontaneous fermentations, but are rapidly replaced by *S. cerevisiae*, which completes the process (Fleet, 2003). Recently, some findings have associated interactions between species with the secretion of certain compounds by yeast during alcoholic fermentation (Ciani and Comitini, 2015; Wang et al., 2015a; Albergaria and Arneborg, 2016), such as some alcohols which are produced at high

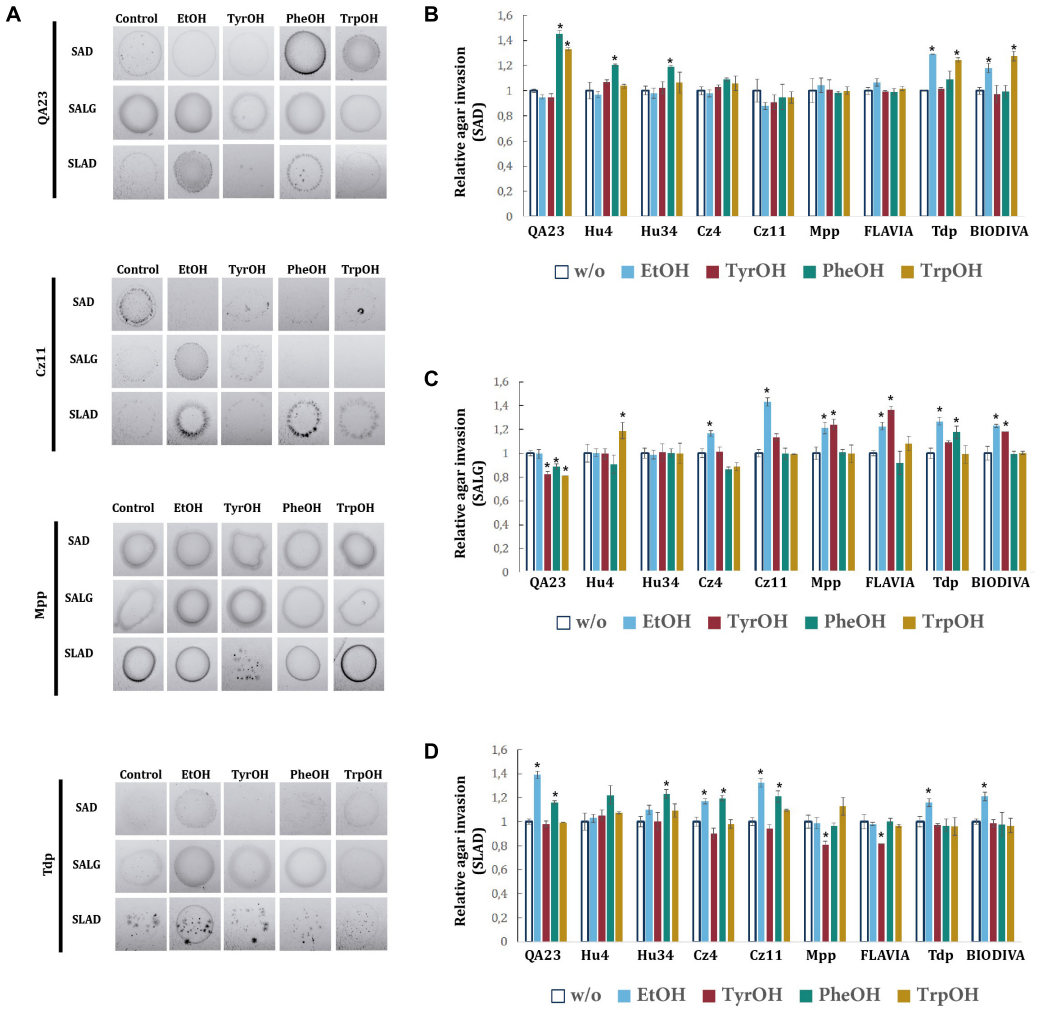

FIGURE 7 | Invasive growth of wine yeast species in the presence of aromatic alcohols and ethanol. **(A)** In a plate washing assay (PWA), equal concentrations of cells were spread on SAD, SALG, and SLAD media in the presence of the aromatic alcohol (TyrOH, PheOH, or TrpOH) at 500 μM or 2% (v/v) EtOH and incubated for 3 days at 28°C. Panel **(A)** shows the results from the washed plate. The invasive growth obtained with different wine yeast species in SAD **(B)**, SALG **(C)**, and SLAD **(D)** was obtained via densitometry. Cells were spotted in triplicate, and the average agar invasion values were calculated. Relative invasion values were obtained by dividing the agar invasion in presence of each compound and the one of the controls (no-supplemented condition, w/o). Statistical analysis was performed comparing the effects of the alcohols in each strain relative to the control, p-value < 0.05.

density by *S. cerevisiae* (Zupan et al., 2013). Our results showed that aromatic alcohols reduced yeast cell growth, especially in non-*Saccharomyces*, where the three fusel alcohols exerted negative effects on GT and maximal growth in most strains, even at low concentrations (100–250 mg/L). Instead, in *S. cerevisiae* strains, only TrpOH exhibited growth inhibition. These aromatic alcohols are produced by wine yeast and are found in alcoholic beverages at concentrations ranging from 4 to 197 mg/L PheOH, 100 to 450 mg/L TrpOH, and 5 to 40 mg/L TyrOH (Swiegers et al., 2005). Non-*Saccharomyces* strains are able to produce these aromatic alcohols, but at lower concentrations than *S. cerevisiae* (Zupan et al., 2013; González, 2017), however, the negative effects on the growth of these alcohols were more pronounced in non-*Saccharomyces*. Thus, the production of aromatic alcohols may play a role in certain yeast interactions, inhibiting the growth of non-*Saccharomyces* strains and even directing the replacement of these species during alcoholic fermentation by the major producer species, *S. cerevisiae*. Nevertheless, in this study, we tested the effects of these alcohols individually, but mixtures of them may have greater impact on yeast growth.

Mel is synthesized from tryptophan and exhibits various biological activities in humans, such as antioxidant activity (Reiter et al., 2001; Anisimov et al., 2006). It has been proved that yeasts generate low concentrations of Mel during alcoholic fermentation (Rodriguez-Naranjo et al., 2012); however, its role in yeast regulation is still unknown. In our study, the presence of Mel in the media did not affect the growth of the yeast strains tested. In contrast to Mel, its precursor, Ser, considerably reduced the maximal growth and doubling time

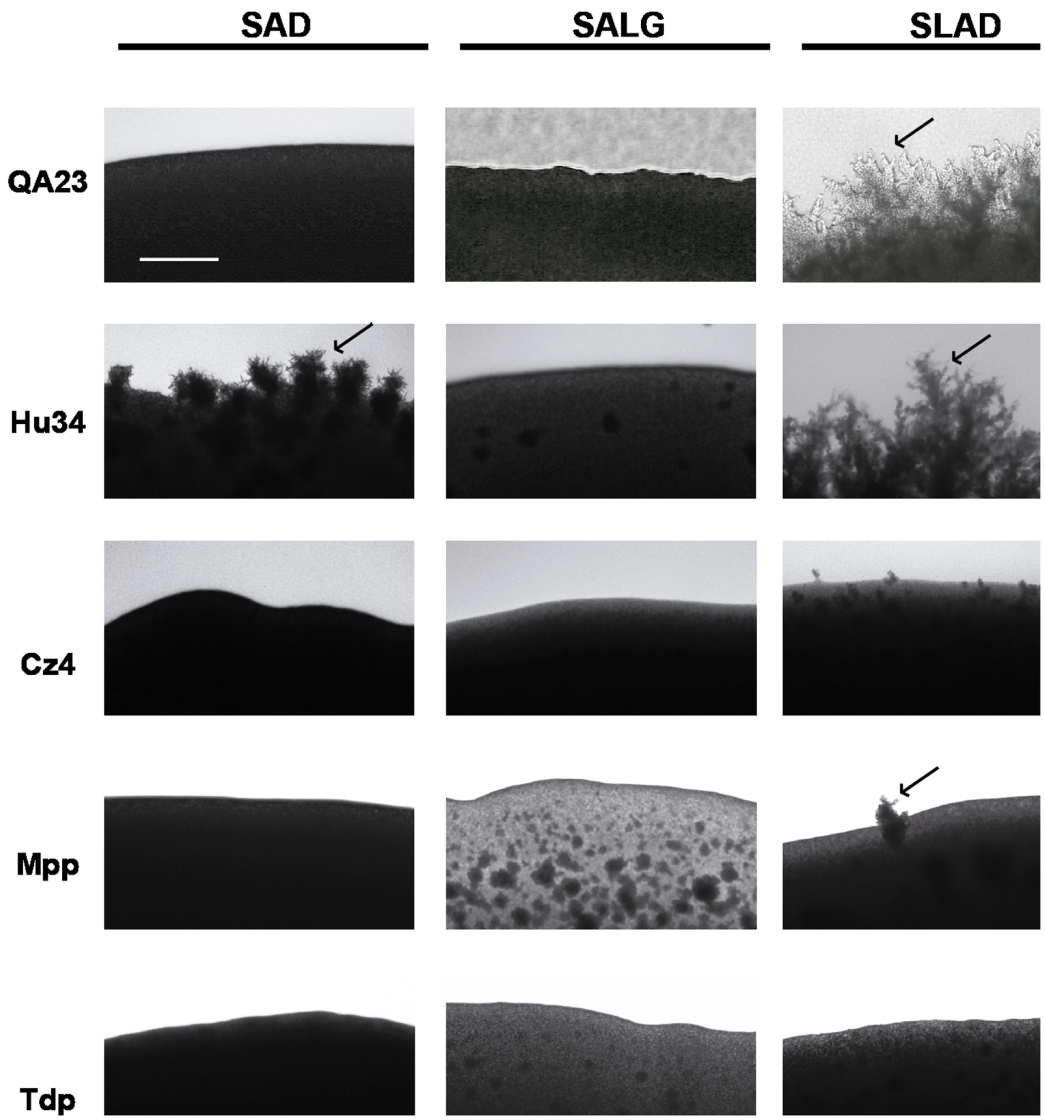

FIGURE 8 | Pseudohyphal growth phenotypes of different wine yeast species in response to ethanol and aromatic alcohols. Cells were spotted on SLAD medium. Colony peripheries were photographed after incubation for 3 days at 28°C. Scale bar is 50 μm.

of all strains tested, and was the most inhibiting compound tested, which indicates that Ser has toxic effects in yeast. Indeed, Ser has previously shown antifungal activity against *Candida* and *Aspergillus* spp. *in vitro* (Lass-Flörl et al., 2002, 2003). On the other hand, Trpm mostly affected the lag phase, being reduced at low concentrations but increased at high concentrations. Trpm levels in wines are usually very low (0.02–0.2 mg/l), and its synthesis largely depends on fermentation temperature but not on supplementation with its precursor amino acid (Lorenzo et al., 2017), Ser is found at very lower concentration at the end of alcoholic fermentation (Fernández-Cruz et al., 2017). Therefore, although Trpm and Ser appear to significantly affect different growth parameters, this does not occur at concentrations usually found in wines.

Recently, the death of non-*Saccharomyces* yeasts in mixed fermentation with *S. cerevisiae* was associated with mechanisms mediated through cell-to-cell contact as well as high cell densities (Nissen et al., 2003, 2004; Pérez-Nevado et al., 2006; Renault et al., 2013). However, the role of cell-to-cell communication through QSM in inhibiting the growth of certain yeast strains during mixed-culture fermentation remains unclear (Wang et al., 2015b; Avbelj et al., 2016). QS in yeasts involves a morphological transition from a filamentous to a yeast form, or *vice versa* (Sprague and Winans, 2006). Yeasts undergo this transition from a unicellular to a filamentous form in response to environmental cues, which may arise from alterations in nutrient concentrations or in the presence of auto-inductive molecules that are secreted by cells (Chen and Fink, 2006). Stimuli that trigger filamentous growth include nitrogen limitation (Gimeno et al., 1992) and

glucose limitation (Cullen and Sprague, 2000). Filamentation is well established in *Saccharomyces* (Chen and Fink, 2006; Cullen and Sprague, 2012) and the dimorphic fungal human pathogen *C. albicans* (Hornby et al., 2001; Chen et al., 2004), but little is known about this type of growth in other genera and species of yeasts (Gori et al., 2011; Pu et al., 2014; van Rijswijck et al., 2015). In our study, we tested two strains each of the major genera involved during wine fermentation to test their ability to penetrate surfaces (invasive growth) or to form pseudohyphae. All strains tested were wild yeasts isolated from wine environments and were able to invade, even in rich media. Indeed, natural yeast isolates exhibit high levels of invasion (Casalone et al., 2005), allowing them to colonize natural niches, such as grapes. According to Pitoniak et al. (2009), yeasts require the filamentous growth pathway and Flo11 to be able to fully colonize this environment. Nutrient limitation also promotes increased invasive growth in non-*Saccharomyces* species. The *S. bacillaris* and *M. pulcherrima* strains increased their invasive growth both under glucose and nitrogen limitation, but they only formed small pseudohyphae with nitrogen limitation. Indeed, the ability to form pseudohyphae and invade agar upon nutrient deprivation provides a selective advantage to yeast cells, facilitating foraging for scarce nutrients at a distance from their initial position (Casalone et al., 2005). On the other hand, *H. uvarum* exhibited a striking behavior because its cells primarily invade the agar under glucose limitation but form a large number of pseudohyphae under nitrogen limitation and, to a lesser extent, in rich media. The ability of these strains to form pseudohyphae in rich media may be an advantage to colonize fruits by adhesion and a possible reason for the wide distribution of this species on natural fruit surfaces; in some studies, *H. uvarum* is the main species found in grape habitats (Pretorius, 2000; Beltran et al., 2002; Cadez et al., 2002; Ocón et al., 2010; Padilla et al., 2016), Finally, *T. delbrueckii* was the only species that did not form pseudohyphae in any of the tested media. Nevertheless, this species was able to invade under nitrogen limitation. This suggests the differential regulation of both phenotypes in this species. A possible explanation for this lack of pseudohyphal growth may be related to its ability to flocculate in liquid medium, especially in YPD medium. Both phenotypic traits are mediated by the same family gene and a recent study demonstrated that variations in the amino acid sequence of the adhesion domain of Flo11 causes different flocculation activities (Barua et al., 2016).

Overall, the two strains of each species tested presented similar behaviors, indicating that filamentous growth is a similar trait in several species. Aromatic alcohols have been reported to possess QS activity, and their effects together with ethanol on *S. cerevisiae* morphology have been thoroughly described (Chen and Fink, 2006; González et al., 2017). In this study, the effects of aromatic alcohols and ethanol were analyzed in three different media, which differed in their glucose and ammonium content. As previously described, PheOH and TrpOH exerted effects on filamentous growth in *S. cerevisiae*. However, these results are not completely in concordance with Chen and Fink (2006), since they observed that PheOH and TrpOH both exerted effects on pseudohyphal growth but only PheOH affected invasive growth, and in our study we observed the opposite. Moreover, we also observed inhibitory effects on pseudohyphae with all aromatic alcohols in low glucose medium. In *H. uvarum*, the sole aromatic alcohol that promoted invasive growth was PheOH, both in rich and in nitrogen-limiting media. A reduction in pseudohyphae formation was observed in the presence of aromatic alcohols, which also occurred with farnesol in *C. albicans* (Hornby et al., 2001). In a recent study, Pu et al. (2014) described the involvement of PheOH in filamentous growth, adhesion, and biofilm formation in *H. uvarum*. On the other hand, TyrOH has been described as an inducer of filamentous growth in *C. albicans* (Chen et al., 2004). However, TyrOH did not affect significantly *S. bacillaris* growth in any of the conditions tested, as it might be expected due to its greater proximity to *C. albicans*. Anyway, this species produced very low concentration of aromatic alcohols, even in a previous study no synthesis was detected (Zupan et al., 2013; González, 2017). Therefore, in this species, other molecules may be the signals that initiate changes in morphogenesis, similar to *C. albicans* with farnesol (Kruppa, 2009). The effects of TyrOH on morphological changes were also observed in *M. pulcherrima*, suggesting a possible signaling role also in this species. Ethanol has been extensively reported to stimulate pseudohyphal growth in *S. cerevisiae* (Lorenz et al., 2000; González et al., 2017). In our study, ethanol affected all species to varying degrees, with the exception of *T. delbrueckii*. However, even in this species, ethanol promoted invasive growth under all tested conditions. As we have previously shown, *T. delbrueckii* did not undergo pseudohyphal growth under any of the tested conditions, but these strains presented flocculent growth in liquid media, which may suppress filamentation, as both responses are controlled by the same gene family (Soares, 2011).

Therefore, the aromatic alcohols appear to be species-specific signaling molecules because different species manifest different responses to these auto-regulatory molecules. This finding was previously observed for *S.cerevisiae* and *C.albicans*: Chen and Fink (2006) demonstrated that different aromatic alcohols exert different effects on the morphogenesis of these two yeast species.

CONCLUSION

We demonstrated that aromatic amino acid-derived compounds produced during alcoholic fermentation by yeast, and at the concentrations found in fermented beverages, modulate the growth of certain yeast species. Among these compounds, aromatic alcohols appear to be the most interesting because yeasts synthesize these compounds at levels that have physiological effects, suggesting a possible role in microbial interaction during wine fermentation. Our study reinforces the idea that these molecules play roles as QSM on both *Saccharomyces* and non-*Saccharomyces* species, as they appear to be able to induce or repress their filamentous and vegetative growth.

AUTHOR CONTRIBUTIONS

BG designed and performed the experiments, analyzed and discussed the results, and wrote the manuscript. JV performed the

experiments and analyzed and discussed the results. PC designed the experiments, discussed the results, and wrote the manuscript. AM, M-JT, and GB designed the experiments, analyzed and discussed the results, wrote the manuscript, and rose the funding.

FUNDING

The authors thank the Ministry of Economy and Competitiveness, Spain (Projects AGL2013-47300-C3-1-R and AGL2016-77505-C3-3-R), and NIH (DE022720 and GM098629) for financial support. GB holds a Fellowship from AGAUR (Government of Catalonia) and JV has a pre-doctoral Fellowship from the Oenological Biotechnology group of the University Rovira i Virgili.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.00670/full#supplementary-material

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

frontiers in Microbiology

ORIGINAL RESEARCH
published: 03 July 2018
doi: 10.3389/fmicb.2018.01460

GPD1 and *ADH3* Natural Variants Underlie Glycerol Yield Differences in Wine Fermentation

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Glycerol is one of the most important by-products of alcohol fermentation, and depending on its concentration it can contribute to wine flavor intensity and aroma volatility. Here, we evaluated the potential of utilizing the natural genetic variation of non-coding regions in budding yeast to identify allelic variants that could modulate glycerol phenotype during wine fermentation. For this we utilized four *Saccharomyces cerevisiae* strains (WE - Wine/European, SA – Sake, NA – North American, and WA – West African), which were previously profiled for genome-wide Allele Specific Expression (ASE) levels. The glycerol yields under Synthetic Wine Must (SWM) fermentations differed significantly between strains; WA produced the highest glycerol yields while SA produced the lowest yields. Subsequently, from our ASE database, we identified two candidate genes involved in alcoholic fermentation pathways, *ADH3* and *GPD1*, exhibiting significant expression differences between strains. A reciprocal hemizygosity assay demonstrated that hemizygotes expressing $GPD1^{WA}$, $GPD1^{SA}$, $ADH3^{WA}$ and $ADH3^{SA}$ alleles had significantly greater glycerol yields compared to $GPD1^{WE}$ and $ADH3^{WE}$. We further analyzed the gene expression profiles for each *GPD1* variant under SWM, demonstrating that the expression of $GPD1^{WE}$ occurred earlier and was greater compared to the other alleles. This result indicates that the level, timing, and condition of expression differ between regulatory regions in the various genetic backgrounds. Furthermore, promoter allele swapping demonstrated that these allele expression patterns were transposable across genetic backgrounds; however, glycerol yields did not differ between wild type and modified strains, suggesting a strong *trans* effect on *GPD1* gene expression. In this line, Gpd1 protein levels in parental strains, particularly $Gpd1p^{WE}$, did not necessarily correlate with gene expression differences, but rather with glycerol yield where low $Gpd1p^{WE}$ levels were detected. This suggests that $GPD1^{WE}$ is influenced by recessive negative post-transcriptional regulation which is absent in the other genetic backgrounds. This dissection of regulatory mechanisms in *GPD1* allelic variants demonstrates the potential to exploit natural alleles to improve glycerol production in wine fermentation and highlights the difficulties of trait improvement due to alternative *trans*-regulation and gene-gene interactions in the different genetic background.

Keywords: wine, yeast, glycerol, alleles, *Saccharomyces*

OPEN ACCESS

Edited by:
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Johan M. Thevelein,
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Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: *19 March 2018*
Accepted: *12 June 2018*
Published: *03 July 2018*

Citation:
Tapia SM, Cuevas M, Abarca V, Delgado V, Rojas V, García V, Brice C, Martínez C, Salinas F, Larrondo LF and Cubillos FA (2018) GPD1 and ADH3 Natural Variants Underlie Glycerol Yield Differences in Wine Fermentation. Front. Microbiol. 9:1460. doi: 10.3389/fmicb.2018.01460

INTRODUCTION

Glycerol production is one of the most important by-products generated during alcohol fermentation. Depending on the quantity and wine type, glycerol can contribute to wine flavor intensity and impact aroma volatility (Gawel et al., 2007; Marchal et al., 2011; Zhao et al., 2015). In budding yeast, glycerol is synthesized by the reduction of dihydroxyacetone phosphate followed by dephosphorylation catalyzed by glycerol-3-phosphate dehydrogenase (*GPD1*) and glycerol-3-phosphatase (*GPP1*) (Albertyn et al., 1994). Genetic modification has been used to engineer yeast that produces more glycerol (Steensels et al., 2014). Despite this, the application of genetically modified organisms (GMOs) in the industry is restricted by the lack of policies that regulate their use and by negative public perception (Steensels et al., 2014). This has inspired the development of alternative strategies for the generation of new strains, such as experimental evolution (Steensels et al., 2014; Tilloy et al., 2014). For example, the wine strain EC1118 has been genetically improved to produce more glycerol through constant exposure to osmotic stress (Tilloy et al., 2014). Nevertheless, the use of experimental evolution to obtain specific phenotypes is time-consuming, and undesirable mutations can complicate industrial applications. Thus, non-invasive nor mutagenic strategies represent an alternative where variants of interest are selected from standing natural genetic variation (Cubillos, 2016). *S. cerevisiae* strains are genotypically and phenotypically highly variable, and thus are an ideal model for studying trait improvement (Thompson and Cubillos, 2017; Peter et al., 2018).

Natural and commercial *S. cerevisiae* isolates differ largely in a series of traits (Crepin et al., 2012; Salinas et al., 2016; Cubillos et al., 2017). In this context, it has been reported that depending on the genetic background, isolates can yield different concentrations of acetic acid, glycerol, ethanol, and other secondary metabolites (Salinas et al., 2012). Efforts aimed at deciphering the genetic basis underlying some of these phenotypic differences in isolate types have demonstrated the existence of a wide set of quantitative trait loci (QTLs), for example: ethanol production (Katou et al., 2009; Pais et al., 2013), ethanol tolerance (Swinnen et al., 2012), glycerol production (Hubmann et al., 2013b), asparagine assimilation (Marullo et al., 2007), low temperature fermentation (Garcia-Rios et al., 2017), and nitrogen assimilation (Brice et al., 2014, 2018; Cubillos et al., 2017). In most of these cases, QTLs are down to non-synonymous changes which significantly impact protein structure and gene function. For example, a series of aminoacidic changes in *SSK1*, *GPD1*, *HOT1*, and *SMP1* genes have been found as responsible for low glycerol and high ethanol yield differences between CBS6412 and Ethanol Red strains (Hubmann et al., 2013a,b). Yet, the molecular mechanisms and the effect of these polymorphisms upon protein activity and stability are unknown.

Although, these regions explain a substantial fraction of the natural phenotypic variation between individuals, a wide set of variants across eukaryotes are located within non-coding regions and finely modulate gene expression and ultimately phenotypes (Wray, 2007). In this context, non-coding regions have been less explored in yeast and could be useful for genetic breeding and industrial applications via the modulation of gene regulation and expression (Thompson and Cubillos, 2017). Previous expression profiles of *S. cerevisiae* isolates obtained from different ecological niches have demonstrated that the genetic control of expression is well-defined (Fay et al., 2004; Kvitek et al., 2008; Ehrenreich et al., 2009; Zhu et al., 2009; Fraser et al., 2010; Cubillos et al., 2012). Additionally, budding yeast can be easily manipulated at the molecular level and represents a great model for genetic improvement and for understanding the consequences of mutations within coding and regulatory regions (Salinas et al., 2016). For example, early QTL mapping on sporulation efficiency between two North-American isolates has validated the role of non-coding regions on natural variation in yeast by showing the effects of a single nucleotide deletion upstream of *RME1* (Gerke et al., 2009). In this context, we have previously demonstrated how widespread Allele Specific Expression (ASE) is across four *S. cerevisiae* isolates representative of different lineages of the species. (Salinas et al., 2016). Interestingly, estimates of the aspartic acid and glutamic acid consumption in the wine fermentation must of two yeast strains from different geographic origins have demonstrated that polymorphisms in both portions (coding and regulatory) of the *ASN1* gene, are partly responsible for nitrogen assimilation differences between genetic backgrounds (Salinas et al., 2016). Moreover, this study provided a catalog of *cis*-variants between strains that directly influence allelic expression and which can be used as tools for the dissection of other phenotypes.

In this study, we utilize the existing standing genetic variation in yeast within non-coding regions to identify natural allelic variants for genes part of the alcoholic fermentation pathways that could impact glycerol production under synthetic wine must (SWM) conditions. For this, we searched our ASE database for genes involved in fermentation, such as alcohol dehydrogenases and in glycerol biosynthesis. From this, we studied two candidate genes, *ADH3* and *GPD1*, with differently expressed alleles between strains. Through reciprocal hemizygosity, allele swapping, along with transcriptional and co-translational profiling across strains, we demonstrate that *ADH3* and *GPD1* allelic variants modulate glycerol yield and could be used as natural sources for genetic improvement and gene expression fine tuning.

MATERIALS AND METHODS

Yeast Strains and Culture Media

The haploid strains Y12 (referred to as Sake, 'SA', *Mat alpha ho::HygMX*, *ura3::KanMX*), YPS128 (referred to as North American, 'NA', *Mat alpha ho::HygMX*, *ura3::KanMX*), DBVPG6044 (referred to as West African, 'WA', *Mat alpha ho::HygMX*, *ura3::KanMX*) and DBVPG6765 (referred to as Wine/European, 'WE', *Mat a, ho::HygMX*, *ura3::KanMX*) together with F1 hybrids (WE x SA, WE x NA, and WE x WA crosses) utilized in this study have been previously described (Cubillos et al., 2009, 2011). Before every experiment, strains

were recovered from frozen glycerol stocks in rich yeast peptone dextrose (YPD) agar media and grown overnight at 28°C.

Fermentation in Synthetic Wine Must (SWM) MS300 and HPLC Analysis

Fermentations were carried out in at least three biological replicates depending on the experiment. Fermentations were conducted using SWM supplemented with 300 mgN/L) (MS300, hereafter referred to as SWM) and 270 g/L of total sugar (glucose and fructose and prepared as previously reported (Rossignol et al., 2003) (Jara et al., 2014). For each experiment, the strains were initially grown with constant agitation in 10 mL of SWM for 16 h at 25°C. Following this, 12 mL of fresh SWM were inoculated to a final concentration of $1x10^6$ cells/mL of yeast (in 15 mL conical tubes) and incubated at 25°C with no agitation. Fermentations were weighed every day to calculate the CO_2 output. The fermentations were maintained until the daily CO_2 lost represented less than 10% of the accumulated CO_2 lost. At the end of the fermentation, the fermented SWMs were centrifuged at $9000 \times g$ for 10 min and the supernatant was collected. From this, the concentration of extracellular metabolites was determined using HPLC. Specifically, 20 μL of filtered must were injected in a Shimadzu Prominence HPLC (Shimadzu, United States) with a Bio-Rad HPX –87H column (Nissen et al., 1997). In this way, the concentrations of glucose, fructose, trehalose, acetic acid, succinic acid, malic acid, ethanol, and glycerol was estimated (results found in **Supplementary Tables S2**, **S4**). Ethanol yield was estimated converting %v/v to g/L utilizing the ethanol density and then dividing by total sugar consumption. Similarly, glycerol yield was estimated by dividing the observed glycerol levels (g/L) by the total amount of sugar consumed.

Reciprocal Hemizygosity Assay

Reciprocal hemizygotes of the *ADH3* and *GPD1* candidate genes were generated as previously described (Cubillos et al., 2013; Jara et al., 2014; Salinas et al., 2016). Briefly, the *URA3* gene previously deleted in the haploid parental strains (Cubillos et al., 2009) was used as a selectable marker for the deletion of each target gene. The haploid versions of the parental strains also contained opposite antibiotic markers in the *HO* locus (Hygromycin B for "Mat *a*" strains and Nourseothricin for "*alpha*" strains), which allowed us to cross the haploid mutant parental strains and construct all possible combinations of single deletions. Thus, mutated parental strains were crossed to generate the reciprocal hemizygote strains, selecting the diploid hybrids in antibiotic plates (300 ug/mL of Hygromycin B and 100 ug/mL of Nourseothricin). Finally, diploids were confirmed by *MAT* locus PCR (Huxley et al., 1990). Primers are listed in **Supplementary Table S1**.

Luciferase Expression Assay (Cloning and Phenotyping)

The *GPD1* genetic constructs carrying the destabilized version of the firefly luciferase reporter gene under the control of the different regulatory allelic variants were assembled using yeast recombinational cloning as previously described (Salinas et al., 2016). Briefly, 700 bp upstream of the ATG start codon (regulatory region) and the firefly luciferase gene (Rienzo et al., 2012) were amplified by PCR using Phusion Flash High-Fidelity PCR Master Mix (Thermo scientific, United States). In addition, the Hygromycin HphMx antibiotic resistance gene was amplified by PCR and was included in the genetic constructs. Overall, the overlap between PCR products was 50 bp and were co-transformed with the linear plasmid pRS426 in the yeast lab strain BY4741 (*MATa*, *his3*Δ*1*, *leu2*Δ*0*, *LYS2*, *met15*Δ*0*, *ura3*Δ*0*). The circular plasmids generated in yeast were transferred to an *E. coli* DH5α strain and confirmed by colony PCR using standard conditions. At least three positives colonies containing the regulatory region, the luciferase gene, and the HphMx cassette were selected for plasmid isolation and sequencing. The sequence identity of the regulatory regions was confirmed using the SGRP2 BLAST database service (Bergstrom et al., 2014). Finally, the parental strains were transformed with the complete genetic constructs, which were amplified by PCR using a Phusion Flash High-Fidelity PCR Master Mix (Thermo scientific, United States). For the latter, 70 bp primers were utilized, which guided direct homologous recombination at the target locus, allowing for the integration of the genetic constructions in the genome. The positive yeast colonies were analyzed by colony PCR with standard conditions.

The use of a destabilized firefly luciferase (Rienzo et al., 2012) allowed quantifying expression of the targeted genes in real-time and, to avoid the effects of copy number and genetic context on gene expression, the genetic constructs were integrated into the original *GPD1* locus, maintaining the genetic context. Additionally, reciprocal hemizygotes were generated with these constructions as previously described. Strains carrying the firefly luciferase constructs were analyzed for luciferase expression using a Cytation3 microplate reader (Biotek, United States). Briefly, the strains were pre-grown in YNB (Yeast nitrogen base, supplemented with 2% glucose) and SWM overnight. The cultures were then diluted 1/100 to inoculate a 96 well plate with 200 uL of fresh culture media containing 0.1 mM of luciferin. The *in vivo* OD_{600}nm and the luminescence intensity of the cell cultures were monitored every 10 min. All the experiments were performed using, at least, three biological replicates.

Allele Swapping

Promoter allele swaps were carried out as previously described (Salinas et al., 2016). Briefly, genetic constructs carrying the regulatory regions of *GPD1* and *ADH3* (700 bp upstream of the ATG start codon) plus the HphMx cassette in the reverse direction (*HphMxRv-*P_{GPD1}) were assembled using yeast recombinational cloning. See details above for full descriptions. Initially, we used the WE strain as a receiver of the promoters coming from the NA, SA, and WA strains. For this, the regulatory region of the target gene was deleted in the WE strain using *URA3* gene as a selectable marker. Then, the construct containing the promoter of interest was amplified by PCR and used for transformation and direct recombination with the regulatory region. The final strains were confirmed by standard colony PCR and sequencing. Furthermore, we used the NA, SA and WA

strains as receivers of the promoter coming from the WE strain following the same procedure.

Additionally, we used the strains carrying the promoter swaps as recipients of the luciferase reporter gene for direct quantification of gene expression in living cells. Again, see above for the full description of the methods. The construct containing the destabilized version of the luciferase gene plus the *URA3* selectable marker (*Luc-URA3*) was amplified by PCR and used for *GPD1* transformation of the strains carrying the promoter swaps. The final strains were confirmed by colony PCR using standard conditions.

Gpd1p Tagging With mCherry

The fusion of the Gpd1 protein with mCherry was carried out using one step PCR and recombination with the 3′end of the *GPD1* ORF, which corresponds to the C-terminal of the Gpd1 protein. This allowed us to remove the stop codon of the *GPD1* gene and fuse its ORF with the mCherry coding sequence (DeLuna et al., 2010). In this way, we generated a construct containing the mCherry sequence plus the hygromycin cassette (mCherry-HphMx). This allowed us to directly tag the *GPD1* ORF and perform selection by hygromycin in each yeast strain. The mCherry-HphMx construct was assembled in a pRS426 plasmid using the above described yeast recombinational cloning method (Oldenburg et al., 1997). The yeast strains carrying the mCherry-HphMx cassette were confirmed using standard yeast colony PCR.

The yeast strains were analyzed in microcultivation with a Cytation 3 microplate reader, which allowed for the dual measurement of OD_{600} and fluorescence of the cell cultures over time. Briefly, the yeast strains were grown overnight in a 96 well plate with 200 uL of YNB or SWM medium. 10 uL of these cultures were used to inoculate a new black 96 well plate containing 300 uL (30-fold dilution) of fresh media. The OD_{600} and the fluorescence were measured every 30 min using 587 nm of excitation and targeting emission wavelengths of 620 nm with a gain of 100 units.

GPD1 Sequence Analysis

GPD1 sequences were obtained from the SGRP2 database (Bergstrom et al., 2014) and regulatory regions together with ORF sequences were compared using Geneious 8.1.5. Transcription factor binding sites were predicted utilizing YeTFaSCo: Yeast Transcription Factor Specificity Compendium (de Boer and Hughes, 2012). The Codon Adaptation Index (CAI) for each allele variant was estimated using the CAIcal server (Puigbo et al., 2008) with default settings and utilizing the standard *Saccharomyces cerevisiae* genome codons usage from the Codon Usage Database (Nakamura et al., 2000).

Data Analysis

The significance of all comparisons was made through non-parametric test depending on whether two groups or multiples groups were compared. Fermentation metabolites results obtained from HPLC were compared across strains utilizing a non-parametric Kruskal-Wallis test and Dunn's Multiple Test Comparison. Similarly, significance in metabolites levels between reciprocal hemizygotes were assessed utilizing a non-parametric Mann Whitney test. Gene expression and protein levels across the four parental strains were evaluated using a Friedman test and Dunn's Multiple Test Comparison. Luciferase expression and glycerol yield was estimated utilizing a Spearman rank correlation test. Finally, gene expression and protein levels across hemizygotes were compared using a Wilcoxon signed rank test. All analyses were performed utilizing GraphPad Prism Software 5.2. In all cases p-values < 0.05 were considered as significant.

RESULTS

Glycerol Production Differs Among *S. cerevisiae* Strains

Through the utilization of the ASE database, we aimed to identify natural allelic variants impacting glycerol production yields when grown in SWM. Thus, we initially characterized the fermentation profiles and ability to produce a series of metabolites in four strains grown in SWM. We estimated the concentrations of glucose, fructose, trehalose, acetic acid, succinic acid, malic acid, ethanol and glycerol after 21 days of micro-fermentations experiments.

Ethanol and glycerol significantly differed among some isolates (**Supplementary Table S2** and **Figure 1A**, p-value < 0.05, and Kruskal-Wallis test). For example, the SA strain showed greater ethanol production than NA (**Figure 1A**). Conversely, the WA and the NA strain showed significant differences for glycerol production, the latter producing lower levels of glycerol (**Figure 1A**, p-value < 0.05, and Kruskal-Wallis test). Since we found relatively high amounts of residual sugars in our fermentations, we estimated yields to accurately measure the quantity of sugar transformed into ethanol and glycerol, respectively (**Figures 1B,C**). From this we observed that the WA strain (a non-domesticated strain) yielded significantly more glycerol than the SA strain (p-value < 0.05, Kruskal-Wallis test), in agreement with their glycerol and ethanol production levels, respectively. Thus, the WA strain produced the highest glycerol yields (**Figure 1C**), while the WE isolate, considered a domesticated strain, did not produce greater ethanol nor glycerol levels/yields compared to WA, demonstrating that other non-domesticated genetic backgrounds may represent potential sources of allelic variants that can be used to boost glycerol production in wine fermentation.

Reciprocal Hemizigosity Assay (RHA) Validates Glycerol Yield Differences Among *ADH3* and *GPD1* Allelic Variants

In order to identify allelic variants that could influence glycerol yield, we utilized the ASE database (Salinas et al., 2016) to find ethanol/glycerol biosynthesis alleles with divergent expression. Additionally, since our focus was the wine fermentation environment, we only selected genes in which the WE allele

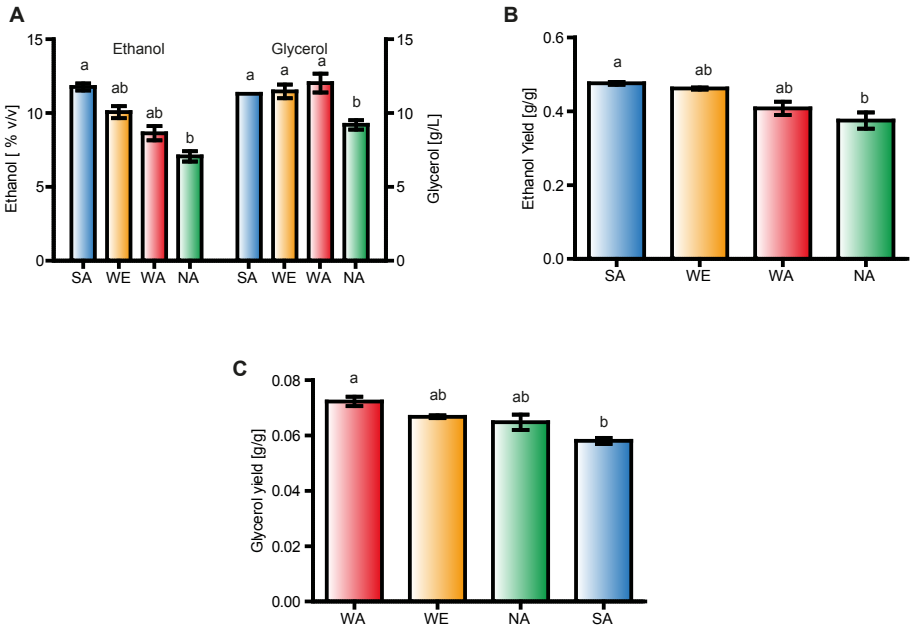

FIGURE 1 | Glycerol production in *S. cerevisiae* strains. **(A)** Glycerol and ethanol levels; **(B)** Ethanol yield and **(C)** glycerol yield in Sake (SA), West African (WA), Wine/European (WE), and NA (North American strains) after fermentation in synthetic wine must. Different letters indicate significant differences between genotypes.

was differently expressed (**Supplementary Table S3**). Here, we found two genes, *ADH3* and *GPD1*, which encode for an alcohol dehydrogenase III and a glycerol-3-phosphate dehydrogenase, respectively (Young and Pilgrim, 1985; Albertyn et al., 1994; de Smidt et al., 2008). Subsequently, to estimate the relative contribution of each genetic variant to ethanol and glycerol yields, we performed a functional analysis to compare the reciprocal hemizygotes derived from the three WE F1 hybrids. For both genes, we observed that hemizygotes carrying a WE variant produced substantially lower glycerol levels (except for *ADH3* - NA × WEΔ, p-value < 0.05, Mann Whitney test) than hemizygotes carrying the WA and SA allelic variants (**Figure 2A**). For example, $ADH3^{WA}$ (*ADH3* hemizygote carrying the WA variant) and $ADH3^{WE}$ hemizygotes produced 15.1 ± 0.4 g/L and 13.5 ± 0.6 g/L of glycerol, respectively. Similarly, this pattern was also observed for glycerol yields, where differences were maximized when sugar consumption was considered (**Figure 2B**). For example, the $GPD1^{WA}$ hemizygote yielded 66% more glycerol than the $GPD1^{WE}$ hemizygote. These results agree with the glycerol levels produced by the WA and WE parental strains, but not with SA strain, suggesting an antagonistic effect of the SA alleles. Interestingly, none of the hemizygotes with significantly greater glycerol yields had lower ethanol yields. Instead, WE hemizygotes produced increased yields of other metabolites including succinic acid and malic acid depending on the hybrid (**Supplementary Table S4**). This suggests quantitative differences in carbon molecules fluxes among strains. Overall, these results indicate that *ADH3* and *GPD1* allelic variants from the WA and SA strains could be used to maximize glycerol yields in wine fermentation; however, we found no evidence that selection of these alleles would affect ethanol production.

Transcriptional Profiling Demonstrates That *GPD1* Expression Levels Are G x E Dependent

We obtained expression profiles for each allelic variant to determine how allelic differences in regulatory regions affected expression levels, timing, and were condition dependent. For this, we focused on *GPD1* since this gene was involved in the greatest glycerol yield differences among hemizygotes. We generated transcriptional fusions in all strains by inserting a destabilized luciferase reporter gene immediately downstream of the regulatory region and replacing the original *GPD1* locus (Salinas et al., 2016). Firstly, the luciferase expression levels were obtained for all parental strains under micro-cultivation conditions in YNB and in SWM to evaluate the strength of the promoters in these two scenarios. From the transcriptional expression profiling, assay we found differences in expression between strains and environments, clearly indicating a G × E interaction (**Figures 3A,B** and **Supplementary Figure S1**). For example, the luminescence of $P_{GPD1}{}^{WE}$-*Luc* (the WE *GPD1* promoter controlling luciferase gene expression) was lower when the strain was cultivated in laboratory media than when cultivated in SWM (p-value < 0.05, Friedman test). Overall, the expression of $P_{GPD1}{}^{WE}$-*Luc* under SWM was highest among all strains (**Figure 3A**, p-value < 0.05, Friedman test). Interestingly, when cultivated in SWM each strain had a unique *GPD1* expression profile with expression levels clearly increasing among strains, WE > SA = WA > NA (p-value < 0.05, Friedman test).

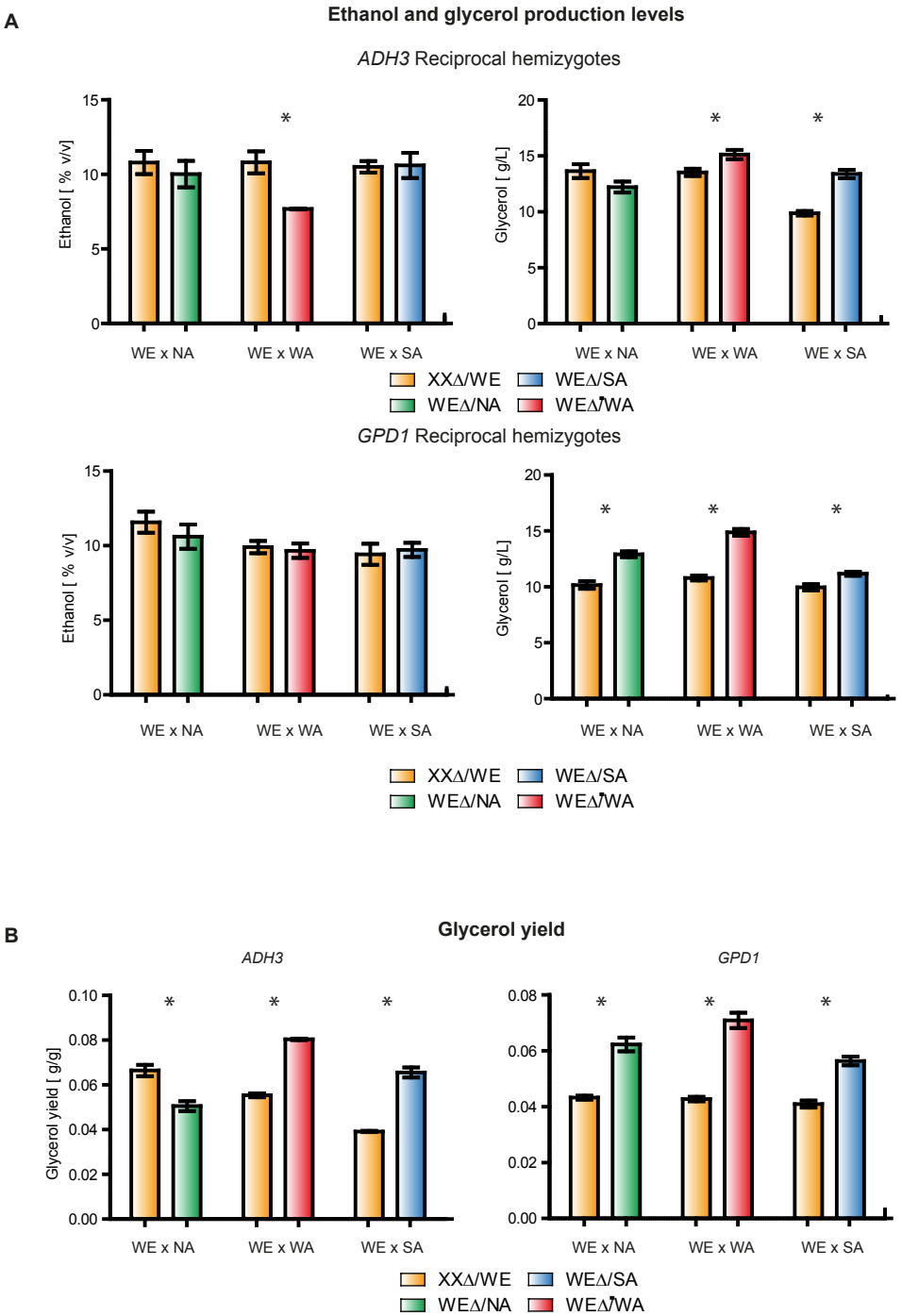

FIGURE 2 | Reciprocal hemizygosity assay for *GPD1* and *ADH3*. **(A)** Ethanol (%v/v) and Glycerol (g/L) levels in WE × NA, WE × SA, and WE × WA reciprocal hemizygotes. The Glycerol yield for the same strains is shown in **(B)**. XX denotes either the NA, WA, or SA genotype depending on the bar. *p-value < 0.05.

Conversely, the results differed when the strains were cultivated in YNB media. Here, $P_{GPD1}{}^{SA}$-*Luc* exhibited the strongest luminescence (**Figure 3B**, p-value < 0.05, Friedman test). It is worth noting that the luciferase expression levels were ∼10 times higher when strains were cultivated in SWM than when cultivated in YNB. These results demonstrate that the expression of *GPD1* is highly induced under fermentative conditions and its strength is dependent on the promoter allelic variant, yet the role of *cis*- and/or *trans* regulation is uncertain. Interestingly, the parental strain expression profiles did not correlate with the glycerol yields previously estimated (**Figure 1** and **Supplementary Table S2**, p-value = 0.2 Spearman rank correlation).

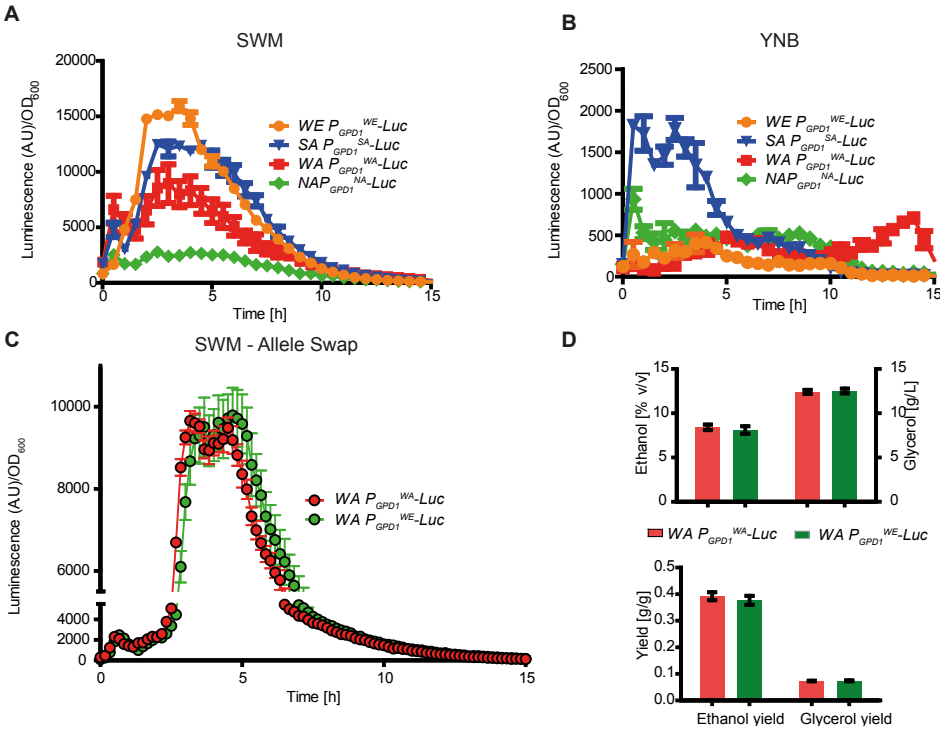

FIGURE 3 | *GPD1* gene expression profile. Luciferase reporter gene expression assay for *GPD1* promoters in their native genetic backgrounds grown in **(A)** synthetic wine must and **(B)** yeast nitrogen base (YNB). **(C)** Allele swap in the WA strain with a luciferase reporter gene controlled by the wild type promoter (red) and the WE promoter (green). **(D)** Metabolite levels in the allele swap assay.

In order to evaluate the role of the *cis*-regulatory region and whether the $P_{GPD1}{}^{WE}$ could sufficiently increase expression levels and thus modify glycerol yield in other genetic backgrounds, we performed an allele swap. Immediately upstream of the luciferase reporter, we replaced the native $GPD1^{WA}$ regulatory region with $P_{GPD1}{}^{WE}$ in the WA strain (700 bp upstream of the ORF). Microcultivation in SWM revealed that the WA strain carrying the $P_{GPD1}{}^{WE}$ variant had greater expression than WA strains with the native promoter (p-value < 0.001, Wilcoxon signed rank test). Expression levels reached a maximum discrepancy of 18% around 5 h of cultivation (**Figure 3C**) and significant differences were found throughout the cultivation period (p-value < 0.001, Wilcoxon signed rank test). This demonstrates the role of *cis*-regions and the potential to increase *GPD1* expression in foreign genetic backgrounds. We next evaluated the impact of a promoter swap on glycerol yield. $P_{GPD1}{}^{WE}$ was introduced in the WA strain controlling the expression of $GPD1^{WA}$ ORF. Fermentation was carried out for 21 days in SWM, and glycerol together with ethanol yields were estimated. No significant differences were found as raw metabolite levels and yields were similar between strains (**Figure 3D**), suggesting that the promoter itself is not sufficient to increase glycerol yield or that we did not have the experimental power to detect minor glycerol yield differences due to experimental noise.

Subsequently, to evaluate the influence of all promoters on gene expression and to avoid polymorphic *trans*-effects that could modulate mRNA levels, we proceeded to examine expression levels in F1 reciprocal hemizygotes. Interestingly, we observed greater expression in the strains with the WE promoter controlling the reporter gene (p-value < 0.001, Wilcoxon signed rank test, **Figure 4** and **Supplementary Figure S2**). Specifically, expression was higher in the $P_{GPD1}{}^{WE}$-*Luc* x WA-*GPD1* (WE x WA F1 hybrid with the WE *GPD1* promoter controlling luciferase expression) and $P_{GPD1}{}^{WE}$-*Luc* x SA-*GPD1* hemizygotes (WE x SA F1 hybrid with the WE *GPD1* promoter controlling luciferase expression gene) compared to the $P_{GPD1}{}^{WA}$-*Luc* x WE-*GPD1* and $P_{GPD1}{}^{SA}$-*Luc* x WE-*GPD1* hemizygotes (**Figure 4**). No significant differences in expression were found for the WE x NA hemizygotes.

To identify putative transcription factor binding sites that could modulate *GPD1* gene expression, we analyzed the *cis*-regulatory region (up to 200 bp upstream of the ATG start site). This was done via sequence alignment and by predicting binding sites utilizing the YeTFaSCo database. (de Boer and Hughes, 2012). Three SNPs are found between the four strains, and two of these were exclusively present in the WE strain. The closest polymorphism to the ATG start site corresponded to a deletion of two nucleotides in the West African and North American strains (*del-32CC*). Nevertheless, this deletion does not yield polymorphic binding sites for this region, and the same transcription factors would bind in all strains. The other two polymorphisms upstream of *del-32CC* encode a thymine instead of a cytosine (*T-180C*) around nucleotide -180 (from the ATG site) and a cytosine instead of a guanine (*C-202G*) around -202

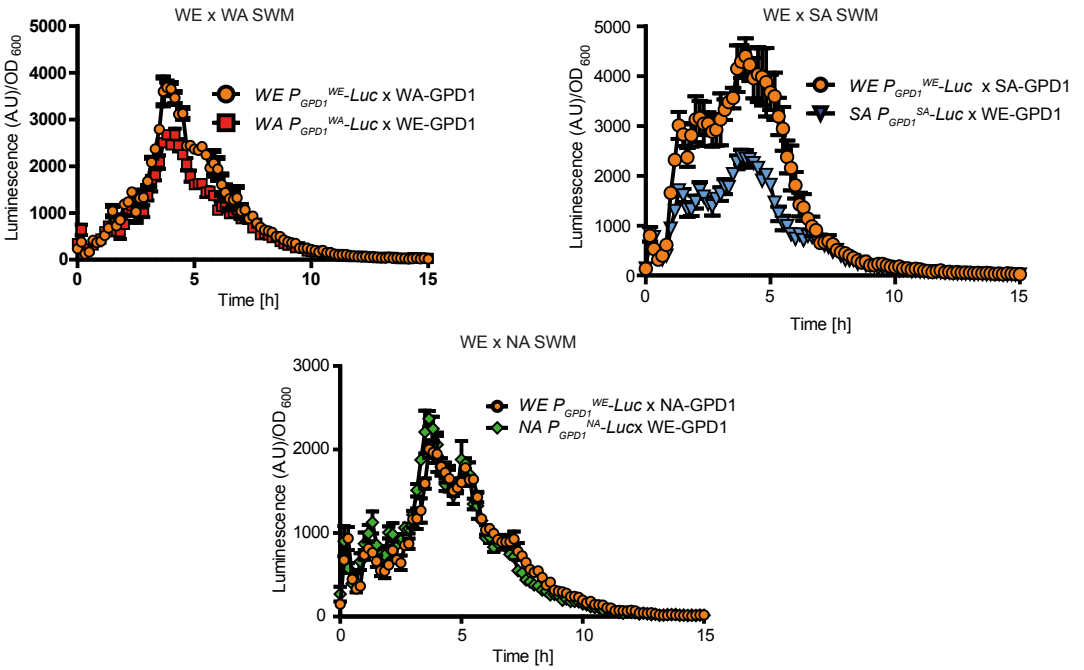

FIGURE 4 | Luciferase reporter assay in *GPD1* reciprocal hemizygotes. Luminescence levels in *GPD1* WE × WA, WE × SA, and WE × NA hemizygotes in micro cultivation in SWM.

in the WE background. The last polymorphism could potentially influence allele specific binding and could alter Crz1 binding, which is a transcription factor associated with the response to ethanol stress (Araki et al., 2009).

Overall, these results suggest the presence of powerful *cis*-factors that increase $GPD1^{WE}$ levels independently of the genetic background. This being said, the lower glycerol yields in WE could be explained by other regulatory mechanisms (negative post-transcriptional regulation, post-translational modifications) or reduced $Gpd1^{WE}$p activity.

Gpd1-mCherry Fusions Suggest Negative Post-transcriptional Regulation on Gpd1p^{WE}

Since a negative correlation was found between *GPD1* expression levels and glycerol yield in the WE strain, we sought to quantify Gpd1 protein levels as a means to detect putative post-transcriptional regulation. For this, we generated a Gpd1p-mCherry fusion in all parental strains by genetically linking mCherry to the Gpd1p C-terminal. Protein levels were estimated in micro-cultivation in SWM. We found significant differences between all strains (p-value < 0.05, Friedman test). During the first hours of incubation, the mCherry fluorescence was strong in the SA and WA strains, whereas the signals in the WE and NA strains were low (**Figure 5A** and **Supplementary Figure S3**). Interestingly, the mCherry fluorescence of the SA and WA strains was two times higher than the WE strain. This indicates that the WE strain produces significantly less Gpd1p, which contrasts with the increased expression of *GPD1* in this strain. Overall, this suggests that post-transcriptional modifications affect protein levels and influence glycerol yields in all strains.

Subsequently, in order to evaluate the role of a dominant or recessive *trans*-factor we quantified protein fluorescence in the WE × WA and WE × SA hemizygotes (**Supplementary Figure S3**). We detected differences in the fluorescence among genotypes; the fluorescence of hemizygotes carrying $Gpd1p^{WA}$ was up to 20% (at 5 h' time point) lower than that of hemizygotes with $Gpd1p^{WE}$. Overall, the fluorescence of $Gpd1p^{WE}$ hemizygote was high throughout the incubation period (**Figure 5B**, p-value < 0.001, Wilcoxon signed rank test). Likewise, after only 3 h of incubation the fluorescence of the WE × SA reciprocal hemizygote carrying $Gpd1p^{WE}$ was 35% greater than that of the hemizygote carrying $Gpd1p^{WE}$, and this trend was significant throughout the entire incubation period (**Figure 5C**, p-value < 0.001, Wilcoxon signed rank test). These results demonstrate the stronger expression induction profile of $pGPD1^{WE}$ and suggests that recessive *trans*-effects, such as post-transcriptional modifications, could negatively impact $Gpd1p^{WE}$ expression levels, which would explain the glycerol yield differences among the *GPD1* allelic variants. We compared the coding *GPD1* sequence between strains and identified six synonymous polymorphisms. Thus, we rule out the possibility of a polymorphism that could be targeted by alternative post-translational modifications (i.e., phosphorylation or acetylation). This being said, one of the synonymous polymorphisms in the $GPD1^{WE}$ allele reduces the CAI respect to the other variants ($GPD1^{WA}$, $GPD1^{NA}$ and $GPD1^{SA}$); specifically, the WE allele carries the ACC codon, whereas the WA, SA and NA alleles carry an ACT codon, yet both code for threonine (T186) (**Figure 5D**).

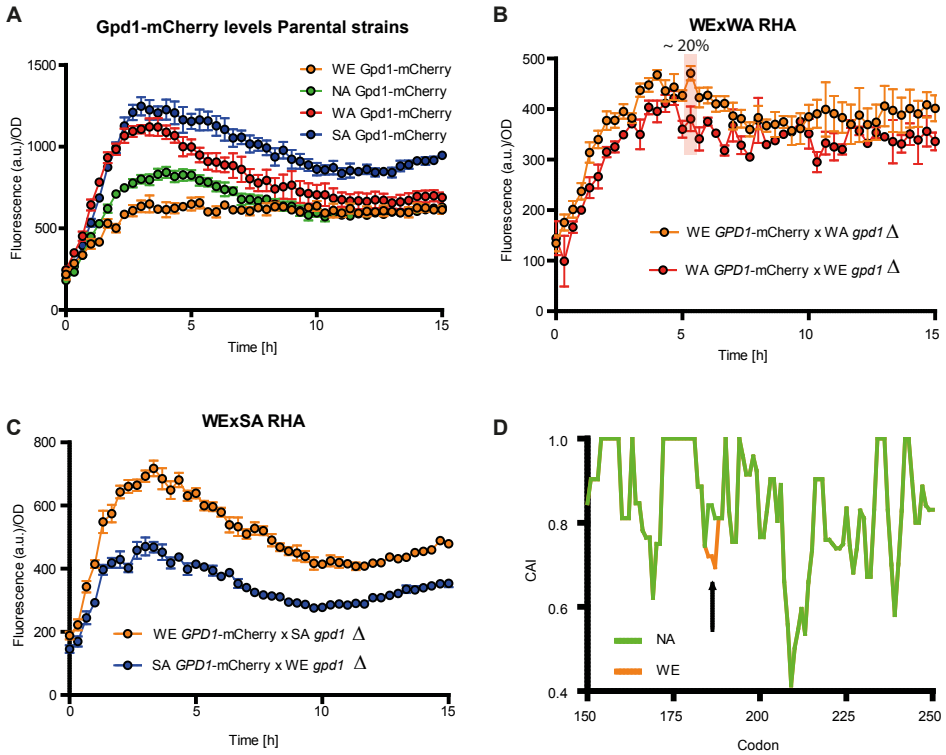

FIGURE 5 | Gpd1p levels in *S. cerevisiae* strains. Gpd1-mCherry levels in **(A)** parental strains, **(B)** WE × WA reciprocal hemizygotes, and **(C)** WE × SA reciprocal hemizygotes in micro cultivation in SWM. **(D)** Codon Adaptation Index (CAI) in NA, WA or SA strains (green) respect to the WE strain for *GPD1* between codons 150 and 250. The arrow indicates the codon for which a CAI difference was found between $GPD1^{NA}$, GPD^{WA} and $GPD1^{SA}$ versus $GPD1^{WE}$.

DISCUSSION

Here, we explored the natural genetic variation of *S. cerevisiae* to determine how ASE can modulate glycerol production. Differences in metabolites production, such as glycerol and ethanol, were found among strains representative of the main genetic clusters recognized for this species. Interestingly, a non-wine strain had greater glycerol yields (**Figure 1B**) yet similar ethanol yields when grown in SWM. This result agrees with previous results by our group for fermentation in high nitrogen concentrations (Salinas et al., 2012). Despite this, here the wine strain yielded more glycerol than that found in the previous study, suggesting that glycerol production is influenced by nitrogen concentration. Indeed, it has been shown that the ratio of carbon to nitrogen in wine musts can significantly alter fermentation performance (Varela et al., 2004), and it is well known that strains differ in their ability to assimilate nitrogen (Crepin et al., 2012; Cubillos et al., 2017; Brice et al., 2018). During fermentation, strains can retain glycerol when under osmotic stress by decreasing the glycerol dissimilation and therefore total extracellular glycerol yields, thus impacting the final product of the fermentation (Nevoigt and Stahl, 1997; Hohmann, 2002). Yet, our results were obtained under static and small volume fermentations, and therefore we believe some differences can be expected when scaling up to larger industrial volumes.

Phenotypic differences between yeast strains can originate from polymorphic coding or non-coding regions (Thompson and Cubillos, 2017). In the analyzed strains, we found only synonymous polymorphisms in the coding portion of *GPD1*. Therefore, it is likely that polymorphisms in the regulatory region are the cause of the observed genotype – phenotype variation, however, we cannot rule out that translation speed could impact protein levels. Indeed, previous reports have demonstrated that expression variants can directly impact phenotypic differences between yeast isolates (Gerke et al., 2009; Salinas et al., 2016; Cubillos et al., 2017). In an earlier study, we have demonstrated that the four strains chosen for this study have different ASE levels (Salinas et al., 2016; Cubillos et al., 2017), and this can directly impact oenological phenotypes such as nitrogen assimilation or fermentation capacity (Salinas et al., 2016). The results presented herein of glycerol yields are consistent with this. The non-wine alleles of *ADH3* and *GPD1* in reciprocal hemizygotes (both exhibit ASE in at least a single cross involving the wine strain, **Supplementary Table S3**) produced higher glycerol levels, consumed lower amounts of sugar, and exhibited greater glycerol yields. In the case of the $ADH3^{SA}$ and $GPD1^{SA}$ alleles, we observed an antagonistic effect relative to the glycerol yields reported in parental strains (**Figures 1**, **2**). Antagonistic alleles and QTLs, refer as those alleles with a different effect from their parental origin, have been extensively described in yeast for different

phenotypes and crosses (Liti and Louis, 2012) and together with other unlinked variants can expand the phenotypic landscape (Cubillos et al., 2011). These results together suggest that strains differ in their metabolic fluxes, and *cis*- and *trans*-regulation significantly impacts glycerol yields. Moreover, our results demonstrate that non-wine alleles can be potential targets of genetic improvement aimed at increasing glycerol yields. Indeed, several studies have targeted *GPD1* over-expression in wine strains to favor glycerol production. For example, introduction of a high copy number vector containing the coding portion of *GPD1* controlled by the *ADH1* promoter into the commercial wine strains K1M, VL1 and BC increases glycerol production by three-fold, while ethanol production is reduced (Cambon et al., 2006). The effective modulation of glycerol and ethanol production was affected by an increase in the production of undesirable secondary metabolites exceeding thresholds allowed for wine. Specifically, the production of acetate, acetaldehyde, and acetoin due to the redox imbalance generated by the overproduction of glycerol confers unacceptable aromas and flavors to wine. Alternatively, the overexpression of *GPD1* complemented by the overexpression of *BDH1* increases acetoin reduction to produce 2,3-butanediol, a compound that has neutral sensory properties (Ehsani et al., 2009). Yet, similar approaches should be targeted for natural variants. Here, we have identified differences in glycerol yields between *GPD1* variants and evaluated their effect in different genetic backgrounds.

Previous QTL mapping efforts have identified *GPD1* variants affecting glycerol and ethanol production, however the effect of these polymorphisms is unclear (Hubmann et al., 2013b). More difficult than generating genetically modified strains, however, is identifying and quantifying the polymorphisms within *GPD1* that underlie phenotypic differences. In this context, regulatory regions are known to finely influence phenotypes (Wray, 2007; Gerke et al., 2009; Salinas et al., 2016), and here we suggest that differences upstream the ATG start site are partly responsible for expression differences between strains. From the luciferase reporter assay we show increased expression in strains containing the wine allele. This result is in contrast with the lower glycerol yields found for reciprocal hemizygotes containing the wine alleles (**Figure 2**). Interestingly, greater expression of $GPD1^{WE}$ was found in parental strains and reciprocal hemizygotes, suggesting a robust *cis* effect (**Figures 3**, **4**). Indeed, our results agree with other reports in model organisms demonstrating that *cis*-variants explain a large proportion of expression differences between alleles (Brem et al., 2002; Yvert et al., 2003; Kliebenstein, 2009; McManus et al., 2010; Goncalves et al., 2012; Cubillos et al., 2014; Thompson and Cubillos, 2017), however, *trans*-eQTLs impact the expression of a greater number of genes (Brem et al., 2002; Yvert et al., 2003). Remarkably, we observed that *GPD1* was only expressed in strains grown in fermentation conditions and not in laboratory settings. The wine strain responded positively to fermentation and activation of the *GPD1* promoter was high; the mRNA levels of this strain exceeded those of other strains (**Figure 3**).

The comparison of allele expression allowed us to identify at least three different *GPD1* regulatory variants (**Figure 3**). The existence of these unique variants indicates that fine-tuning gene expression utilizing natural variants is possible. While significant differences in expression among variants were evident from the gene expression profiles (**Figure 3**), these expression patterns did not fully reflect the relative glycerol yield differences when the alleles were introduced into other strains (**Figure 3D**). One possibility for this discrepancy is that our experimental approach was insufficient to identify mild phenotypic differences due to *cis* regulation; thus, more sensitive experiments should be conducted in the future. Also, *cis*-regulatory variants can be found up to10 kb from the targeted gene and therefore by only considering 700 bp upstream the ATG start site we might be missing variants with a stronger effect upon glycerol production (Zheng et al., 2010), yet variants with stronger effects upon gene expression and phenotypes are mostly found nearby regulated genes. A more likely hypothesis is the existence of a *trans*-factor, which would agree with the patterns observed in the allele swap experiments and the lack of a positive correlation between *GPD1* expression levels, Gpd1p and glycerol yields. From the Gpd1p-mCherry fusions we found low protein levels only in the parental WE strain and not in the reciprocal hemizygotes (**Figure 3**). This suggests that a *cis*-active module strongly induces *GPD1* mRNA expression in the WE strain however a recessive post-transcriptional *trans*-acting factor could be downregulating Gpd1p decreasing glycerol yields. One could argue that technical settings could be responsible for differences between the *pGPD1-Luc* expression patterns (estimated under 200 μL in microcultivation conditions) and glycerol yields (estimated in fermentations utilizing 12 mL), however, it has been previously demonstrated that biomass and cells physiological states under microcultivation conditions correlate with larger volumes cultures (Warringer and Blomberg, 2003) and many of these findings are relevant under wine fermentation conditions (Gutierrez et al., 2013; Ibstedt et al., 2015; Brice et al., 2018; Peltier et al., 2018) and many other environments (DeLuna et al., 2010 #1056). In this context, we found a positive correlation between our Gpd1p-mCherry fusions and glycerol yields, suggesting that both set-ups would be comparable. Indeed, our findings are in line with several studies that demonstrate that mRNA levels do not necessarily correlate with protein levels due to post-transcriptional regulation that directly impacts the phenotypic outcome. As such, low protein levels can result from accelerated mRNA degradation (Liu et al., 2016). Indeed, post-transcriptional regulation of glucose production has been demonstrated in yeast, where gluconeogenic mRNA targets, such as *FBP1* and *PCK1*, are degraded (Yin et al., 2000). Apparent *GPD1* epistatic interactions have also been observed in other similar studies (Hubmann et al., 2013b). Overall, the identification of the mechanisms regulating *GPD1* ASE is a challenge. This being said, our luciferase kinetics approach did allow us to determine expressions pattern through time. It has recently been reported that time-resolved experiments are significantly more informative than genetic

perturbations for inferring metabolic adaptation (Goncalves et al., 2017); thus, we were able to profile how *GPD1* regulatory regions respond to environmental perturbations through time in different genetic backgrounds.

Based on our gene expression and protein fusions assays conducted in SWM, we show that natural *GPD1* variants produce different glycerol levels and yields. Depending on the strain, this variation in glycerol production is controlled by *cis* and/or *trans* regulators, thought the *trans*-factors involved remain to be identified. These *trans*-factors likely module mRNA degradation decreasing overall Gpd1p levels. Identification of these factors requires further approaches, such as QTL mapping or Genome wide association studies involving a large number of wine strains. Indeed, our previous findings demonstrate that *RIM15* is responsible for differences in glycerol production in a WE x SA recombinant population (Salinas et al., 2012; Kessi-Perez et al., 2016). Future studies of epistatic interactions could help to determine whether differences among strains are due to *trans*-factors. Nevertheless, the set of *GPD1* regulatory variants characterized here can be used in different strains to modulate *GPD1* expression (in fermentation conditions) and glycerol production. It remains to be explored if these observations can be applied in the wine industry under larger fermentations and real industrial settings.

AUTHOR CONTRIBUTIONS

FS, VG, and FC designed the experiments. ST, MC, FS, VA, VD, CB, and VR performed the experiments. ST, FS, VG, CM, LL, and FC discussed the results and experiments. FS and FC wrote the paper. CM, LL, and FC provided reagents.

FUNDING

This work was supported by grants from Comisión Nacional de Investigación Científica y Tecnológica CONICYT FONDECYT (Grant 11140097) to FC, CONICYT FONDECYT (Grant 1150522) to CM, CONICYT FONDECYT (11130148) to VG, CONICYT FONDECYT (11170158) to FS. This research is funded by MIISSB Iniciativa Científica Milenio-MINECON to FC and LL. LL is an International Research Scholar of the Howard Hughes Medical Institute. Experiments in Cyation3 were funded by FONDEQUIP (EQM130158). VD was supported by CONICYT/Ph.D. scholarship (6313018).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.01460/full#supplementary-material

FIGURE S1 | *GPD1* gene expression profiles in **(A)** parental SWM, **(B)** parental YNB, and **(C)** allele swapped strains in SWM. Raw luminescence and OD values are provided.

FIGURE S2 | *GPD1* gene expression profiles in reciprocal hemizygotes. Raw luminescence and OD values are provided.

FIGURE S3 | Gpd1p protein levels in parental and reciprocal hemizygote strains. Raw fluorescence and OD values are provided.

TABLE S1 | Primers used in this study.

TABLE S2 | Physico-chemical characteristics of must after fermentations.

TABLE S3 | Genes involved in ethanol and glycerol production in yeast and evaluated for ASE.

TABLE S4 | Reciprocal hemizygotes' physico-chemical characteristics of must after fermentations.

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

The reviewer PM and handling Editor declared their shared affiliation.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 13 July 2018
doi: 10.3389/fmicb.2018.01563

Quantifying the Effects of Ethanol and Temperature on the Fitness Advantage of Predominant *Saccharomyces cerevisiae* Strains Occurring in Spontaneous Wine Fermentations

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OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: 03 April 2018
Accepted: 25 June 2018
Published: 13 July 2018

Citation:
Ganucci D, Guerrini S, Mangani S, Vincenzini M and Granchi L (2018) Quantifying the Effects of Ethanol and Temperature on the Fitness Advantage of Predominant Saccharomyces cerevisiae Strains Occurring in Spontaneous Wine Fermentations Front. Microbiol. 9:1563. doi: 10.3389/fmicb.2018.01563

Different *Saccharomyces cerevisiae* strains are simultaneously or in succession involved in spontaneous wine fermentations. In general, few strains occur at percentages higher than 50% of the total yeast isolates (predominant strains), while a variable number of other strains are present at percentages much lower (secondary strains). Since *S. cerevisiae* strains participating in alcoholic fermentations may differently affect the chemical and sensory qualities of resulting wines, it is of great importance to assess whether the predominant strains possess a "dominant character." Therefore, the aim of this study was to investigate whether the predominance of some *S. cerevisiae* strains results from a better adaptation capability (fitness advantage) to the main stress factors of oenological interest: ethanol and temperature. Predominant and secondary *S. cerevisiae* strains from different wineries were used to evaluate the individual effect of increasing ethanol concentrations (0-3-5 and 7% v/v) as well as the combined effects of different ethanol concentrations (0-3-5 and 7% v/v) at different temperature (25–30 and 35°C) on yeast growth. For all the assays, the lag phase period, the maximum specific growth rate (μ_{max}) and the maximum cell densities were estimated. In addition, the fitness advantage between the predominant and secondary strains was calculated. The findings pointed out that all the predominant strains showed significantly higher μ_{max} and/or lower lag phase values at all tested conditions. Hence, *S. cerevisiae* strains that occur at higher percentages in spontaneous alcoholic fermentations are more competitive, possibly because of their higher capability to fit the progressively changing environmental conditions in terms of ethanol concentrations and temperature.

Keywords: ***Saccharomyces cerevisiae*** **strains, spontaneous wine fermentation, fitness advantage, temperature, ethanol**

INTRODUCTION

Spontaneous grape juice fermentation into wine is carried out by the yeast populations naturally occurring on the grape surface and in the winery environment (Sabate et al., 2002; Bisson, 2012). In this process, in the vats filled at the beginning of the vintage, non-*Saccharomyces* yeast species usually predominate in the early stages and later, with ethanol increasing, they are replaced by *Saccharomyces cerevisiae* because of higher resistance of this yeast species to alcohol (Pretorius, 2000; Bisson, 2005; Querol and Fleet, 2006; Albergaria and Arneborg, 2016). This substitution may be explained by the competitive exclusion of the less efficient yeasts species (Arroyo-López et al., 2011). Although ethanol production has been the cause traditionally accepted for explaining the imposition of *S. cerevisiae* on non-*Saccharomyces* yeast species, other death-inducing mechanisms have been proposed as responsible for its competitive advantage, including the production of antimicrobial compounds, such as SO_2 and peptides, the cell-to-cell contact, and the temperature increase during alcoholic fermentation (Goddard, 2008; Salvadó et al., 2011; Perrone et al., 2013; Branco et al., 2015; Williams et al., 2015; Albergaria and Arneborg, 2016; Pérez-Torrado et al., 2017). Therefore, as the fermentation progresses, the grape must becomes a more selective environment representing a highly specialized ecological niche (Salvadó et al., 2011). Nevertheless, *S. cerevisiae* populations generally display a high polymorphism in spontaneous wine fermentations. Indeed, numerous studies, carried out by molecular techniques on the population dynamics of *S. cerevisiae* during spontaneous wine fermentations in several regions all over the world, have established that different strains are simultaneously or in succession involved during the whole fermentation process (Querol et al., 1994; Pramateftaki et al., 2000; Augruso et al., 2005; Schuller et al., 2005; Agnolucci et al., 2007; Csoma et al., 2010; Orlić et al., 2010; Capece et al., 2011, 2012; Mercado et al., 2011; Bisson, 2012). In some cases *S. cerevisiae* strains were able to dominate the alcoholic fermentation in all vats of the same winery, independently of the grapevine cultivar (Frezier and Dubourdieu, 1992; Guillamón et al., 1996), whereas other times the yeast strains were found to be specific for each grape variety (Blanco et al., 2006). In general, few *S. cerevisiae* strains occur at higher percentages (more than 30–50% of the total yeast isolates) while a variable number of strains are present at lower percentages. Therefore, these strains can be differentiated in "predominant" and "secondary" strains, respectively (Versavaud et al., 1995). In addition, the predominant strains can sometimes persist in alcoholic fermentations carried out in the same winery in consecutive years and can be described as "recurring" strains (Gutièrrez et al., 1999; Bisson, 2012). Since *S. cerevisiae* strains, participating in alcoholic fermentations, may differently affect the chemical and sensory qualities of resulting wines (Fleet, 2003; Romano et al., 2003; Villanova and Sieiro, 2006; Lopandic et al., 2007; Barrajón et al., 2011; Knight et al., 2015; Bokulich et al., 2016; Callejon et al., 2016), it is of great importance to assess whether the predominant strains retain the dominant behavior after their isolation from grape must fermentations. Furthermore, it could be noteworthy to investigate whether the predominance of these *S. cerevisiae* strains on others results from a different adaptation capability (fitness advantage) to some stress factors of oenological interest. Recently, two studies concerning competition between strains of *S. cerevisiae* species suggest that the dominance of one strain over another is dependent on the different SO_2 production and resistance and on the cell-to-cell contact in mixed cultures, i.e., in the same environment (Perrone et al., 2013; Pérez-Torrado et al., 2017). With the exception of studies on killer factors (Jacobs and van Vuuren, 1991; Pérez et al., 2001), to our knowledge, other surveys on the competition among strains of *S. cerevisiae* species are lacking. Considering that temperature and ethanol during alcoholic fermentation are held responsible for the ability of *S. cerevisiae* to dominate on non-*Saccharomyces* yeasts or on other *Saccharomyces* spp. (Goddard, 2008; Williams et al., 2015; Alonso del-Real et al., 2017; Henriques et al., 2018), the adaptability to these two factors could be also involved in determining the dominance of predominant on secondary *S. cerevisiae* strains. Moreover, the predominance of *S. cerevisiae* strains with particular resistance capability to these two stress factors could contribute to the construction of an ecological niche typical of each fermentation tank and possibly winery.

Therefore, the aim of this study was to investigate whether the predominance of some *S. cerevisiae* strains during spontaneous alcoholic fermentation results from a better adaptation capability of these strains to ethanol and temperature as stress factors. At first, dynamics of *S. cerevisiae* strains during spontaneous wine fermentations carried out in six Tuscan wineries were monitored to identify one predominant and one secondary *S. cerevisiae* strains from each winery. After that, the predominant and secondary strains of each winery were tested in synthetic media to compare their growth capability when subjected to stress of ethanol and temperature. Finally, the fitness advantage (as defined by Salvadó et al., 2011) was calculated to verify if the predominant strains owned a better adaptation capability than the secondary strains to the two main stress factors of oenological interest.

METHODS

Isolation of *Saccharomyces cerevisiae* From Spontaneous Wine Fermentations

Spontaneous wine fermentations were carried out under industrial conditions during the same vintage in six wineries (A, B, C, D, E, and F) producing DOC and DOCG red wines in Tuscany region (Central Italy). In all the winery except the E, commercial starter yeasts were never used. In each winery, various fermentation tanks (6 in winery A; 2 in B; 8 in C; 6 in D; 4 in E; 6 in F) were filled with musts from different grape varieties (S: *Sangiovese*; CA: *Cabernet*; N: *Pinot Nero*; M: *Merlot*; V: *Vermentino*). Yeasts were isolated by plating the must/wine samples on WL Nutrient Agar medium (Oxoid Ltd, Basingstoke, Hampshire, UK) containing sodium propionate (2 g/L) and streptomycin (30 mg/L) to inhibit mold and bacterial growth, respectively. Plates were incubated for 48 h at 30°C, under aerobic conditions. *S. cerevisiae* isolates were identified

by PCR-RFLP analysis of the rDNA Internal Transcribed Spacer (ITS) according to Esteve-Zarzoso et al. (1999).

About 25 isolates from each fermentation tank belonging to *S. cerevisiae* species were stored in liquid cultures containing 50% (v/v) glycerol at −80°C until use.

Genotypic Characterization of *S. cerevisiae* Isolates

Genotypic differentiation of *S. cerevisiae* isolates was performed by mitochondrial DNA restriction analysis (mtDNA-RFLP) and the restriction endonucleases *Rsa*I and *Hinf*I (Granchi et al., 2003). The restriction DNA fragments were separated on 0.8%(w/v) agarose gels containing ethidium bromide (1 mg mL^{-1}) by electrophoresis in 1X-TBE buffer (90 mMTris-borate, 2 mM, EDTA pH 8.0) at 4 Vcm^{-1} for 6 h. The RFLP patterns were submitted to pairwise comparison using the Dice coefficient (SD) (Sneath and Sokal, 1973) and cluster analysis with unweighted pair group method (UPGMA) by Gel Compar 4.0 software (Applied Math, Kortrijk, Belgium). *S. cerevisiae* diversity in each winery was quantified by using the two indices "H" and "e" as proposed by Shannon–Weaver (Shannon and Weaver, 1963).

Laboratory-Scale Fermentations to Verify the Predominance Behavior of *S. cerevisiae* Strains

The medium used for laboratory scale fermentation was the chemically defined grape juice medium reported in the **Table 1** of the RESOLUTION OIV-OENO 370 (2012). The synthetic medium was buffered to pH 3.3 using HCl 1N and sterilized by filtration. Fermentation experiments were carried out in triplicate in 250-mL Erlenmeyer flasks containing 160 mL of the medium. Each flask was inoculated with two *S. cerevisiae* strains at the same concentration (10^4 cells mL^{-1}) from pre-cultures grown for 24 h in the same medium. After inoculation, the flasks were sealed with a Müller trap previously filled with sulphuric acid to allow only CO_2 to outflow and they were incubated at 28°C. The fermentation progress was followed by determining the weight loss due to CO_2 release until the weight remained constant. At the end of fermentation, chemical analysis were performed by HPLC (Schneider et al., 1987; Granchi et al., 1998). Viable counts of the yeasts were performed, after 24 h and 10 days from inoculation, on WL Nutrient Agar medium (Oxoid Ltd, Basingstoke, Hampshire, UK) incubated 48 h at 30°C in aerobic conditions. To calculate isolation frequencies of the two *S. cerevisiae* strains inoculated together in the each fermentation flask, a significant number of colonies from WL Nutrient Agar medium were assayed using mtDNA-RFLP as reported above.

Effect of Ethanol on the *S. cerevisiae* Growth

The medium used to assay the effect of ethanol on the growth of the different *S. cerevisiae* strains was Yeast Nitrogen Base (Difco) integrated with glucose (20 g L^{-1}) and increasing concentrations of ethanol (0-3-5 and 7% v/v). Fermentation trials were carried out at 28°C in triplicate in 100-mL Erlenmeyer flasks containing synthetic medium (50 mL each flask) inoculated with 2×10^6 cells mL^{-1} (axenic cultures) from pre-cultures of the various *S. cerevisiae* strains grown for 24 h in the same medium without ethanol. Fermentation progress was monitored every 2 h quantifying by HPLC sugar degradation (Lefebvre et al., 2002). On the same samples, viable cells were determined by Thoma counting chamber and fluorescence microscopy to monitor the yeast growth as reported by Granchi et al. (2006). The decimal logarithms of viable counts detected during the time course of each fermentation after 8 and 24 h were fitted both to Baranyi and Roberts (1994) function and to reparametrized Gompertz equation proposed by Zwietering et al. (1990) by using Combase-DMfit software and GraphPadPrism 5, respectively. Finally, the area under the growth curve of each strain was calculated as reported by Arroyo-López et al. (2009, 2010) and Castilleja et al. (2017), using GraphPadPrism 5.

Combined Effect of Ethanol and Temperature on *S. cerevisiae* Growth

To evaluate the combined effect of temperature and ethanol on the *S. cerevisiae* growth, a Box-Wilson Central Composite Design with two variables and three levels was used. The medium used was Yeast Nitrogen Base (Difco) integrated with 20 g mL^{-1}. The range of temperatures was 25–35°C, while the range of ethanol was 0–7% (v/v). As reported in the previous experiment, fermentation progress was monitored every 2 h (for 24 h) quantifying by HPLC the sugar degradation, while the viable yeast cells were determined by Thoma counting chamber and fluorescence microscopy. The decimal logarithms of viable yeast cells detected during the time course of each fermentation were fitted to Gompertz function using GraphPadPrism 5. The area under the growth curve of each strain was calculated using GraphPadPrism 5.

RESULTS

Predominant *S. cerevisiae* Strains in Spontaneous Wine Fermentations

Dynamics of yeasts in spontaneous wine fermentations carried out during the same vintage in six wineries producing DOC and DOCG red wines in Tuscany region (Italy) were monitored. For each winery, from two to eight fermentation tanks were filled with musts obtained from different grape variety (*Sangiovese*, *Cabernet*, *Pinot Nero*, *Merlot*, *Vermentino*), and allowed to ferment naturally. When the yeast population reached the maximum growth yield, 637 isolates belonging to *S. cerevisiae* species (about 25 isolates from each fermentation tank) were analyzed by mitochondrial DNA (mt-DNA) restriction analysis. The mt-DNA profiles obtained for each tank in the different wineries and the relative frequencies of isolation expressed as percentages are reported in table 1. Results revealed that, independently of the winery and the grape variety, each spontaneous wine fermentations was carried out by one or two predominant *S. cerevisiae* strains at high frequency, ranging from about 30–90%, in association with a variable number of secondary strains at low frequency. Moreover, some predominant strains were shared by different grape varieties fermented in

TABLE 1 | Isolation frequencies, expressed as percentages, of mt-DNA profiles of *S. cerevisiae* from 32 spontaneous wine fermentations carried out in different tanks during the same vintage in six wineries in Tuscany (Italy).

Sample code	mt-DNA profiles	Isolation frequency (%)
AS1	**AIV**- AV - AVI - AVII - AXII - AXIII	**46** - 17 - 17 - 4 - 8 - 8
AS2	**AIV** - AV - AVI - AVII - AXII - AXIII	**29** - 8 - 21 - 17 - 12.5 - 12.5
AM1	AI - AIV - AV - AVII - **AX** - **AXII** - AXIV	4 - 4 - 13 - 13 - **36** - **26** - 4
AM2	AVI - AVII - **AX** - AXIV	4 - 12.5 - **79.5** - 4
AP1	**AX** - AXI	**90** - 10
AP2	AIV - AX - AXI - **AXII** - AXIII	16.5 - 16.5 - 8.5 - **42** - 16.5
BS1	BI - **BII** - BIII - **BIV** - BVII - BVIII - BIX	10 - **35** - 5 - **35** - 5 - 5 - 5
BS2	BII - BIV - BV - BVI - BVII - BVIII - BX BXI - BXII - BXIII - BXIV - BXV - BXVI	20.2–3.8 - 3.8–15.2 - 11.4–7.6–3.8 3.8 - 15.2 - 3.8 - 3.8 - 3.8 - 3.8
CS1	CI - CII - CIII - CIV - CVI - **CVII**	10 - 5 - 10 - 25 - 5 - **45**
CS2	CI - CII - **CIII** -CIV - CV - CVII	10 - 5 - **60** - 5 - 5 - 15
CS3	CI - **CIII** - CIV - CV	10.6 - **73.5** - 10.6 - 5.3
CS4	CI - **CIII**	10 - **90**
CM1	CI - **CIII**	10 - **90**
CM2	CI - **CIII**	5 - **95**
CM3	CI - **CIII**	16 - **84**
CM4	CI - **CIII**	5 - **95**
DS1	DI - DIV - DVI - DX	4.2 - 83.2 - 4.2 - 8.4
DS2	**DIV** - DVI - DVII	**69.6** - 17.4 - 13
DS3	DI - DII - **DIV** - DVI - DVII - DXI	12.5 - 4.2 - **41.6** - 12.5 - 25 - 4.2
DS4	DI - DIII - **DIV**- DVI -DVII - DVIII	22.5 - 9 - **32.5** - 13.5–18 - 4.5
DM1	**DI** - DII - DIII - DIV - DV	**50** - 5 - 5 - 35 - 5
DM2	DI - DIII - **DIV** - DVI - **DVII** - DIX	4.25 - 4.25 - **46** - 4.25 - **37** - 4.25
ES1	**EI** - **EVII**	**40** - **60**
EC2	**EI** - EIII - EVII	**71.5** - 9.5 - 19
EM1	**EI** - EII - EIII - EIV - EV - EVI	**68.5** - 14 - 7 - 3.5 - 3.5 - 3.5
EM2	**EI** - EIII	**96** - 4
FVN1	FI - **FII** - FIII - FIV - FV	**42** - 8 - 8 - **34** - 8
FVN2	FI - **FII** - FIV	**60** - 10 - **30**
FVN3	FI - **FIII** - FIV - FVII - FVIII	**44** - 25 - 19 - 6 - 6
FVB1	FI - **FIII** - FIV - FVII	13.5 - **40** - **40** - 6.5
FVB2	FI - **FIII** - FIV	6.5 - **53.5** - **40**
FVB3	FI - FII - FIII - **FIV** - FV	13.5 - 6.5 - 26.5 - **47** - 6.5

The sample codes indicate the winery (A, B, C, D, E and F), the grape variety (Sangiovese: S, Cabernet: C, Pinot Nero: P, Merlot: M, Vermentino nero: VN and Vermentino bianco: VB), and the number of the fermentation tank. The predominant S. cerevisiae strains are in bold and underlined.

various tanks (**Table 1**). By calculating the isolation frequency of each different mt-DNA profile occurring in each winery, a total of 58 *S. cerevisiae* strains out 637 isolates from the six wineries were obtained (**Table 2**). Then, they were distributed in three frequency classes: strains at low frequency (<10%), strains at frequency ranging from 10 to 30% and predominant strains at frequency>30% (**Table 3**). Although according to Shannon's index "*H*," estimating the richness of *S. cerevisiae* strains found in the six wineries, different diversity level was observed, only one *S. cerevisiae* strain emerged as clearly predominant in each winery except for the cellar B. Indeed, the evenness index "*e*," ranging between 0 and 1 and that increases with the decreasing of the number of isolates showing the same mt-DNA, assumed the highest value in the winery B in which the predominant strain occurred at the lowest frequency value found. All the mt-DNA profiles corresponding to the different *S. cerevisiae* strains were also analyzed using UPGMA clustering analysis and the resulting dendrogram is reported in **Figure 1**. In this elaboration were also included the mt-DNA profiles of six commercial starter strains most commonly used in Tuscany. The *S. cerevisiae* strains, at 60% of similarity, grouped into 13 clusters mainly based on the winery where they come from, independently on the grape variety used. In particular, the *S. cerevisiae* strains isolated from the winery B were included in the clusters 6-7-8 and 9, while the commercial starter strains grouped in the same cluster.

In conclusion, according to these results, for each winery one predominant strain, indicated by the code HF (High Frequency), and one secondary strain, indicated by the code LF (Low Frequency), were chosen with the aim to compare their behavior in subsequent trials. The HF-*S. cerevisiae* strains displayed the following mt-DNA profiles: AX – BII – CIII – DIV – EI and FIV,

TABLE 2 | Distribution of different mt-DNA profiles of *S. cerevisiae* in the six wineries (A, B, C, D, E, and F) in the same vintage.

Winery code											
A		B		C		D		E		F	
mt-DNA profiles	Isolation frequency (%)	mt-DNA profiles	Isolation frequency (%)	mt-DNA profiles	Isolation frequency (%)	mt-DNA profiles	Isolation frequency (%)	mt-DNA profiles	Isolation frequency (%)	mt-DNA profiles	Isolation frequency (%)
AI	1	BI	4.3	CI	9.5	DI	14.5	EIV	1.0	FI	25
AIV	18	BII	**26**	CII	2.0	DII	1.5	EII	4.0	FII	3
AV	7.5	BIII	2.2	CIV	5.0	DIII	3.0	EIII	5.0	FIII	29
AVI	8.5	BIV	17.4	CIII	**74**	DIV	**52**	EI	**71**	FIV	**36**
AVII	9.5	BV	2.2	CV	1.3	DV	0.7	EV	1.0	FV	2
AX	**32**	BVI	8.7	CVI	0.7	DVI	8.7	EVI	1.0	FVII	3
AXI	2	BVII	8.7	CVII	7.5	DVII	16	EVII	17	FVIII	2
AXII	14	BVIII	6.5			DVIII	0.7				
AXIII	6	BIX	2.2			DIX	0.7				
AXIV	1.5	BX	2.2			DX	1.5				
		BXI	2.2			DXI	0.7				
		BXII	8.7								
		BXIII	2.2								
		BIV	2.2								
		BXV	2.2								
		BXVI	2.2								

Predominant strains are in bold and underlined.

TABLE 3 | Number of mt-DNA profiles of *S. cerevisiae* at different isolation frequency in the six wineries and related indices Shannon and Weaver (1963).

Winery	Frequency (%)			*H*	*e*
	<10	10–30	>30		
A	7	2	1	1.86	0.81
B	14	2	-	2.35	0.85
C	6	-	1	1.02	0.49
D	8	2	1	1.73	0.61
E	5	1	1	1.01	0.46
F	4	2	1	1.44	0.74

H, biodiversity index; e, evenness.

while the LF-*S. cerevisiae* strains corresponded to the mt-DNA profiles AI – BI – CVI – DXI – EVI and FV.

Laboratory Scale Fermentation to Verify the Predominance Behavior of HF-*S. cerevisiae* Strains on LF-*S. cerevisiae* Strains

To verify whether the *S. cerevisiae* strains identified as HF were actually able to dominate on the strains identified as LF, laboratory-scale co-fermentations were performed. One HF and one LF strain isolated from each winery were co-inoculated in synthetic must at the same cell concentration (10^4 CFU/mL). This value was chosen in order to simulate the low *S. cerevisiae* cell densities usually found in spontaneous alcoholic fermentation. Co-fermentations carried out at 28°C by the strains from the wineries A, C, D, and F were completed in about 10 days, even if the strains from the wineries D and F showed lower fermentation rates than those from the wineries A and C (data not shown). On the contrary, the strains from winery B were unable to complete alcoholic fermentation (20% w/v of reducing sugars). During the fermentations, samples were taken at two different times (after 24 h and 10 days from the inoculation) in order to assess mt-DNA patterns of the *S. cerevisiae* isolates as well as their isolation frequencies. **Figure 2** shows the isolation frequencies of HF and LF strains assayed for each fermentation after 24 h and 10 days from the inoculation. Although the starting inoculum of HF and LF strains was at the same cell concentration, after 24 h the isolation frequencies of the LF strains were lower than 35% in all the fermentations. After 10 days the HF strains isolated from A, B, D, and F winery showed isolation percentages of 100%, while HF strains from C and E of 96%. Therefore, the results demonstrated that during the fermentative process all the HF-*S. cerevisiae* strains occurred progressively at higher percentages demonstrating to retain in laboratory the "predominance behavior" displayed in industrial fermentations.

Effect of Ethanol on the Growth Performance and the Fitness Advantage of HF and LF-*S. cerevisiae* Strains

The HF and LF-*S. cerevisiae* strains of the experiment previously described were also used to perform axenic fermentations in synthetic media containing various concentrations of ethanol (0-3-5 and 7% v/v). The aim of these trials was to investigate on

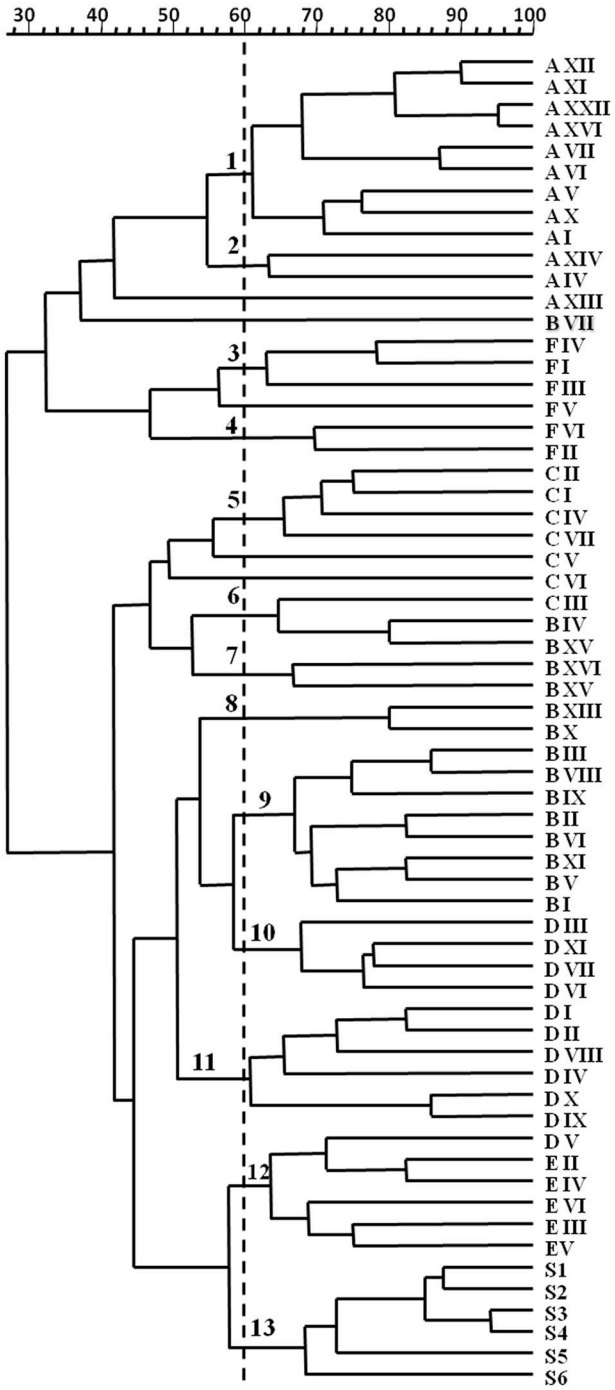

FIGURE 1 | Dendrogram from UPGMA clustering analysis, based on Dice coefficient of mt-DNA *Rsa*I restriction patterns of the *S. cerevisiae* isolates from 32 spontaneous wine fermentations carried out in six different wineries (A, B, C, D, E, and F) in Tuscany (Italy). S1-S6 indicate commercial starter cultures. Arabic numerals at 60% similarity indicate the different clusters.

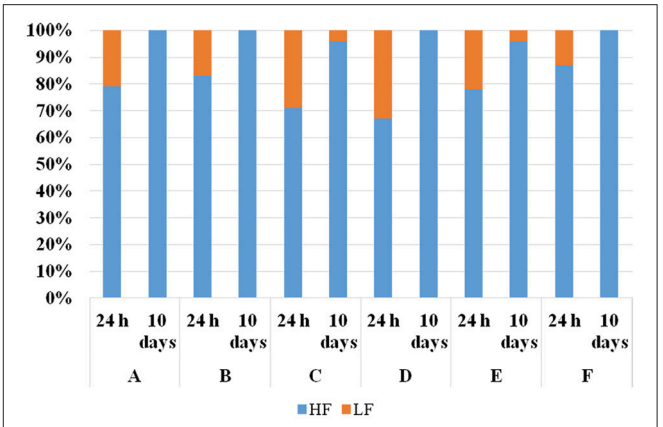

FIGURE 2 | Isolation frequencies of one "high frequency"(HF)-*S. cerevisiae* strain and one "low frequency " (LF) *S. cerevisiae* strain, representative of each winery (A, B, C, D, E and F) after 24 h and 10 days in co-fermentations in synthetic must at 28°C. The "HF" and "LF" strains were inoculated at the same cell concentration (10^4 cell/mL).

the growth performance and on the fitness of HF and LF-strains, in order to detect any behavior justifying the different isolation frequencies observed during the spontaneous fermentations.

Baranyi and Roberts-model was used to estimate the fermentative performance of the strains in terms of lag phase (λ), maximum specific growth rate (μmax) and maximum cell densities at the end of fermentations (**Table 4**). The goodness of fit of this model was appropriate for all the strains assayed, R^2 values being, higher than 0.90 (data not shown). The findings pointed out that the μ_{max} values of HF- strains from each winery were significantly higher than the μ_{max} values of the LF-strains, at least in the presence of one of the ethanol concentrations considered. In particular, the HF-strains coming from A, B, E, and F wineries showed higher values in the presence of 5% ethanol, while HF-strains from wineries C and D, in the presence of 7 and 3% ethanol, respectively. Moreover, HF-strains from five wineries (A, C, D, E, and F) showed a higher growth yield than the respective LF-strains in synthetic medium containing 3 or 5% ethanol, indicating a higher alcohol tolerance of the HF-strains than LF-*S. cerevisiae* strains. On the contrary, only the HF-strains from the winery C and F showed a lag phase shorter than that of the respective LF-strains, when ethanol concentration was 3 or 5% (**Table 4**). To assess the overall effect of ethanol on the HF and LF-strains from each winery, the inhibition percentages of the growth due to ethanol was estimated comparing the area under the growth curve of the positive control (absence of ethanol) with the areas of the other conditions (presence of ethanol at different concentrations: 3, 5, and 7%). Therefore, for each strain, the percentage of inhibition determined by the different ethanol concentrations was calculated using the following formula: = [1 – (Area under the growth curve in presence of ethanol/Area under the curve without ethanol)]*100 (**Table 4**). This parameter is shown to be inversely related to the lag phase and linearly related to both the maximum exponential growth rate and maximum cell densities reached and thus is appropriate to assess the overall yeast growth (Arroyo-López et al., 2010). HF-*S. cerevisiae* strains isolated from four winery (A, C, E, and F) showed an inhibition percentages significantly lower than the LF-strains coming from

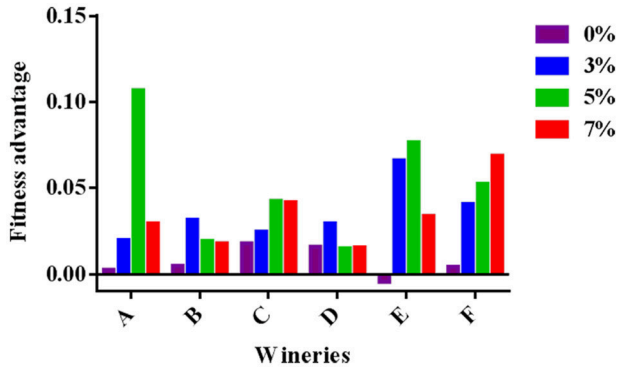

FIGURE 3 | Fitness advantage at different ethanol concentrations calculated for each pair of HF/LF-*S. cerevisiae* strains from the six different wineries (A, B, C, D, E, and F) considering the average growth rate calculated between 0 and 8 h in synthetic medium at 28°C.

the same wineries at all the concentrations of ethanol tested, while in the case of remaining two wineries, B and D, this difference was observed only at 5 and 3% of ethanol, respectively.

Finally, to quantify how increasing ethanol concentrations affects competition between HF- and LF-strains isolated from each winery, the concept of fitness advantage (Goddard, 2008; Salvadó et al., 2011) was used. Two main factors affect the yeast fitness: the maximum specific growth rate (μ_{max}) and the duration of the lag phase (Buchanan and Solberg, 1972; Swinnen et al., 2004; Oxman et al., 2008). Therefore, in order to consider both factors, the fitness advantage was calculated taking into account the average growth rate (v) between 0 and 8 h using the following mathematical formula: fitness advantage (m) = v HF – v LF. In **Figure 3**, the data obtained at different ethanol concentrations, ranging from 0.01 to 0.11 (h^{-1}), are shown. Independently on ethanol concentration, values resulted positive, demonstrating the fitness advantage of HF-*S. cerevisiae*—strains.

Combined Effect of Temperature and Ethanol on the Growth Performance and the Fitness Advantage of HF and LF-*S. cerevisiae* Strains

Temperature and ethanol can considerably affect yeast growth and thus the wine fermentation kinetics. The contemporary presence of these two factors could play an important role in niche construction favoring some strains of *S. cerevisiae* compared to others in wine fermentation. To prove this selective effect, the combined effect of these two parameters on HF and LF-strains was studied in laboratory scale fermentation planning the experiment according to a central composite design with two variable (ethanol and temperature) and three-level. In particular, the three conditions of temperatures were 25, 30, and 35°C, while the three concentrations of ethanol were 0, 3.5, and 7%, obtaining nine combinations in total. Gompertz model was used to estimate the performances of the strains in terms of lag phase period, maximum specific growth rate (μ_{max}) and maximum cell densities of the various fermentation kinetics (**Table 5**). The goodness of fit of this model was appropriate for all the strains assayed, R^2 values being, higher than 0.95 (data not shown). The comparison between the growth performances of each pair of strains representative of the six wineries showed that, when significant differences occurred they were always in favor of HF instead of LF strains (shorter lag phase, higher maximum specific growth rate, higher maximum cell densities). Similarly, the inhibition percentages due to the combined effect of ethanol and temperature, when differences were statistically significant, were always higher for the LF strains compared to the HF-strains.

Finally, the fitness advantage between the HF and LF strains was calculated for each winery taking into account the average growth rate between 0 and 8 h (v) using the mathematical formula reported above. As shown in **Figure 4**, the advantage of HF-*S. cerevisiae* strains was pointed out in most cases, with few exceptions (5 in total).

Theoretical Time Required to Achieve Dominance of HF on LF-*S. cerevisiae* Strains

Fitness advantage in a specific competitive environment can explain why a given strain outcompetes another. Therefore, the values of fitness advantage reported in **Figure 4** can be used to calculate the theoretical time (t) needed for HF-strains to dominate on LF-strains. The equation to calculate "t" was developed by Hartl and Clark (1997) and recently were used by Goddard (2008) and García-Ríos et al. (2014):

$$t = \frac{1}{m} \ln \frac{ptq0}{qtp0}$$

where "m" was the fitness advantage, "p" the frequency of HF-*S. cerevisiae* strains, "q" the frequency of LF-*S. cerevisiae* strains. In particular, $p0$ and $q0$ were the initial frequencies, while pt and qt were the final frequencies. The initial frequencies of both HF and LF-strains were imposed at 0.50, while the final frequencies were 0.90 and 0.10 for HF and LF-strains, respectively. In **Figure 5** are reported the theoretical times required in each winery to achieve dominance of HF-*S. cerevisiae* on LF-strains. Theoretically, the assayed HF-*S. cerevisiae* strains takes an average of 14 to almost 50 h to dominate on LF-*S. cerevisiae* strains according to the winery considered. These theoretical values were in agreement with the experimental data obtained from laboratory-scale co-fermentations carried out by one HF and one LF strain that were inoculated in synthetic must at the same initial concentrations corresponding to frequencies at 0.50. Indeed, in each of six co-fermentations the HF-*S. cerevisiae* strain dominated on LF-strain within the first 24 h.

DISCUSSION

In spontaneous wine fermentation, different yeast species as well as various strains of the same species, usually coexist interacting with each other and the environmental conditions (Albergaria and Arneborg, 2016; Ciani et al., 2016; Morrison-Whittle and Goddard, 2018). Since during the alcoholic fermentation progress many changes occur in grape must becoming wine,

TABLE 4 | Growth parameters and inhibition percentages to ethanol of the HF and LF-*S. cerevisiae* strains in synthetic media at different ethanol concentrations.

Ethanol (% v/v)	Lag phase (h)		μ (h^{-1})		(cell/mL)*10^6		Inhibition percentages to ethanol	
	HF	LF	HF	LF	HF	LF	HF	LF
Winery A								
0	2.858 ± 0.442	2.705 ± 0.389	0.179 ± 0.003	0.164 ± 0.005	12.00 ± 0.25	11.75 ± 0.15	–	–
3	3.288 ± 0.237	4.349 ± 0.593	0.162 ± 0.011	0.149 ± 0.002	10.19 ± 0.06^S	8.69 ± 0.06	16.99 ± 1.72^S	36.00 ± 0.63
5	4.796 ± 0.274	4.187 ± 0.555	0.158 ± 0.008^S	0.058 ± 0.013	7.93 ± 0.18^S	3.37 ± 0.12	47.66 ± 1.68^S	76.93 ± 2.13
7	4.989 ± 0.076	n.f.*	0.044 ± 0.035	n.f.*	2.68 ± 0.06	2.12 ± 0.12	66.23 ± 4.56^S	96.42 ± 2.07
Winery B								
0	2.763 ± 0.309	2.523 ± 0.523	0.173 ± 0.007	0.176 ± 0.001	15.70 ± 0.50	15.10 ± 0.10	–	–
3	3.763 ± 0.498	3.782 ± 0.727	0.179 ± 0.020	0.155 ± 0.029	11.70 ± 0.50	9.10 ± 0.50	32.69 ± 9.61	32.24 ± 5.70
5	4.086 ± 0.287	3.765 ± 0.230	0.114 ± 0.002^S	0.097 ± 0.003	5.60 ± 0.01^S	4.80 ± 0.01	54.12 ± 0.48^S	58.04 ± 0.42
7	4.665 ± 2.423	5.451 ± 0.390	0.073 ± 0.049	0.045 ± 0.019	3.00 ± 0.10	2.60 ± 0.10	83.54 ± 3.50	89.63 ± 0.22
Winery C								
0	2.317 ± 0.185	1.751 ± 0.071	0.181 ± 0.010	0.159 ± 0.005	19.90 ± 0.70	17.25 ± 0.75	–	–
3	2.142 ± 0.059^S	3.249 ± 0.063	0.137 ± 0.001	0.135 ± 0.005	11.35 ± 0.15^S	9.32 ± 0.42	23.43 ± 1.02^S	46.83 ± 3.85
5	3.243 ± 0.254	3.130 ± 0.202	0.122 ± 0.011	0.090 ± 0.012	7.45 ± 0.05^S	5.30 ± 0.20	47.98 ± 4.79^S	61.13 ± 2.10
7	4.176 ± 0.774	4.236 ± 0.822	0.044 ± 0.006^S	0.025 ± 0.001	3.50 ± 0.25	2.50 ± 0.10	66.64 ± 0.96^S	88.61 ± 4.52
Winery D								
0	1.659 ± 0.036	1.501 ± 0.189	0.156 ± 0.005	0.143 ± 0.001	17.75 ± 0.35^S	15.63 ± 0.12	–	–
3	1.920 ± 0.211	1.479 ± 0.074	0.142 ± 0.001^S	0.112 ± 0.003	14.55 ± 0.45^S	11.50 ± 0.10	14.80 ± 0.46^S	19.00 ± 0.78
5	1.936 ± 0.001	1.921 ± 0.352	0.077 ± 0.004	0.068 ± 0.005	6.93 ± 0.31	6.15 ± 0.25	40.47 ± 0.42	43.39 ± 2.08
7	1.982 ± 0.031	1.995 ± 0.144	0.049 ± 0.006	0.045 ± 0.005	4.25 ± 0.25	3.75 ± 0.15	63.89 ± 3.46	73.62 ± 1.26
Winery E								
0	2.568 ± 0.008	2.341 ± 0.201	0.169 ± 0.001	0.169 ± 0.006	17.90 ± 0.30	18.55 ± 0.25	–	–
3	3.677 ± 0.005	3.706 ± 0.460	0.153 ± 0.001^S	0.102 ± 0.002	9.44 ± 0.19^S	5.57 ± 0.32	37.06 ± 1.17^S	60.19 ± 2.06
5	3.346 ± 0.257	4.062 ± 0.717	0.156 ± 0.010^S	0.077 ± 0.009	7.15 ± 0.20^S	3.87 ± 0.12	59.05 ± 1.81^S	78.74 ± 4.43
7	4.042 ± 0.077	5.124 ± 1.200	0.038 ± 0.001^S	0.016 ± 0.001	2.87 ± 0.12^S	2.19 ± 0.06	82.39 ± 1.17^S	96.30 ± 2.34
Winery F								
0	0.340 ± 0.210^S	1.436 ± 0.223	0.136 ± 0.005	0.136 ± 0.004	18.00 ± 0.10	17.40 ± 0.40	–	–
3	0.790 ± 0.236^S	1.878 ± 0.023	0.123 ± 0.006	0.117 ± 0.001	13.00 ± 0.40^S	9.37 ± 0.37	19.17 ± 3.22^S	35.52 ± 3.20
5	1.342 ± 0.144^S	2.464 ± 0.090	0.097 ± 0.004^S	0.075 ± 0.007	7.81 ± 0.18^S	5.14 ± 0.24	45.80 ± 1.50^S	63.86 ± 0.10
7	4.175 ± 0.306	4.670 ± 0.744	0.105 ± 0.004^S	0.055 ± 0.007	5.14 ± 0.24^S	2.96 ± 0.16	77.30 ± 0.38^S	88.77 ± 3.48

The growth parameters were calculated using Baranyi and Roberts-model (Combase DMfit software), the inhibition percentages to ethanol were calculated using the following formula: = [1- (Area under the growth curve in presence of ethanol/Area under the curve without ethanol)] 100. All the results are expressed as mean ± standard deviation; S, significant different (t-Test; $p<0.05$); n.f., no significant fit.

the environmental conditions turn out to be more selective, and different yeast species and strains undergo sequential substitution in relation to their fitness for such harsh conditions (Bisson, 2012; Perrone et al., 2013; Williams et al., 2015; Ciani et al., 2016; Brice et al., 2018; Henriques et al., 2018). Different studies have raised evidence that the dominance of *S. cerevisiae* on non-*Saccharomyces* yeast species, that usually takes place in the first stages of spontaneous wine fermentation, is dependent on, not only higher tolerance to ethanol, but also on temperature (Goddard, 2008; Salvadó et al., 2011; Alonso del-Real et al., 2017), and other factors such as cell-to-cell contact mechanism (Nissen and Arnebor, 2003). On the other hand, few studies have investigated the dominance of *S. cerevisiae* strains during spontaneous or induced wine fermentation (Perrone et al., 2013; García-Ríos et al., 2014; Pérez-Torrado et al., 2017).

In this work, the influence of ethanol and temperature on the dominance of different *S. cerevisiae* strains, occurring in several spontaneous alcoholic fermentations carried out at industrial level in six wineries in Tuscany (Italy), was assayed by using the concept of fitness advantage (García-Ríos et al., 2014). The predominant *S. cerevisiae* strains were differentiated by RFLP-mtDNA method and according to their isolation frequency. The results obtained, by analyzing 637 isolates, confirmed the genetic polymorphism expected for *S. cerevisiae* population in spontaneous wine fermentations and the high variability between the isolation frequencies of different strains (Bisson, 2012; Schuller et al., 2012; Tofalo et al., 2013). In particular, independently of the grape variety, five out six wineries considered, showed only one predominant *S. cerevisiae* strain, with an isolation frequency ranging from 32

TABLE 5 | Growth parameters and inhibition percentages to ethanol of the HF and LF-*S. cerevisiae* strains in synthetic media at different ethanol concentrations.

Ethanol (% v/v)	Temperature (°C)	Lag phase (h)		μ (h^{-1})		C (Log cell/mL)		Inhibition percentages	
		HF	LF	HF	LF	HF	LF	HF	LF
Winery A									
0	25	1.173 ± 0.004^{S}	1.478 ± 0.058	0.1478 ± 0.0001	0.1493 ± 0.0027	1.429 ± 0.001	1.444 ± 0.002	–	–
3.5	25	1.094 ± 0.014^{S}	2.060 ± 0.056	0.1028 ± 0.0001^{S}	0.0810 ± 0.0001	1.240 ± 0.001	1.245 ± 0.014	26.14 ± 0.24^{S}	49.12 ± 1.08
7	25	2.468 ± 0.044^{S}	5.100 ± 0.356	0.0646 ± 0.0009	0.0643 ± 0.0015	1.183 ± 0.011	1.165 ± 0.0034	64.98 ± 0.14^{S}	83.37 ± 1.82
0	30	1.004 ± 0.071^{S}	1.841 ± 0.040	0.1861 ± 0.0027	0.2081 ± 0.0075	1.331 ± 0.006^{S}	1.280 ± 0.002	–	–
3.5	30	1.040 ± 0.052^{S}	1.886 ± 0.116	0.1028 ± 0.0007^{S}	0.08164 ± 0.0019	1.236 ± 0.005^{S}	1.132 ± 0.006	34.13 ± 0.54^{S}	56.22 ± 0.05
7	30	1.705 ± 0.062^{S}	3.576 ± 0.007	0.0656 ± 0.0002^{S}	0.04307 ± 0.0007	0.677 ± 0.001^{S}	0.498 ± 0.004	66.33 ± 1.04^{S}	87.11 ± 2.57
0	35	0.248 ± 0.041^{S}	1.732 ± 0.136	0.1245 ± 0.0015^{S}	0.1650 ± 0.0075	1.295 ± 0.004^{S}	1.137 ± 0.002	–	–
3.5	35	1.040 ± 0.053^{S}	1.886 ± 0.016	0.06763 ± 0.0005^{S}	0.0607 ± 0.0009	0.813 ± 0.012^{S}	0.734 ± 0.002	48.38 ± 0.55	48.98 ± 0.07
7	35	1.463 ± 0.302	2.798 ± 0.456	0.03562 ± 0.0019	0.0300 ± 0.0026	0.321 ± 0.021	0.184 ± 0.006	79.96 ± 0.57^{S}	87.55 ± 1.11
Winery B									
0	25	1.685 ± 0.010	1.643 ± 0.086	0.1862 ± 0.0045	0.2073 ± 0.0055	1.264 ± 0.002^{S}	1.232 ± 0.004	–	–
3.5	25	1.642 ± 0.224	1.522 ± 0.319	0.0920 ± 0.0002^{S}	0.0797 ± 0.0006	1.135 ± 0.012^{S}	1.030 ± 0.0082	45.06 ± 0.20^{S}	54.63 ± 0.88
7	25	2.698 ± 0.115^{S}	4.654 ± 0.044	0.0515 ± 0.0002	0.0574 ± 0.0018	1.065 ± 0.058	1.077 ± 0.209	68.60 ± 0.76	78.37 ± 2.96
0	30	1.324 ± 0.090	1.775 ± 0.050	0.2394 ± 0.0022	0.2296 ± 0.0041	1.240 ± 0.006	1.246 ± 0.002	–	–
3.5	30	1.899 ± 0.068	2.172 ± 0.075	0.1552 ± 0.0030^{S}	0.1419 ± 0.0023	1.116 ± 0.008^{S}	0.982 ± 0.005	31.29 ± 0.76^{S}	42.59 ± 0.46
7	30	1.960 ± 0.046^{S}	2.760 ± 0.043	0.0459 ± 0.0050	0.0398 ± 0.0017	0.675 ± 0.012	0.609 ± 0.013	72.34 ± 1.73	72.45 ± 0.99
0	35	1.789 ± 0.066	1.635 ± 0.018	0.2360 ± 0.0145	0.2343 ± 0.0009	1.220 ± 0.018	1.238 ± 0.001	–	–
3.5	35	1.900 ± 0.378	3.065 ± 0.002	0.1336 ± 0.0087	0.1523 ± 0.0031	1.045 ± 0.020^{S}	0.705 ± 0.011	39.00 ± 2.13^{S}	58.31 ± 0.65
7	35	2.336 ± 0.075	3.140 ± 0.218	0.0223 ± 0.0010	0.0243 ± 0.0005	0.636 ± 0.119	0.451 ± 0.036	82.78 ± 0.66	84.66 ± 0.96
Winery C									
0	25	0.755 ± 0.039^{S}	1.132 ± 0.072	0.1418 ± 0.0006	0.1464 ± 0.0032	1.453 ± 0.002^{S}	1.321 ± 0.007	–	–
3.5	25	0.719 ± 0.067^{S}	2.351 ± 0.214	0.1197 ± 0.0011	0.1306 ± 0.0066	1.345 ± 0.001^{S}	1.147 ± 0.003	59.12 ± 1.09^{S}	69.31 ± 0.52
7	25	1.129 ± 0.152^{S}	15.140 ± 0.262	0.0880 ± 0.0030	0.0999 ± 0.0017	1.151 ± 0.001^{S}	0.868 ± 0.067	84.79 ± 1.03^{S}	92.52 ± 0.38
0	30	1.044 ± 0.143	1.023 ± 0.082	0.1571 ± 0.0042	0.1647 ± 0.0024	1.277 ± 0.001	1.267 ± 0.002	–	–
3.5	30	1.102 ± 0.027^{S}	1.023 ± 0.002	0.1438 ± 0.0002^{S}	0.1295 ± 0.0019	1.062 ± 0.009^{S}	0.993 ± 0.004	47.88 ± 0.18^{S}	55.32 ± 1.21
7	30	1.474 ± 0.090^{S}	8.693 ± 1.092	0.0815 ± 0.0011^{S}	0.0553 ± 0.0038	0.9602 ± 0.005	0.801 ± 0.269	82.25 ± 0.21^{S}	93.73 ± 0.94
0	35	0.734 ± 0.101	0.830 ± 0.067	0.1616 ± 0.0042	0.1580 ± 0.0019	1.107 ± 0.009	1.128 ± 0.002	–	–
3.5	35	0.734 ± 0.174^{S}	3.464 ± 0.237	0.1352 ± 0.0006	0.1382 ± 0.0083	1.041 ± 0.040	0.941 ± 0.001	85.97 ± 2.86	82.73 ± 2.01
7	35	0.818 ± 0.055^{S}	7.352 ± 0.247	0.05821 ± 0.0003	0.0511 ± 0.0057	0.755 ± 0.001^{S}	0.259 ± 0.015	94.72 ± 0.21	97.68 ± 0.02
Winery D									
0	25	2.983 ± 0.073^{S}	2.436 ± 0.019	0.2043 ± 0.0027^{S}	0.1592 ± 0.0020	1.254 ± 0.007	1.234 ± 0.016	–	–
3.5	25	4.631 ± 0.061	5.138 ± 0.207	0.1638 ± 0.0016	0.1349 ± 0.0111	0.992 ± 0.009	1.158 ± 0.045	33.92 ± 0.15^{S}	51.12 ± 0.67
7	25	5.654 ± 0.129^{S}	6.979 ± 0.029	0.0872 ± 0.0056	0.0867 ± 0.0078	0.990 ± 0.018	1.001 ± 0.030	74.40 ± 0.01	90.49 ± 4.57
0	30	2.108 ± 0.017^{S}	2.225 ± 0.001	0.1905 ± 0.0020	0.1903 ± 0.0010	1.253 ± 0.001^{S}	1.264 ± 0.002	–	–

(Continued)

TABLE 5 | Continued

Ethanol (% v/v)	Temperature (°C)	Lag phase (h)		μ (h^{-1})		C (Log cell/mL)		Inhibition percentages	
		HF	LF	HF	LF	HF	LF	HF	LF
3.5	30	3.156 ± 0.071	3.193 ± 0.027	0.1403 ± 0.0019^{S}	0.1126 ± 0.0014	1.187 ± 0.011^{S}	1.092 ± 0.011	43.33 ± 0.52^{S}	55.47 ± 0.03
7	30	4.811 ± 0.049^{S}	6.947 ± 0.193	0.0924 ± 0.0022	0.0801 ± 0.0073	0.927 ± 0.010	1.022 ± 0.034	84.52 ± 1.11	89.77 ± 1.63
0	35	2.692 ± 0.026^{S}	2.467 ± 0.025	0.1849 ± 0.0011^{S}	0.1639 ± 0.0015	1.229 ± 0.011^{S}	1.090 ± 0.004	–	–
3.5	35	6.020 ± 0.036^{S}	5.696 ± 0.016	0.0717 ± 0.0003^{S}	0.0601 ± 0.0001	1.013 ± 0.046	1.449 ± 0.279	44.05 ± 0.23^{S}	58.44 ± 0.98
7	35	7.212 ± 0.013^{S}	8.311 ± 0.015	0.0661 ± 0.0037	0.0473 ± 0.0031	1.039 ± 0.006^{S}	0.635 ± 0.019	78.88 ± 1.44^{S}	95.49 ± 1.40
Winery E									
0	25	2.148 ± 0.003	2.318 ± 0.161	0.1603 ± 0.0021	0.1713 ± 0.0099	1.252 ± 0.0035	1.231 ± 0.0095	–	–
3.5	25	2.597 ± 0.016^{S}	3.695 ± 0.109	0.1180 ± 0.0016	0.1343 ± 0.0053	1.186 ± 0.005	1.125 ± 0.035	41.47 ± 0.10^{S}	57.86 ± 0.21
7	25	4.051 ± 0.003^{S}	6.372 ± 0.063	0.06499 ± 0.0018	0.08217 ± 0.0302	1.043 ± 0.008	1.018 ± 0.142	75.15 ± 0.12^{S}	87.19 ± 1.46
0	30	1.456 ± 0.156	1.958 ± 0.065	0.1924 ± 0.0055^{S}	0.1584 ± 0.0042	1.254 ± 0.013	1.139 ± 0.001	–	–
3.5	30	2.008 ± 0.075^{S}	3.069 ± 0.067	0.1161 ± 0.0003^{S}	0.0998 ± 0.0030	1.109 ± 0.008^{S}	0.947 ± 0.008	34.07 ± 0.58^{S}	51.87 ± 0.49
7	30	4.715 ± 0.072	5.407 ± 0.586	0.0392 ± 0.0015	0.03448 ± 0.0005	2.233 ± 0.033^{S}	1.295 ± 0.177	79.29 ± 0.43	87.89 ± 2.05
0	35	0.7815 ± 0.020^{S}	1.686 ± 0.116	0.1171 ± 0.0016	0.1399 ± 0.0052	1.147 ± 0.014	1.107 ± 0.024	–	–
3.5	35	1.450 ± 0.061^{S}	3.015 ± 0.097	0.07534 ± 0.0051	0.08448 ± 0.0018	0.932 ± 0.051	0.8141 ± 0.021	47.08 ± 0.43^{S}	53.24 ± 0.19
7	35	2.177 ± 0.452	3.446 ± 0.814	0.0350 ± 0.0038^{S}	0.007538 ± 0.010	0.4133 ± 0.009^{S}	0.1737 ± 0.007	85.05 ± 1.35^{S}	95.11 ± 1.28
Winery F									
0	25	1.516 ± 0.015	1.582 ± 0.096	0.1610 ± 0.0009	0.1613 ± 0.0017	1.229 ± 0.002	1.244 ± 0.008	–	–
3.5	25	2.547 ± 0.003^{S}	3.450 ± 0.017	0.1250 ± 0.0015	0.1220 ± 0.0021	1.105 ± 0.007	1.020 ± 0.033	13.20 ± 1.08^{S}	36.41 ± 1.26
7	25	4.057 ± 0.043^{S}	5.597 ± 0.347	0.0925 ± 0.0029	0.1145 ± 0.0123	0.890 ± 0.003^{S}	0.733 ± 0.004	41.28 ± 0.82^{S}	85.72 ± 1.61
0	30	1.623 ± 0.012	1.712 ± 0.017	0.1880 ± 0.0022	0.1972 ± 0.0016	1.223 ± 0.005	1.175 ± 0.024	–	–
3.5	30	1.741 ± 0.132^{S}	2.726 ± 0.031	0.1226 ± 0.0051	0.1225 ± 0.0010	1.012 ± 0.017^{S}	0.8961 ± 0.007	11.48 ± 0.08^{S}	48.16 ± 1.66
7	30	3.273 ± 0.043	4.957 ± 0.755	0.08269 ± 0.0222	0.0808 ± 0.0235	0.987 ± 0.058	0.948 ± 0.348	50.83 ± 0.19^{S}	91.62 ± 1.24
0	35	0.939 ± 0.001	1.424 ± 0.042	0.1686 ± 0.0003	0.1717 ± 0.0010	1.187 ± 0.007^{S}	0.9929 ± 0.002	–	–
3.5	35	1.905 ± 0.035^{S}	2.570 ± 0.084	0.1206 ± 0.0070	0.1185 ± 0.0079	0.7066 ± 0.016^{S}	0.5607 ± 0.027	13.21 ± 2.20^{S}	59.33 ± 0.38
7	35	3.306 ± 0.466	4.879 ± 0.548	0.0430 ± 0.0061	0.0227 ± 0.0020	0.6424 ± 0.033^{S}	0.3017 ± 0.035	47.02 ± 0.86^{S}	98.09 ± 0.99

The growth parameters were calculated using Gompertz function (GraphPadPrism software), the inhibition percentages to ethanol at different temperatures were calculated using the following formula: = [1 – (Area under the growth curve in presence of ethanol at a 25, 30 or 35°C/Area under the curve without ethanol at a 25, 30, or 35°C)] 100. All the results are expressed as mean ± standard deviation; S, significant different (t-Test; $p<0.05$); n.f., no significant fit.

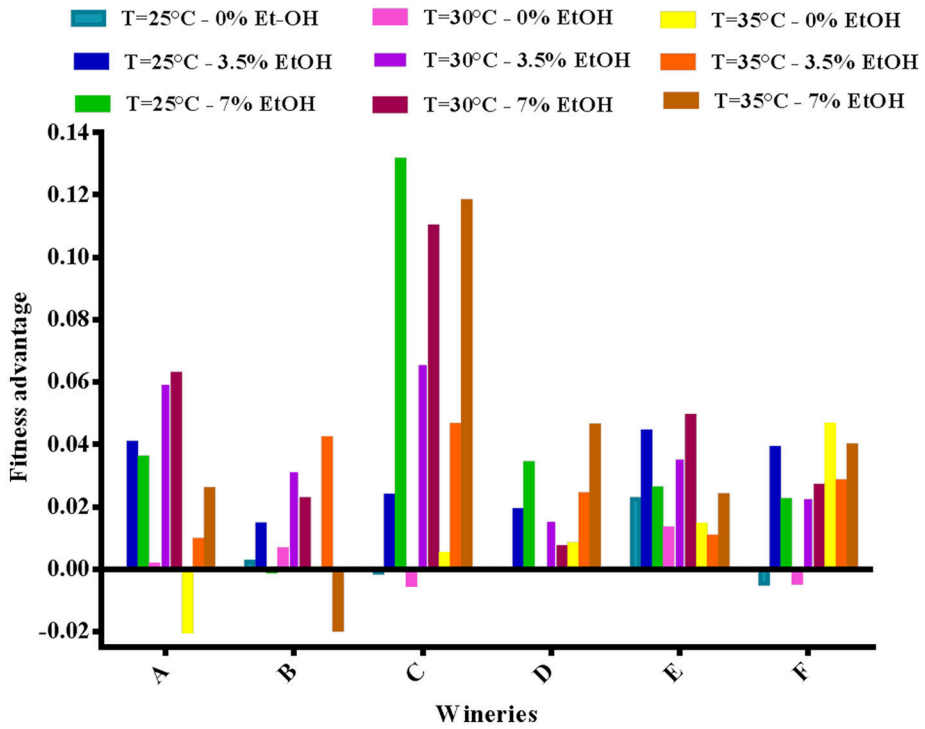

FIGURE 4 | Fitness advantage at different concentrations of ethanol and temperatures calculated for each pair of HF/LF-*S. cerevisiae* strains from the six different wineries (A, B, C, D, E, and F), considering the average growth rate calculated between 0 and 8 h in synthetic medium.

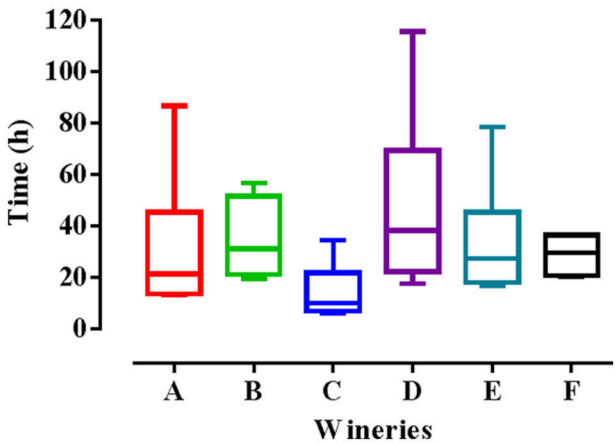

FIGURE 5 | Theoretical time required by HF-*S. cerevisiae* strains to dominate on LF-*S. cerevisiae* strains in the six wineries studied (A, B, C, D, E, and F).

to 74%, while a variable number of strains (from 4 to 14) was characterized by an isolation frequency lower than 10%. These finding were consistent with those reported by other Authors (Versavaud et al., 1995; Gutiérrez et al., 1997; Egli et al., 1998; Sabate et al., 1998) although in some cases *S. cerevisiae* strains predominating the fermentative process, were not found (Vezinhet et al., 1992; Torija et al., 2001). In agreement with other studies (Versavaud et al., 1995; Barrajón et al., 2010), the indigenous *S. cerevisiae* strains were differentiated in two groups: strains at high frequency (HF) or "predominant" and strains at low frequency (LF) or "secondary" strains. Moreover, our results demonstrated that the *S. cerevisiae* strains dominating spontaneous wine fermentations were not related to the grape variety used to perform alcoholic fermentations; instead, they were representative of different wineries, strengthening the idea of the occurrence of yeast strains possessing better fitness to the specific winemaking conditions used in each winery (Cocolin et al., 2004). Probably, during the usual cellar operations, yeast strains spread throughout the environment and those that were better adapted to certain conditions occurred at higher frequencies, becoming the dominant yeast strains in the winery. In the literature, in some cases *S. cerevisiae* strains were found to be capable of dominating the alcoholic fermentation in all vats of the same winery, independently of the grapevine cultivar (Frezier and Dubourdieu, 1992; Guillamón et al., 1996), whereas other times the yeast strains were found to be specific for each grape variety (Blanco et al., 2006; Schuller et al., 2012).

In any case, cluster analysis with the Dice coefficient and the UPGMA method grouped the profiles of predominant (HF) and secondary (LF) *S. cerevisiae* strains in clusters according to the winery where they came from. Furthermore, the yeast commercial strains assayed in this study, chosen because they are the most frequently used in Tuscany as starter cultures,

grouped into a distinct cluster indicating that they were significantly different from the indigenous strains, probably because of they were isolated from French oenological areas. Mercado et al. (2011), by using two molecular methods (RFL-mtDNA and *interdelta* PCR) observed a clear separation between *S. cerevisiae* strains isolated from vineyard and commercial strains, while in other study on cellar-associated *S. cerevisiae* population structure "only 7% of cellar strains were found to be related to commercial strains usually used" as starter cultures (Börlin et al., 2016). On the contrary, Martiniuk et al. (2016) found that commercial and commercial-related yeasts occurred in spontaneous fermentations of a Canadian winery, although they did not dominate the *S. cerevisiae* populations that were unrelated to commercial strains present in the same fermentations. Concerning this work, it should be emphasized that five out of six wineries here taken into account never used commercial yeast strains and only the winery E used the S1 strain as starter some years before the survey.

The occurrence of specific *S. cerevisiae* strains in each winery supports the potential role of these microorganisms in determining distinctive wine characteristics and their selection could represent a resource to contribute in preserving the typicality of wines (Vezinhet et al., 1992; Augruso et al., 2008; Aponte and Blaiotta, 2016; Bokulich et al., 2016). Recent studies suggested the concept of "the so-called microbial *terroir*" demonstrating that indigenous yeast strains can be associated to a given viticultural region (Bokulich et al., 2016; Morrison-Whittle and Goddard, 2018). However, according to our results, specific *S. cerevisiae* strains seem to be representative of single winery rather than of an oenological area: three out six wineries (A, B, and C) were situated within 10 km radius, and showed *S. cerevisiae* grouped in three different *clusters*. Therefore, data suggested the idea of the "winery effect" or a microbial *terroir* at a smaller scale. Nevertheless, in order to assess the existence of certain relationship between indigenous *S. cerevisiae* strains and single winery, further surveys in consecutive years in the same wineries located in different oenological areas should be carried out.

The further step was addressed to confirm, in laboratory-scale fermentations, the dominant behavior, exhibited by *S. cerevisiae* strains at high frequency (HF) in the spontaneous alcoholic fermentations in each winery. Co-fermentations were carried out by inoculating at the same cell densities (10^4 cell/mL) one HF-strain and one LF-strain coming from the six wineries, and the ability of one strain to dominate over another was assayed by using the RFLP-mtDNA method. The data obtained raised evidence that after 24 h in co-fermentations total yeast population reached values of 10^7 CFU/mL and that in all our trials the "HF" *S. cerevisiae* strain occurred at frequency ranging from 70 to 87%, confirming the dominance behavior observed in industrial spontaneous fermentations in the six wineries. Other Authors (Barrajón et al., 2010; Perrone et al., 2013; Pérez-Torrado et al., 2017) that assayed the competition between indigenous "dominant" *S. cerevisiae* strains and commercial yeasts or between one "dominant" and one "non-dominant" strain by using co-fermentations, reported similar results. This dominance phenomenon has been mainly attributed to a cell-to-cell contact mechanism or microenvironment contact, conditions in which cells compete for space when are in high densities and in cell-to-cell contact, so that the non-dominant yeast strain arrests its growth (Ciani et al., 2016). Moreover, a differential sulphite production and resistance and the killer activity seemed to be involved in dominant behavior of the yeast strains (Perrone et al., 2013; Pérez-Torrado et al., 2017). It is noteworthy that no killer activity was detected in HF-strains assayed in this study and no significant differences in sulphite production were found (data not shown).

Nevertheless, the competition degree of each strain, which determine the capacity of one strain to out-compete another, is influenced by other factors including pH, temperature, ethanol, osmotic pressure, nitrogen available (Ciani et al., 2016). Indeed, our findings concerning the influence of ethanol and temperature on the growth performance and the fitness advantage of High frequency (HF) *S. cerevisiae* strains, support the important role that these two factors may play in determining the dominance of one strain over another in wine fermentations. By considering the single effect of ethanol on growth performance, "HF" strains showed significant lower inhibition percentages than "LF" strains although in the presence of different ethanol concentrations (from 3 to 7%). The inhibition percentages, calculated as reported by Arroyo-López et al. (2009, 2010), was an appropriate indicator of the overall yeast growth as this parameter was inversely related to the lag phase, but linearly related to both the maximum specific growth rate (μ_{max}) and the maximum cell densities at the end of growth. Consequently, the fitness advantage, which according to Salvadó et al. (2011) represents the difference in μ_{max} between competitors for a specific environmental condition, resulted higher for "HF" strains, suggesting their better adaptability to increasing ethanol concentrations in comparison with "LF" strains. However, this capability resulted to be a strain-dependent characteristic as the fitness advantage showed values ranging from 1 to 6% per hour and from 1 to 10% per hour in the presence of 3 and 5% ethanol, respectively. Indeed, each *S. cerevisiae* strains may display different stress responses to ethanol as the effects of increasing ethanol concentrations on the yeast cell include different changes such as in membrane composition and in gene expression, synthesis of heat shock proteins, increases in chaperons proteins etc. (Ding et al., 2009). Recently, a study aimed to assess fitness advantages of four commercial wine yeast strains has stressed that fermentation temperature might be an important factor in determining the dynamics of the *S. cerevisiae* strain population (García-Ríos et al., 2014). In fact, ethanol and high temperature affect synergistically the membrane integrity and permeability causing a decrease in the growth of yeast populations (Alexandre et al., 1994; Albergaria and Arneborg, 2016). The data related to the combined effect of increasing ethanol concentrations and different temperatures on the growth performance and the fitness advantages of six couple of HF and LF-*S. cerevisiae* here considered, confirmed that these two factors could play an important role in niche construction favoring some strains of *S. cerevisiae* compared to others in wine fermentation. According to some studies, the competitive advantage of *S. cerevisiae* on non-*Saccharomyces* yeasts in spontaneous alcoholic fermentations seems to be related to both

ethanol and temperature adaptation (Goddard, 2008; Salvadó et al., 2011; Ciani et al., 2016; Alonso del-Real et al., 2017). Therefore, similar competition mechanisms might be responsible for interaction among indigenous *S. cerevisiae* strains. Our results proved that the six "HF" strains had always fitness advantage in comparison with relative LF strains when temperature was 25 or 30°C in the presence of ethanol concentrations of 3.5 and 7% v/v. These conditions typically occur in the early stages of alcoholic fermentations and suggest that they can affect the competition among different *S. cerevisiae* strains during the first 2 days of the fermentative process.

Taking into account values of fitness advantage obtained at different temperature and ethanol concentrations was calculated the hypothetical time needed for each "HF"-*S. cerevisiae* to achieve dominance on the relative "LF"-*S. cerevisiae* strain in a theoretical mixed population in which each strain was equally represented (50%) (García-Ríos et al., 2014). Results showed that assayed "HF"-*S. cerevisiae* strains took an average of 14 to almost 50 h to dominate on "LF"-*S. cerevisiae* strains based in relation to the winery where they originated.

In conclusions, these findings support the key role of ethanol and temperature in determining fitness advantage of some *S. cerevisiae* strains and contribute to the understanding of predominance of *S. cerevisiae* strains in spontaneous wine fermentations, even though other factor and or mechanisms can be involved. Moreover, these yeast strains could be exploited to develop new wine *starters* able to guarantee a high fermentative performance in grape musts even under stressful conditions and a positive metabolites production in the final wine (Bonciani et al., 2016). Recently, in order to achieve this goal, the construction of hybrid *S. cerevisiae* strains has been performed through selection programs based on the adaptive evolution strategy or a multi-phase approach (Bonciani et al., 2018), valuable tools to obtain improved and suitable yeast strains in the modern oenology.

AUTHOR CONTRIBUTIONS

SG, DG, SM, and LG conceived and designed the experiments. SM contributed to perform chemical analysis. DG contributed to perform microbiological analysis and genotyping characterization of *S. cerevisiae* isolates. SG and LG contributed to statistical analysis and interpretation of data for the work, to draft the work and revising it. MV contributed to the revision of the work.

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

frontiers in Microbiology

ORIGINAL RESEARCH
published: 13 February 2018
doi: 10.3389/fmicb.2018.00196

Altered Fermentation Performances, Growth, and Metabolic Footprints Reveal Competition for Nutrients between Yeast Species Inoculated in Synthetic Grape Juice-Like Medium

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OPEN ACCESS

Edited by:
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Université de Bordeaux, France

Reviewed by:
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Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: *29 November 2017*
Accepted: *29 January 2018*
Published: *13 February 2018*

Citation:
Rollero S, Bloem A, Ortiz-Julien A, Camarasa C and Divol B (2018) Altered Fermentation Performances, Growth, and Metabolic Footprints Reveal Competition for Nutrients between Yeast Species Inoculated in Synthetic Grape Juice-Like Medium. Front. Microbiol. 9:196. doi: 10.3389/fmicb.2018.00196

The sequential inoculation of non-*Saccharomyces* yeasts and *Saccharomyces cerevisiae* in grape juice is becoming an increasingly popular practice to diversify wine styles and/or to obtain more complex wines with a peculiar microbial footprint. One of the main interactions is competition for nutrients, especially nitrogen sources, that directly impacts not only fermentation performance but also the production of aroma compounds. In order to better understand the interactions taking place between non-Saccharomyces yeasts and S. cerevisiae during alcoholic fermentation, sequential inoculations of three yeast species (*Pichia burtonii*, *Kluyveromyces marxianus*, *Zygoascus meyerae*) with *S. cerevisiae* were performed individually in a synthetic medium. Different species-dependent interactions were evidenced. Indeed, the three sequential inoculations resulted in three different behaviors in terms of growth. *P. burtonii* and *Z. meyerae* declined after the inoculation of *S. cerevisiae* which promptly outcompeted the other two species. However, while the presence of *P. burtonii* did not impact the fermentation kinetics of *S. cerevisiae*, that of *Z. meyerae* rendered the overall kinetics very slow and with no clear exponential phase. *K. marxianus* and *S. cerevisiae* both declined and became undetectable before fermentation completion. The results also demonstrated that yeasts differed in their preference for nitrogen sources. Unlike *Z. meyerae* and *P. burtonii*, *K. marxianus* appeared to be a competitor for *S. cerevisiae* (as evidenced by the uptake of ammonium and amino acids), thereby explaining the resulting stuck fermentation. Nevertheless, the results suggested that competition for other nutrients (probably vitamins) occurred during the sequential inoculation of *Z. meyerae* with *S. cerevisiae*. The metabolic footprint of the non-*Saccharomyces* yeasts determined after 48 h of fermentation remained until the end of fermentation and combined with that of *S. cerevisiae*. For instance, fermentations performed with *K. marxianus* were characterized by the formation of phenylethanol and phenylethyl acetate, while those performed with *P. burtonii* or *Z. meyerae* displayed higher production of isoamyl alcohol

and ethyl esters. When considering sequential inoculation of yeasts, the nutritional requirements of the yeasts used should be carefully considered and adjusted accordingly. Finally, our chemical data suggests that the organoleptic properties of the wine are altered in a species specific manner.

Keywords: *S. cerevisiae*, non-*Saccharomyces* yeasts, yeast interactions, nutrient competition, fermentative aromas, wine

INTRODUCTION

Spontaneous alcoholic fermentation is a complex microbial process that involves diverse yeast species. These yeast species are mostly characterized by large and predominant populations of non-*Saccharomyces* species in grape juice and at early stages of fermentation. Thereafter, *Saccharomyces cerevisiae* dominates and completes the fermentation (Fleet, 1993, 2003). Until recently, non-*Saccharomyces* have been associated with spontaneous and unpredictable fermentation which may lead to stuck or sluggish fermentations. However, some of these species have now garnered interest in winemaking practices because of their positive impact on the wine quality and complexity (Ciani et al., 2006; Fleet, 2008; Anfang et al., 2009; Viana et al., 2009, 2011; Andorrà et al., 2010; Jolly et al., 2014) and in an attempt to reach new consumer's markets.

As the majority of the non-*Saccharomyces* yeasts found in grape juice are unable to ferment to dryness, the use of controlled mixed or sequential fermentations of non-*Saccharomyces* yeasts together with *S. cerevisiae* appears to be an appropriate process to combine a diversification of the wine styles and a reliable and complete fermentation (Romano et al., 1997; Sadoudi et al., 2012; Gobbi et al., 2013). Although a massive amount of cells of *S. cerevisiae* is typically used for inoculation, many studies have shown that indigenous or commercial non-*Saccharomyces* strains are not completely suppressed, and may persist during other fermentative stages (Ciani et al., 2010; Medina et al., 2012; Lopez et al., 2014; Wang et al., 2015).

The main concern about the use of mixed/sequential cultures of different yeasts is the probable occurrence of complex interactions between the organisms (Fleet, 2003; Alexandre et al., 2004; Barbosa et al., 2015). These interactions can have a desirable or a detrimental effect on the fermentation process and the organoleptic properties of wines. The main positive influence of the mixed/sequential inoculation of non-*Saccharomyces* yeasts with *S. cerevisiae* is the increase in the concentration of desirable compounds, such as esters (Moreira et al., 2005, 2008; Viana et al., 2009; Renault et al., 2015). In 2006, Howell et al. showed different profiles of compounds in wines obtained by co-culture fermentation from those made in mono-culture (Howell et al., 2006). These authors also demonstrated that the combination of volatile aromas found in mixed cultures of *Saccharomyces* yeasts was distinctly different from that obtained by blending together mono-culture wines indicating a clear metabolic interaction between the yeasts. Nevertheless, the initial rapid growth of some non-*Saccharomyces* strains may have a negative impact on the metabolism and physiology of *S. cerevisiae* leading to sluggish or stuck fermentations. Competition for nutrients seemed to be one of the main causes for incomplete fermentations in non-*Saccharomyces*/*Saccharomyces* co-cultures. A more complete understanding of nutrient requirements for the non-*Saccharomyces* yeasts is necessary to better conduct the mixed and sequential fermentations in terms of nutrition to avoid sluggish or stuck fermentations. The impact of nutrient limitation on mixed/sequential cultures wine fermentation has been poorly studied. However, deficiency in nitrogen and some vitamins such as thiamine and pantothenic acid has been associated with sluggish wine fermentations performed with *S. cerevisiae* (Bataillon et al., 1996; Bisson, 1999; Blateyron and Sablayrolles, 2001; Wang et al., 2003; Bohlscheid et al., 2007). Medina et al. (2012) were among the first authors to highlight competition for nitrogen between *S. cerevisiae* and *Hanseniaspora vinae* or *Metschnikowia pulcherrima*, especially when the initial nitrogen content was too low. Nevertheless, in the latter study, only the total yeast assimilable nitrogen (YAN) was monitored and not the individual consumption of each nitrogen source by non-*Saccharomyces* yeasts. In 2014, Taillandier et al. reported a similar result when *Torulaspora delbrueckii* was inoculated together with *S. cerevisiae*. The presence of *H. guilliermondii* had a strong influence on the gene expression of *S. cerevisiae*, in particular on genes involved in the biosynthesis of vitamins as well as uptake and biosynthesis of amino acids (Barbosa et al., 2015). These results underlined the importance of competition for nitrogen and vitamins between yeast species. Kemsawasd et al. (2015b) showed that certain nitrogen sources were beneficial for all yeast species while others were only beneficial to specific species. Overall, the influence of nitrogen sources on yeast growth and fermentation performance differed between species, with *T. delbrueckii* and *H. uvarum* being the most similar to *S. cerevisiae*. Recently, Gobert et al. (2017) determined the order of uptake of nitrogen sources of three non-*Saccharomyces* yeast strains (*M. pulcherrima*, *Starmerella bacillaris,* and *Pichia membranifaciens*) inoculated as pure cultures in grape juice. Species-dependent differences were evidenced, but these did not impact *S. cerevisiae*'s fermentation and growth performances in sequential cultures. However, the consumption of different concentrations of nitrogen sources by the non-*Saccharomyces* yeasts impacted the organoleptic properties of the final wines.

Rollero et al. (submitted) have recently determined the preferences in terms of nitrogen sources for 10 non-conventional wine yeasts isolated from South African grape juices. This work highlighted some differences with *S. cerevisiae* as the consumption of GABA or few amount of ammonium (as *Zygoascus meyerae* or *Pichia burtonii*) but some strains,

Kluyveromyces marxianus for instance, displayed the same preferences than *S. cerevisiae*. Specific aroma profiles for these strains were also identified in pure culture and could be interesting for the organoleptic properties of wines. In summary, the few studies published in literature suggest that, when non-*Saccharomyces* yeasts are co-inoculated with *S. cerevisiae*, competition for nutrients occurs and may have dire impact on fermentation.

The aim of our study was to evaluate the effect of nitrogenous nutrient consumption in a synthetic fermentation broth by three non-*Saccharomyces* strains (*P. burtonii*, *Z. meyerae,* and *K. marxianus*) selected during a previous study (Rollero et al. submitted) during sequential inoculation with *S. cerevisiae* on their growth, fermentation performances, and aroma production. Possible interactions and competitions for nutrients, in particular nitrogen sources, between *S. cerevisiae* and selected non-*Saccharomyces* yeasts were also assessed in order to optimize sequential fermentations, to manage nutrient supplementation adequately and ultimately prevent stuck or sluggish fermentations.

MATERIALS AND METHODS

Yeasts Strains and Preculture Conditions

The fermentations were performed with the commercial wine strain *Saccharomyces cerevisiae* Lalvin EC1118® (Lallemand SA, Montreal, Canada) and three non-*Saccharomyces* yeasts isolated from South African grape juices (IWBT collection, Stellenbosch, South Africa), namely *Kluyveromyces marxianus* IWBT Y885, *Zygoascus meyerae* IWBT Y826, and *Pichia burtonii* IWBT Y951. The cryopreserved yeast cultures were thawed at room temperature and streaked on Yeast Peptone Dextrose (YPD) agar (Biolab-Merck, Modderfontein, South Africa). Starter cultures of all yeast strains were prepared by inoculating a single colony into 5 ml YPD broth for each strain. The cultures were incubated at 30°C on a test tube rotating wheel for 24 h. These starter cultures were used to inoculate YPD precultures at an initial cell density of 1×10^6 cells/ml which were incubated at 30°C with shaking (125 rpm) for 9 h. In an attempt to deplete the reserves of nitrogen sources present in the cells, the yeasts were incubated for 4 h (*P. burtonii*), 6 h (*K. marxianus*), or 8 h (*Z. meyerae* and *S. cerevisiae*) in YNB containing neither amino acid nor ammonium (Difco Laboratories) supplemented with 20 g/l of glucose at 30°C with shaking (125 rpm). The growth in this medium was monitoring every 2 h until the end of growth corresponding to the depletion in nitrogen.

Sequential mixed cultures were performed with the inoculation of one of the non-*Saccharomyces* yeasts 48 h before *S. cerevisiae* yeast. A pure culture with only *S. cerevisiae* was also carried out. All the strains were inoculated from the preculture at 1×10^6 cells/ml.

Fermentations Conditions and Sampling

Fermentations were carried out in synthetic medium (SM) that simulates standard grape juice (Bely et al., 1990). The SM used in this study contained 230 g/l of sugar (115 g/l of glucose and 115 g/l of fructose); 2.5 g/l of potassium L-tartrate; 3 g/l of malic acid; 0.2 g/l of citric acid; 1.14 g/l of potassium hydrogen phosphate; 0.44 g/l of magnesium sulfate heptahydrate; 1.23 g/l of calcium chloride dehydrate; vitamins (mg/l): myo-inositol (100), calcium pantothenate (1), thiamin hydrochloride (0.5), nicotinic acid (2), pyridoxine hydrochloride (2), biotin (0.125), PABA.K (0.2), riboflavin (0.2), folic acid (0.2); trace elements (μg/l): manganese (II) chloride tetrahydrate (200), zinc chloride (135), iron chloride (30), copper chloride (15), boric acid (5), cobalt nitrate hexahydrate (1), sodium molybdate dehydrate (25), potassium iodate (10).

The nitrogen sources comprised ammonium chloride and amino acids. The composition of the stock solution of amino acids and ammonium was (in g/l): tyrosine (1.8), tryptophan (17.9), isoleucine (3.2), aspartate (4.4), glutamate (12.0), arginine (37.4), leucine (4.8), threonine (7.5), glycine (1.8), asparagine (5.3), glutamine (50.5), alanine (14.5), valine (4.4), methionine (3.1), phenylalanine (3.7), serine (7.8), histidine (3.2), lysine (1.7), GABA (14), cysteine (1.3), proline (61.2), and ammonium chloride (46). To obtain 200 mg/l of yeast assimilable nitrogen in the SM, 6.57 ml of this solution was added to the 1 l of medium.

Instead of adding ergosterol (yeast sterol) as described previously (Bely et al., 1990), SM medium was initially supplemented with anaerobic factors composed of phytosterols (85451, Sigma Aldrich), sterols naturally present in the grape juice (Le Fur et al., 1994), and Tween 80 for a final concentration of 10 mg/l. The stock solution was composed of 5 g/l of phytosterols in Tween 80 and ethanol (1:1, v/v).

The pH of the synthetic medium was adjusted to 3.3 with potassium hydroxide (Saarchem, Krugersdorp, South Africa). The trace elements, vitamins, nitrogen sources, and anaerobic factors were filtered through a 0.22-μm syringe filter (Starlab Scientific, Cape Town, South Africa) and added into the autoclaved synthetic medium.

Each fermentation was performed in triplicate. The fermentations were carried out in cylindrical fermenters of 3.5 cm diameter and 10 cm height. The fermenters contained 70 ml of medium, so that the headspace occupied 30% of the volume of the fermenters. In order to maintain anoxic conditions, the fermenters were equipped with fermentation locks filled with water, at 25°C, with orbital agitation (125 rpm). The fermentation progress was monitored by determination of CO_2 release extrapolated from the measurement of the weight loss throughout the process.

At the end of each fermentation, different samples were centrifuged at 4000 g for 5 min, after which the supernatants were filtered through a 0.22-μm syringe filter (Starlab Scientific, Cape Town, South Africa) and stored at −20°C for further chemical analysis.

Additions of Nitrogen Sources

For some fermentations where *Z. meyerae* or *K. marxianus* were sequentially inoculated with *S. cerevisiae*, nitrogen sources (ammonium, mixture of amino acids, or FermaidO®, Lallemand SAS, Canada) were added at the same time as the inoculation of *S. cerevisiae* to reach the yeast assimilable nitrogen concentration of 200 mg/l.

Monitoring of Yeast Population

During the first 48 h, the yeast cell populations were monitored by plating each day the appropriate dilutions onto YPD nutrient agar (Biolab-Merck, Modderfontein, South Africa). After the *S. cerevisiae*'s inoculation, the viability of yeasts was monitored throughout the fermentation by plating on a selective medium which was identified before inoculation. The three non-*Saccharomyces* yeasts were enumerated on YPD agar supplemented with 5 mg/l cycloheximide, which was the lowest concentration found to suppress *S. cerevisiae* growth while allowing that of the other species. *S. cerevisiae*'s population was determined by plating the appropriate dilutions on YPD agar plates and by subtracting the yeasts enumerated on YPD + cycloheximide plates. Plates were incubated at 30°C, generally for 2–3 days, until colonies were formed.

Quantification of Residual Sugars and Ammonium by Enzymatic Assays

For the residual glucose, fructose, and ammonium concentrations, 400 µl of filtered sample was enzymatically analyzed using the Arena 20XT (Thermo Fisher Scientific, Waltham, MA) which makes use of automated spectrophotometric readings to determine the concentrations of the various compounds. The different enzymatic assay kits utilized were the following: Enzytec™ Fluid D-Glucose (Id-No: 5140, R-BiopharmAG, Germany) for glucose, Enzytec™ Fluid D-Fructose (Id-No: 5120, R-BiopharmAG, Germany) for fructose, and Enzytec™ Fluid Ammonia (Id-No: 5390, R-BiopharmAG, Germany) for ammonium.

Quantification of Individual Amino Acids

Quantification of individual amino acids was performed by high performance liquid chromatography (HPLC), Agilent 1100 (Agilent Technologies, Waldbronn, Germany) using pre-column derivatization and fluorescence detection based upon a method previously described (Henderson and Brooks, 2010) with some modifications to the derivatization and injection. A Poroshell HPH-C18 column (4.6 × 150 mm, 2.7 µm particle size; Agilent Technologies) was used following derivatization of the amino acids. Derivatization was performed using three different reagents: iodoacetic acid (Sigma Aldrich) for cysteine, o-phthaldialdehyde (OPA, Sigma Aldrich) for primary amino acids, and fluorenylmethyloxycarbonyl chloride (Sigma Aldrich) for secondary amino acids. Internal standards, norvaline (Sigma Aldrich), and sarcosine (Sigma Aldrich) were spiked to each sample prior to derivatization. One milliliter of each filtered sample was analyzed.

Analysis of Major Volatile Compounds

The quantification of major volatiles (i.e., a selection of higher alcohols, acetate esters, fatty acids, fatty acid ethyl esters) was carried out by gas chromatography equipped with a flame ionization detector (GC-FID) using the Agilent GC System HP 6890 Series (Agilent, Little Falls, Wilmington, USA) as described previously (Louw et al., 2009) with minor modifications. Five milliliters of each of the filtered samples were used with 100 µl of 4-methyl-2-pentanol (internal standard). Diethyl ether (1 ml) was added to the mixture which was then placed in an ultrasonic bath for 5 min to extract the volatile compounds. Thereafter, the samples were centrifuged at 4000 g for 3 min. Sodium sulfate was added to remove any water from the non-polar layer. HP Chemstation software was used for data analysis.

Statistical Analysis

Statistical analyses were performed using the R software, version 3.2.3 (http://cran.r-project.org/). Each variable was then tested using a one-way analysis of variance (ANOVA) with the uptake concentration of each nitrogen source as a factor to describe the diversity between the different strains to detect a global effect at a *p*-value threshold of 0.05. For each parameter, normality of residual distributions and homogeneity of variance were studied using standard diagnostic graphics; no violation of the assumptions was detected. As the effect was significant at a *p*-value threshold of 0.05, all pairwise comparisons for agitation speed were tested using Tukey's honestly significant difference (HSD) test. The principal component analysis (PCA) was carried out with the FactoMineR package (Le et al., 2008).

RESULTS

This work aimed to compare the outcomes of fermentation (fermentation performances and aroma production) by three non-*Saccharomyces* strains during sequential inoculations with *S. cerevisiae* and highlighted the possible competition for nutrients.

Fermentation Kinetics and Population Dynamics

As expected, the pure culture of *S. cerevisiae*, considered as control, was the only yeast to reach dryness (i.e., residual sugars below 2 g/l), while fermentations conducted by the three non-*Saccharomyces* yeast pure cultures got stuck with residual sugars of 104, 184, and 190 g/l (data not shown) for *K. marxianus*, *P. burtonii,* and *Z. meyerae*, respectively (**Figures 1A–C**). Concerning the population dynamics, *S. cerevisiae* reached its maximal population (1.1×10^8 cfu/ml) after 32 h of fermentation, while the three non-*Saccharomyces* species reached theirs after 48 h (6.10×10^7, 5.8×10^7, and 3.0×10^7 cfu/ml, for *K. marxianus*, *Z. meyerae,* and *P. burtonii*, respectively). No loss of viability was observed in pure culture (**Figures 1D–F**).

Concerning the sequential inoculations, three different behaviors were observed according to the yeasts inoculated with *S. cerevisiae*. Only the sequential inoculation of *K. marxianus* together with *S. cerevisiae* did not reach dryness and got stuck with 48 g/l of residual sugar (data not shown), while the sequential fermentations with *P. burtonii* or *Z. meyerae* exhausted all the sugars (**Figures 1A–C**). However, the kinetic profiles were different. Indeed, the fermentation with *Z. meyerae* displayed a longer fermentation duration (around 15 days) with a slow average fermentation rate even after the inoculation of *S. cerevisiae* (0.35 g CO_2/l/h) (**Figure 1B**). However, the fermentation performed with *P. burtonii* finished in 10 days displaying after the inoculation of *S. cerevisiae* the same average

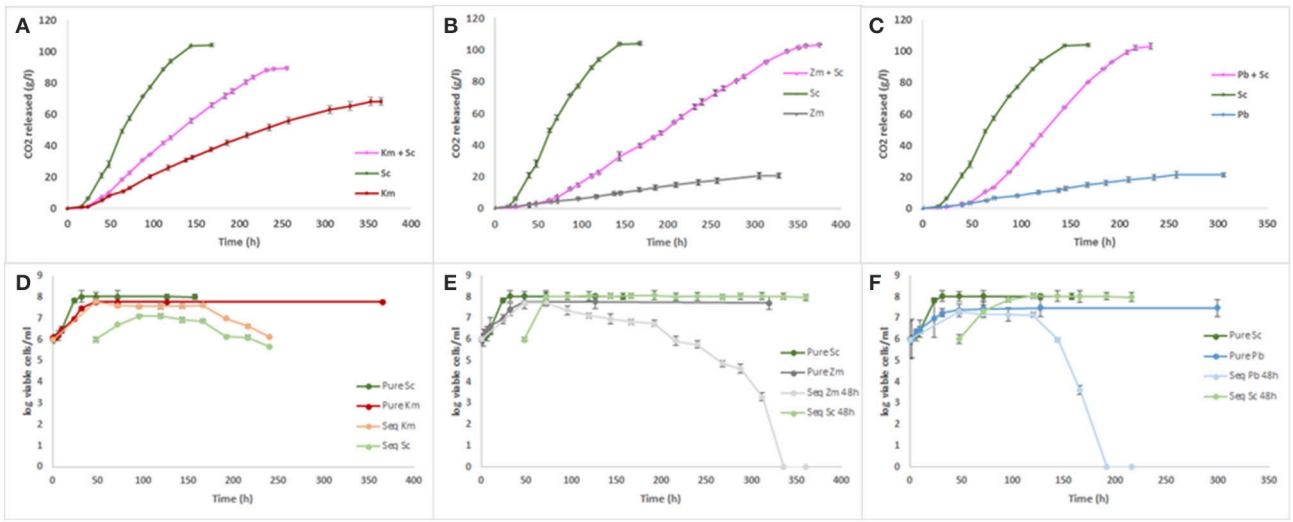

FIGURE 1 | Fermentation kinetics and yeast growth of pure and sequential cultures of *K. marxianus* **(A,D)**, *Z. meyerae* **(B,E)**, and *P. burtonii* **(C,F)** with *S. cerevisiae*.

fermentation rate (0.93 g CO_2/l/h) as the *S. cerevisiae*'s pure culture (**Figure 1C**).

The population dynamics during sequential fermentation presented two distinct patterns. For sequential inoculations with *Z. meyerae* or *P. burtonii*, a mortality of these yeasts was observed within the hours following the inoculation of *S. cerevisiae* until they were no longer detectable, while *S. cerevisiae*'s population reached the same maximal population than its pure culture (**Figures 1E,F**). Concerning the sequential culture with *K. marxianus*, a very weak implantation of *S. cerevisiae* was observed associated with a decrease of the non-*Saccharomyces* yeast population (**Figure 1D**). When fermentation stopped, both species were undetectable.

Consumption of Nitrogen Sources

Consumption of ammonium and amino acids was determined at 48 h and at the end of fermentation during the sequential inoculations (**Table 1**). After 48 h of fermentation, the nature and the quantity of nitrogen sources consumed differed for each strain. *S. cerevisiae* displayed the highest uptake for the majority of nitrogen sources (55% of the assimilable nitrogen) except for γ-aminobutyric acid (GABA) and arginine which were taken up in greater amounts by *Z. meyerae* and *P. burtonii* for GABA and by *Z. meyerae* for arginine. *K. marxianus* was able to consume almost 40% of the assimilable nitrogen present in the medium within the first 48 h and displayed the same preferences than *S. cerevisiae*, except for ammonium which was poorly consumed by *K. marxianus*. On the other hand, *P. burtonii* and *Z. meyerae* were able to consume around 20% of the nitrogen. Interestingly within the first 48 h, *Z. meyerae* did not consume threonine at all. At the end of the sequential fermentations and for all the yeasts, nitrogen was completely depleted (except for GABA and in a lesser extent glycine).

Production of Major Volatile Compounds during Sequential Fermentations

The major volatile compounds formed by yeasts during alcoholic fermentation were determined after 48 h of fermentation and in the final wines (**Figure 2A,B**, Table SD1). First, it is interesting to note that the triplicates were well grouped on the PCA and three groupings were identifiable: *Z. meyerae* and *P. burtonii*, *K. marxianus* and finally *S. cerevisiae*. After 48 h of fermentation, different aroma profiles can be identified according to the yeast species inoculated. Fermentations performed with *K. marxianus* were characterized by the enhanced production of isobutanol, phenylethylacetate, ethyl acetate, and acids (fusel and medium chain fatty acids), while *Z. meyerae* and *P. burtonii* produced the highest amount of phenylethanol, isoamyl alcohol, and ethyl esters (**Figure 2A**). Isoamyl acetate, ethyl hexanoate, hexanoic, and propionic acids characterized the fermentations conducted by *S. cerevisiae* alone (**Figure 2A**). Interestingly, *Z. meyerae* and *P. burtonii* did not produce any fatty acid (short or medium chain) within the first 48 h, but at the end of the sequential fermentation, the concentrations were higher than the pure culture of *S. cerevisiae* (with just a few exceptions). In the final wines, the groupings remained the same than after 48 h, and were still characterized by the same aromatic profiles according the non-*Saccharomyces* used to perform the sequential inoculation with *S. cerevisiae* with some notable exceptions such as isoamyl alcohol, acetoin, and some fatty acids (**Figure 2B**).

Higher alcohols can be formed by the catabolism of certain amino acids (via the Ehrlich pathway) but also by the sugar metabolism. In an attempt to estimate the amount of these compounds which was directly formed through amino acid metabolism (in contrast to that formed through carbon metabolism), the molar ratio of higher alcohol produced over the amino acid precursor consumed was calculated (**Figure 3**). At 48 h, this ratio was systematically higher for *Z. meyerae*

TABLE 1 | Uptake of individual amino acids and ammonium (in mg/l) for the 4 strains after 48 h and at the end of fermentation during sequential inoculations for the non-*Saccharomyces* yeasts and during pure culture for *S. cerevisiae*.

	Initial content	After 48 h of fermentation				End of fermentation			
		S. cerevisiae	*K. marxianus*	*Z. meyerae*	*P. burtonii*	*S. cerevisiae*	*K. marxianus*	*Z. meyerae*	*P. burtonii*
NH4	104.89	60.94 ± 0.54^{a}	24.07 ± 0.28^{b}	27.41 ± 1.29^{c}	22.93 ± 1.19^{b}	104.89 ± 0.21^{a}	104.88 ± 0.32^{a}	104.92 ± 0.43^{a}	104.86 ± 0.54^{a}
GLU	80.30	46.39 ± 0.15^{a}	28.12 ± 1.28^{b}	5.47 ± 0.67^{c}	14.70 ± 1.72^{d}	80.31 ± 0.02^{a}	80.29 ± 1.83^{a}	78.81 ± 0.74^{b}	80.30 ± 1.29^{a}
GLN	303.34	205.53 ± 0.07^{a}	156.55 ± 6.56^{b}	52.86 ± 1.50^{c}	95.73 ± 1.80^{d}	303.34 ± 10.23^{a}	298.92 ± 10.76^{a}	303.34 ± 18.96^{a}	303.34 ± 4.87^{a}
ARG	229.10	77.89 ± 0.43^{a}	49.06 ± 4.22^{b}	99.10 ± 0.15^{c}	35.60 ± 0.12^{d}	229.09 ± 11.23^{a}	223.64 ± 12.50^{a}	228.16 ± 19.50^{a}	229.10 ± 20.37^{a}
ASP	28.40	19.67 ± 0.19^{a}	13.48 ± 0.16^{b}	3.99 ± 0.30^{c}	9.03 ± 0.09^{d}	28.40 ± 0.11^{a}	28.39 ± 0.79^{a}	28.32 ± 3.06^{a}	28.40 ± 0.52^{a}
ASN	41.14	19.50 ± 0.03^{a}	18.12 ± 0.45^{b}	6.42 ± 0.16^{c}	11.19 ± 0.52^{d}	41.14 ± 1.21^{a}	39.22 ± 2.24^{a}	40.24 ± 4.44^{a}	41.14 ± 1.83^{a}
HIS	24.56	23.86 ± 0.06^{a}	10.21 ± 0.34^{b}	1.28 ± 0.56^{c}	4.99 ± 0.23^{d}	24.56 ± 0.32^{a}	24.56 ± 0.78^{a}	24.56 ± 3.58^{a}	24.56 ± 0.03^{a}
GLY	16.06	8.01 ± 0.06^{a}	0.85 ± 0.40^{b}	1.51 ± 0.08^{c}	4.18 ± 0.26^{d}	16.06 ± 0.35^{a}	13.52 ± 2.31^{b}	14.01 ± 3.19^{b}	16.06 ± 0.93^{a}
ALA	92.14	36.89 ± 0.26^{a}	24.88 ± 2.52^{b}	16.13 ± 0.78^{c}	14.84 ± 1.42^{c}	92.14 ± 5.03^{a}	90.46 ± 4.94^{a}	90.48 ± 8.78^{a}	92.14 ± 4.98^{a}
GABA	142.82	12.18 ± 1.01^{a}	17.92 ± 1.87^{b}	47.26 ± 0.69^{c}	19.07 ± 2.45^{d}	34.09 ± 7.43^{a}	37.99 ± 9.87^{a}	88.93 ± 11.85^{b}	41.96 ± 7.86^{c}
LYS	12.11	11.24 ± 0.13^{a}	10.93 ± 0.11^{c}	4.05 ± 0.10^{b}	11.39 ± 0.21^{c}	12.11 ± 0.97^{a}	12.11 ± 0.31^{a}	12.11 ± 1.62^{a}	12.11 ± 0.39^{a}
SER	56.01	43.85 ± 0.09^{a}	28.89 ± 0.65^{b}	7.64 ± 0.25^{c}	16.98 ± 0.52^{d}	56.11 ± 0.02^{a}	56.09 ± 0.21^{a}	56.01 ± 0.96^{a}	56.07 ± 0.24^{a}
THR	45.53	36.57 ± 0.05^{a}	23.63 ± 0.84^{b}	0.02 ± 0.24^{c}	15.32 ± 0.04^{d}	45.59 ± 0.19^{a}	45.53 ± 0.81^{a}	45.51 ± 0.64^{a}	45.56 ± 0.16^{a}
TYR	13.94	11.40 ± 0.06^{a}	7.36 ± 0.06^{b}	2.48 ± 0.12^{c}	2.97 ± 0.26^{d}	13.99 ± 0.34^{a}	13.94 ± 0.22^{a}	13.91 ± 0.23^{a}	13.92 ± 0.84^{a}
VAL	34.32	28.27 ± 0.26^{a}	25.76 ± 0.14^{b}	5.78 ± 0.09^{c}	7.52 ± 0.60^{d}	34.33 ± 0.18^{a}	34.32 ± 0.25^{a}	34.30 ± 0.15^{a}	34.30 ± 0.58^{a}
MET	16.88	17.12 ± 0.32^{a}	14.81 ± 0.21^{b}	8.61 ± 0.27^{c}	3.92 ± 0.16^{d}	16.90 ± 0.17^{a}	16.88 ± 0.12^{a}	16.88 ± 0.67^{a}	16.88 ± 0.03^{a}
TRP	118.46	75.17 ± 0.07^{a}	43.61 ± 1.88^{b}	27.31 ± 0.28^{c}	31.96 ± 0.11^{d}	118.46 ± 1.43^{a}	116.48 ± 1.57^{a}	118.46 ± 1.06^{a}	118.46 ± 2.93^{a}
PHE	30.69	29.66 ± 0.06^{a}	27.55 ± 0.25^{b}	5.66 ± 0.25^{c}	3.17 ± 0.67^{d}	30.71 ± 0.76^{a}	30.69 ± 0.10^{a}	30.68 ± 0.90^{a}	30.70 ± 0.41^{a}
ILE	20.16	19.50 ± 0.08^{a}	19.22 ± 0.06^{a}	3.95 ± 0.15^{b}	7.94 ± 0.31^{c}	20.16 ± 0.32^{a}	20.18 ± 0.06^{a}	20.12 ± 0.75^{a}	20.13 ± 0.24^{a}
LEU	32.66	32.79 ± 0.02^{a}	31.64 ± 0.08^{b}	4.78 ± 0.22^{c}	15.33 ± 0.37^{d}	32.66 ± 1.34^{a}	32.61 ± 0.25^{a}	32.63 ± 0.42^{a}	32.68 ± 0.30^{a}
Total	1443.5*	816.43	576.66	331.71	348.76	1335.04	1320.7	1382.38	1342.67

*Mean values ± standard deviation Strains sharing the same letter for a nitrogen sources are not significantly different at a 0.05 threshold. *1443.5 mg/l corresponds to 200 mg/l of assimilable nitrogen. Proline was not consumed by the different yeast strains (data not shown). Due to the HPLC method, cysteine concentration was not correctly assessed.*

and *P. burtonii* (e.g., 12 and 4 times higher for isoamyl alcohol/leucine, respectively) than that calculated for the pure culture of *S. cerevisiae*, while *K. marxianus* and *S. cerevisiae* displayed the same ratio (**Figure 3A**). At the end of fermentation, the differences between the yeast strains were less important, but some differences were still visible. *S. cerevisiae* presented the lowest ratio for the three higher alcohols (**Figure 3B**). The ratios for isobutanol and phenylethanol were higher for the wines obtained with *K. marxianus* (2 and 1.5 times higher respectively, **Figure 3B**) than those obtained for *S. cerevisiae*'s pure culture. Concerning isoamyl alcohol, the highest ratio was reached with *Z. meyerae* and then *P. burtonii* (1.5 and 1.2 time higher, respectively). The same trends were observed for the fusel acids and acetate esters (data not shown).

At the end of the fermentation, the concentrations of isobutanol, phenylethanol, and isoamyl alcohol were generally much higher in sequential fermentations than in those where non-*Saccharomyces* yeasts were absent (**Figure 4**). The figure shows that the contribution of non-*Saccharomyces* yeasts extends beyond the concentrations produced during the first 48 h, such as the production of isobutanol with *K. marxianus* or *Z. meyerae*.

Influence of Additions of Various Nitrogen Sources

As *K. marxianus* consumed 70% of the assimilable nitrogen available during the first 48 h of fermentation and considering the fact that *S. cerevisiae* was not able to grow, nitrogen additions (with ammonium or mixture of amino acids and ammonium used in the synthetic must) were performed (**Figure 5A**). With nitrogen supplementations, the fermentation performed with *K. marxianus* sequentially inoculated with *S. cerevisiae* was able to reach dryness regardless of the nature of the nitrogen source added (**Figure 5A**).

When *Z. meyerae* was sequentially inoculated with *S. cerevisiae*, the fermentation reached dryness but only after 15 days due to a slower fermentation rate compared to the *S. cerevisiae*'s pure culture or the sequential inoculation *P. burtonii*/*S. cerevisiae* (**Figure 1**). To determine the reason of this slower fermentation, some nutrient additions were performed: mixture of amino acids, ammonium or a commercial nutrient (yeast autolysate), FermaidO®. No impact on the fermentation process was observed with the addition of amino acids and ammonium, while the fermentation was shorter with the addition of the commercial nutrient (**Figure 5B**). The limitation of the fermentation performance was not due to nitrogen deficiency.

DISCUSSION

The inoculation of non-*Saccharomyces* yeasts sequentially with *S. cerevisiae* is becoming a common practice to alter the organoleptic properties of wine (Hu et al., 2016; Liu et al., 2016;

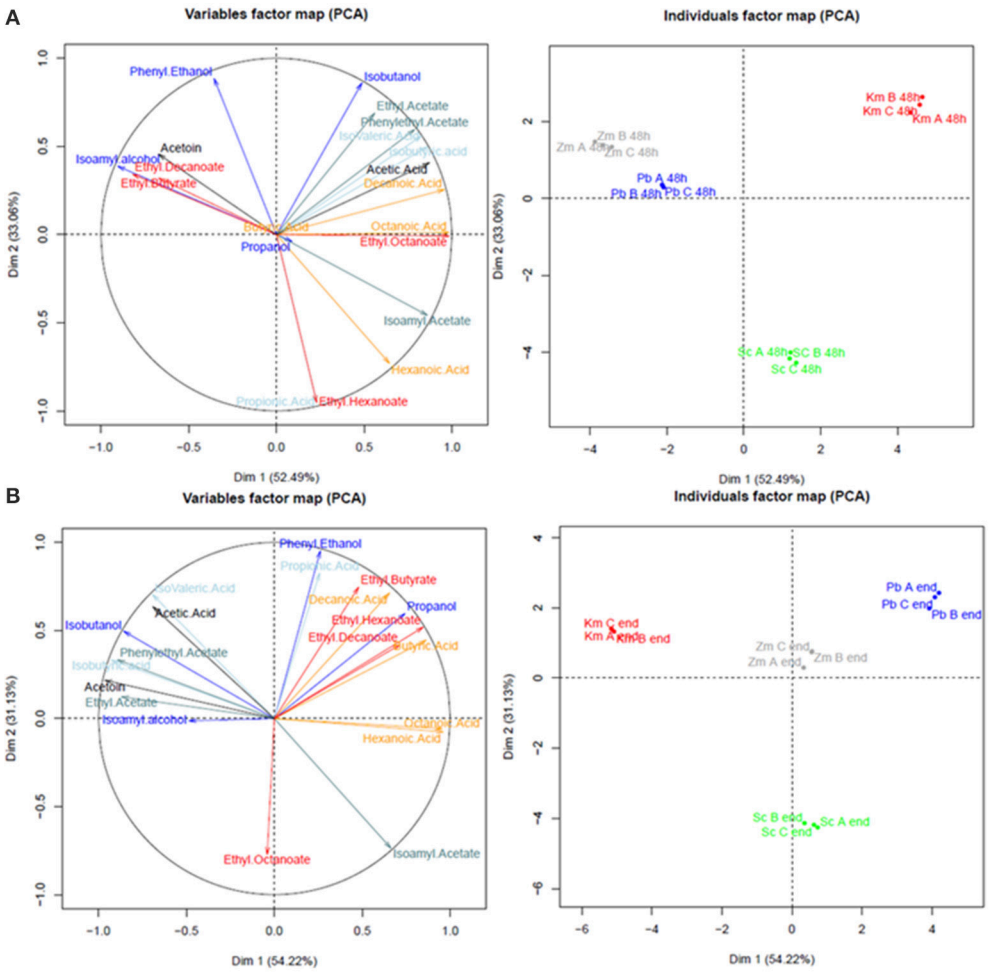

FIGURE 2 | Principal component analysis of major volatile compound production after 48 h **(A)** and at the end of fermentation **(B)** during sequential fermentations. Dark blue, higher alcohols; light blue, acetate esters; gray blue, fusel acids; red, small and medium chain fatty acids; orange, ethyl esters.

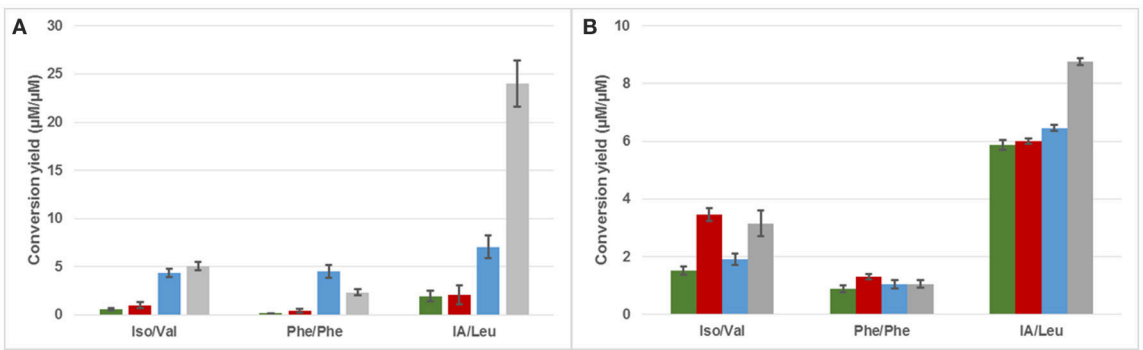

FIGURE 3 | Conversion yield of amino acid precursors into their higher alcohols at 48 h **(A)** and at the end of fermentation **(B)**. ISO, isobutanol; Phe, phenylethanol; IA, isoamyl alcohol; Val, valine; Phe, phenylalanine; Leu, leucine. Green: *S. cerevisiae*, Red: *K. marxianus*, Blue: *P. burtonii*, Gray: *Z. meyerae*.

Lleixà et al., 2016; Renault et al., 2016; Gobert et al., 2017). However, the yeast-yeast interactions and possible competition for nutrients arising from this new style of inoculation require further investigations (Ciani and Comitini, 2011; García et al., 2016). Previously, it was suggested that the growth of non-*Saccharomyces* yeasts resulted in the depletion in nutrients,

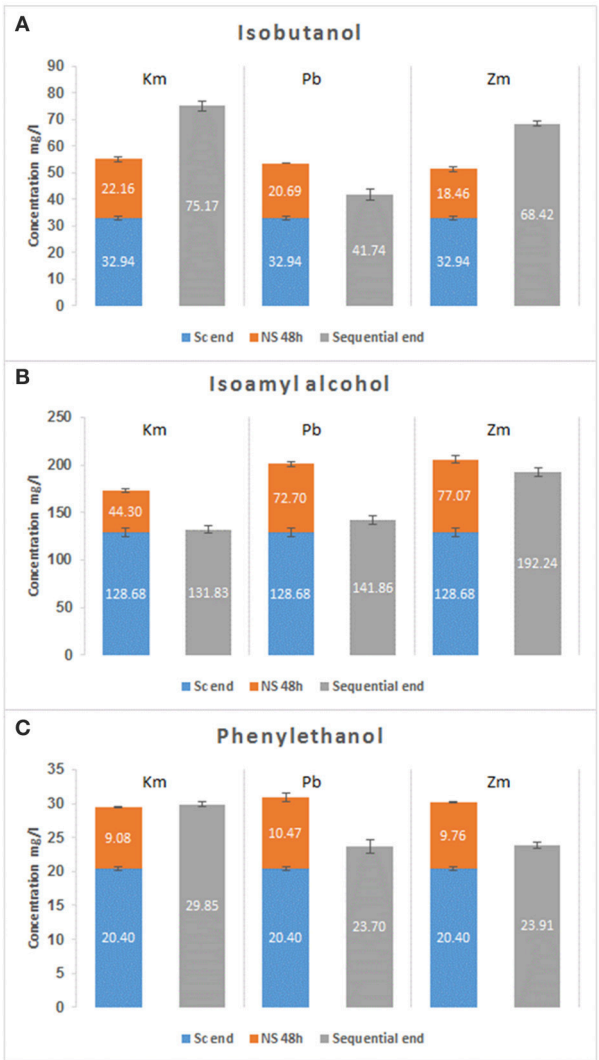

FIGURE 4 | Contribution of non-Saccharomyces yeasts for isobutanol **(A)**, isoamyl alcohol **(B)**, and phenylethanol **(C)** production during sequential fermentations.

especially in assimilable nitrogen, and in an unfavorable medium for *S. cerevisiae* proliferation (Fleet, 1993; Bataillon et al., 1996). The aim of our study was to evaluate the effect of sequential *S. cerevisiae* and non-*Saccharomyces* cultures on nitrogen source consumption and fermentative aroma production in order to determine the extent of competition for nitrogen sources among the different microorganisms involved and ultimately the consequence on fermentation outcomes.

Our results clearly demonstrate that *S. cerevisiae* had an antagonistic impact upon *P. burtonii* and *Z. meyerae* as the populations of these two species were rapidly decimated after the inoculation of *S. cerevisiae*. According to literature, several mechanisms underlying these interactions occur and depend on the *S. cerevisiae*/non-*Saccharomyces* pair used. Previous studies hypothesized that the premature death of non-*Saccharomyces* yeasts was induced by the production of toxic compounds by *S. cerevisiae* such as killer toxins or antimicrobial peptides (Pérez-Nevado et al., 2006; Albergaria and Arneborg, 2016; Wang et al., 2016). Other authors concluded that the early death of non-*Saccharomyces* yeasts can be due to a cell-to-cell contact mechanism (Nissen et al., 2003; Renault et al., 2013; Kemsawasd et al., 2015a). It is important to note that in the present work, non-*Saccharomyces* yeasts and *S. cerevisiae* were inoculated at the same concentration (10^6 cells/ml). Several studies highlighted that the increase of the ratio of inoculation in favor of the non-*Saccharomyces* yeasts improved their persistence in the medium in presence of *S. cerevisiae* (Pérez-Nevado et al., 2006; Comitini et al., 2011; Domizio et al., 2011). Changing this ratio could be a lever to enhance the persistence of these non-*Saccharomyces* yeasts in our fermentations but it would also increase the possible competition for nutrients without the guarantee of a better fermentation performance of non-*Saccharomyces* yeasts which displayed a very low sugar consumption in pure culture (<40 g/l).

The decline and premature death of *K. marxianus* during the sequential fermentation could be explained by its difficulty to overcome the ethanol increase and the impact on its plasmic membrane. Indeed, Diniz et al. (2017) demonstrated that the expression of some gene-encoding enzymes related to unsaturated fatty acid and ergosterol biosynthesis decreased upon ethanol exposure, and free fatty acid and ergosterol measurements demonstrate that their content in *K. marxianus* did not change under this stress.

Competition for nutrients may have a negative impact on *S. cerevisiae*'s growth and fermentation performance. Indeed, the uptake of nutrients by non-*Saccharomyces* yeasts may hinder *S. cerevisiae*'s growth and ultimately affect its fermentation performance. Non-*Saccharomyces* species growing early in the fermentation could strip the medium of amino acids and vitamins, limiting the subsequent growth and fermentation performances of *S. cerevisiae* (Bisson, 1999; Medina et al., 2012; Taillandier et al., 2014; Barbosa et al., 2015). In our study, three different behaviors of *S. cerevisiae* were observed depending on the non-*Saccharomyces* yeasts used in the sequential culture and can be explained by the competitions for nutrients. These species-dependent interactions were not observed by Gobert et al. (2017). Indeed, in the latter author' study, the performances of *S. cerevisiae* remained similar, regardless of the non-*Saccharomyces* inoculated. These differences in the behavior of *S. cerevisiae* could be explained by the differing experimental conditions between the two studies: (1) the species and the medium used were different, and (2) in this study, prior to inoculation into the fermentation medium, the yeasts were starved of nitrogen which probably greatly impacted their nitrogen uptake. During the sequential fermentation with *P. burtonii*, *S. cerevisiae* displayed the same performances than its pure culture and it can be concluded that no nutrient competition occurred between these two strains, corroborated by the very low amino acid and ammonium consumption of *P. burtonii*. While *S. cerevisiae* grew poorly and declined rapidly during the sequential culture with *K. marxianus* leading to an incomplete fermentation. The competition for nitrogen between *K. marxianus* and *S. cerevisiae* was suspected because

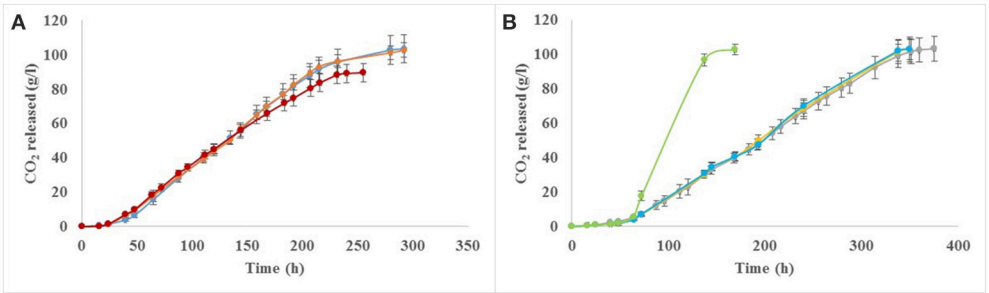

FIGURE 5 | Impact of nitrogen additions on fermentation performances of *K. marxianus* **(A)** and *Z. meyerae* **(B)**. Ammonium addition (orange), amino acid mixture addition (blue), FermaidO® addition (green), no addition with *K. marxianus* (red), or *Z. meyerae* (gray).

of the amino acid consumption pattern of *K. marxianus* was very close to that of *S. cerevisiae* and then confirmed experimentally when ammonium or amino acid additions led to a complete fermentation. This observation was consistent with previous studies conducted on other species which demonstrated this competition between *T. delbrueckii* (Taillandier et al., 2014) or *L. thermotolerans* (Ciani et al., 2006; Gobbi et al., 2013) and *S. cerevisiae*. Concerning the sequential culture *Z. meyerae*/*S. cerevisiae*, competition for another nutrient different from nitrogen was evidenced. The addition of amino acids or ammonium did not change the fermentation rate of *S. cerevisiae*. However, the addition of a more complex nutrient led to a faster fermentation, thereby suggesting that the strains competed for lipids, vitamins or minerals. Indeed, literature in reference to the vitamin requirements for growth and fermentation performances by wine yeasts is very limited. Bataillon et al. (1996) showed that *K. apiculata* was very efficient at shipping thiamine and removed this vitamin from the medium more rapidly than *S. cerevisiae* leading to deficient growth of *S. cerevisiae*. Recently, Medina et al. (2012) also highlighted the importance of vitamin availability during mixed cultures. Moreover, the use of complex nutrients can also be an alternative to restore a certain balance between the various nutrients, especially by providing lipids. Indeed, it was previously shown that a deficiency in lipids leads to stuck or sluggish fermentations and the addition of lipids allows to re-establish a complete fermentation, a better growth and viability of cells (Casalta et al., 2013; Ochando et al., 2017).

Most of the studies with co-inoculation or sequential inoculation of non-*Saccharomyces*/*S. cerevisiae* species have highlighted the differences in the aromatic profiles obtained in these final wines compared with monocultures of *S. cerevisiae* (Comitini et al., 2011; Andorrà et al., 2012; Renault et al., 2015; Gobert et al., 2017). However, none of the latter studies clearly established that the aroma compounds produced by the non-*Saccharomyces* yeasts within the first 48 h (prior to *S. cerevisiae* inoculation) allow to distinguish the final wines from each other in a species-dependent manner. In this context, our study clearly demonstrated that the aromatic footprint of the non-*Saccharomyces* yeasts visible after 48 h was still present at the end of the sequential culture, irrespective of the survival or decline of these yeasts. For instance, *P. burtonii* and *Z. meyerae* were associated with a higher production of higher alcohols. These compounds can have both a positive and negative impact on the aroma and flavor of a wine depending on their final concentration (Beltran et al., 2005). It has been reported that concentrations below 300 mg/l add a desirable level of complexity to wine, whereas concentrations that exceed 400 mg/l can have a detrimental effect. Both the sequential cultures *P. burtonii*/*S. cerevisiae* and *Z. meyerae*/*S. cerevisiae* never exceeded this concentration (Table SD1). These strains also significantly increased the synthesis of ethyl esters that impart fruity flavors to wine associated with the increase of short and medium chain fatty acids, precursors of these esters. The sequential fermentation with *K. marxianus* presented significant increases in compounds, which can impact positively on the aroma such as phenylethanol and phenylethyl acetate, which contribute to a desirable floral (rose) aroma, consistent with previous observations about this species (Gethins et al., 2015). The final result of these fermentations will be a higher complexity, yet further studies including sensorial analysis should be performed. Nevertheless, we cannot certify the origin of this higher complexity. The different aromatic patterns of wines can be due to: (i) the production of volatile compounds throughout the fermentation by the non-*Saccharomyces* yeasts even after the inoculation of *S. cerevisiae*, or (ii) the interaction between non-*Saccharomyces* yeast and *S. cerevisiae* which impacted the metabolism of the latter which will then produce more aromas than in pure culture. Gobert et al. (2017) also suggested the existence of these two mechanisms which appeared to be volatile compound- and strain-dependent in their study.

Higher alcohols can be formed from the degradation of specific amino acids or from sugar metabolism (Hazelwood et al., 2008). Previous studies on *S. cerevisiae* showed that only a small fraction (5%) of higher alcohols were produced from the catabolism of amino acids (Crépin et al., 2017; Rollero et al., 2017). Our results suggested that this fraction was even smaller for *Z. meyerae* and *P. burtonii* because of their very low consumption of valine, leucine and phenylalanine and comparatively high production of the corresponding higher alcohols (**Table 1**), while it remained similar for *K. marxianus*, except for the phenylalanine/phenylethanol ratio which seemed to be higher. Since phenylethanol may be produced through

the degradation of compounds arising from the pentose phosphate pathway, this observation is in accordance with the transcriptomic results reported in Diniz et al. (2017). Indeed, the latter authors showed that the genes involved in the pentose phosphate pathway seemed to be overexpressed in the presence of 6% of ethanol. A complete quantitative study of the fate of amino acids is required to better characterize the role of amino acids in the aroma production.

CONCLUSION

The use of sequential yeast cultures in industrial wine production is currently under scrutiny. In this study, we demonstrated that the nitrogen consumption by non-*Saccharomyces* yeasts during sequential fermentations with *S. cerevisiae* can lead to stuck or sluggish fermentation due to species-dependent competition for nitrogen sources but also for other nutrients, thereby highlighting the importance of monitoring nutrient concentrations closely in these inoculation scenarios. Nevertheless, our study also showed that the use of non-*Saccharomyces* yeasts led to a more complex and aromatic wine that the monoculture of *S. cerevisiae*. These benefits could justify the selection of appropriate non-*Saccharomyces* yeasts whose production of detrimental products is low and that they interact correctly with *S. cerevisiae*. Thus, a better understanding of the nutrient consumption is required for industrial environments in order to adapt nitrogen management according to the yeast pair considered.

AUTHOR CONTRIBUTIONS

SR: Performing the experiments, writing of the manuscript, discussion of results, analysis of results. AB, CC, and BD: Design of experiments, analysis of results, discussion of results, writing of the manuscript. AO-J: Design of experiments, discussion of the results.

FUNDING

This project was funded by Lallemand SAS and Stellenbosch University.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.00196/full#supplementary-material

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

frontiers in Microbiology

ORIGINAL RESEARCH
published: 02 February 2018
doi: 10.3389/fmicb.2018.00088

Saccharomyces cerevisiae and *S. kudriavzevii* Synthetic Wine Fermentation Performance Dissected by Predictive Modeling

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OPEN ACCESS

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

Received: *04 October 2017*
Accepted: *15 January 2018*
Published: *02 February 2018*

Citation:
Henriques D, Alonso-del-Real J, Querol A and Balsa-Canto E (2018) Saccharomyces cerevisiae and S. kudriavzevii Synthetic Wine Fermentation Performance Dissected by Predictive Modeling. Front. Microbiol. 9:88. doi: 10.3389/fmicb.2018.00088

Wineries face unprecedented challenges due to new market demands and climate change effects on wine quality. New yeast starters including non-conventional *Saccharomyces* species, such as *S. kudriavzevii*, may contribute to deal with some of these challenges. The design of new fermentations using non-conventional yeasts requires an improved understanding of the physiology and metabolism of these cells. Dynamic modeling brings the potential of exploring the most relevant mechanisms and designing optimal processes more systematically. In this work we explore mechanisms by means of a model selection, reduction and cross-validation pipeline which enables to dissect the most relevant fermentation features for the species under consideration, *Saccharomyces cerevisiae* T73 and *Saccharomyces kudriavzevii* CR85. The pipeline involved the comparison of a collection of models which incorporate several alternative mechanisms with emphasis on the inhibitory effects due to temperature and ethanol. We focused on defining a minimal model with the minimum number of parameters, to maximize the identifiability and the quality of cross-validation. The selected model was then used to highlight differences in behavior between species. The analysis of model parameters would indicate that the specific growth rate and the transport of hexoses at initial times are higher for *S. cervisiae* T73 while *S. kudriavzevii* CR85 diverts more flux for glycerol production and cellular maintenance. As a result, the fermentations with *S. kudriavzevii* CR85 are typically slower; produce less ethanol but higher glycerol. Finally, we also explored optimal initial inoculation and process temperature to find the best compromise between final product characteristics and fermentation duration. Results reveal that the production of glycerol is distinctive in *S. kudriavzevii* CR85, it was not possible to achieve the same production of glycerol with *S. cervisiae* T73 in any of the conditions tested. This result brings the idea that the optimal design of mixed cultures may have an enormous potential for the improvement of final wine quality.

Keywords: *Saccharomyces* species, temperature, wine fermentation, dynamic modeling, parameter estimation, cross-validation

INTRODUCTION

Wine is obtained through the fermentation of grape must, a complex media composed by a rich blend of amino acids, sugars, organic acids, vitamins and the list goes on. Modern wine industry selects specific yeasts to inoculate the grape must and to perform controlled fermentations. This approach reduces the risk of wine contamination while increasing reproducibility and enabling the production of wines with specific aromas or other compounds of interest. Selecting appropriate yeast species may contribute to face the challenges brought by climate change, but also to increase the variety and quality of wines, as consumers and market demand.

Most of the commercial yeasts belong to the *Saccharomyces cerevisiae* species, therefore being the most frequently used in wine making, as well as the most studied species. However, other yeasts, such as non-*Saccharomyces* species, have shown their potential to solve the new challenges of the wine making industry (Ciani et al., 2016; Pérez-Torrado et al., 2017). Interestingly, species of the *Saccharomyces* genus, such as *Saccharomyces kudriavzevii*, exhibit promising physiological properties. *S. kudriavzevii* ferments at lower temperatures (Salvadó et al., 2011), produces less ethanol and more glycerol (Oliveira et al., 2014; Pérez-Torrado et al., 2016) with no increase in the acetic acid levels in wine (Alonso-del Real et al., 2017), and generates a higher content of aromatic superior alcohols (Stribny et al., 2016).

Temperature is one of the most important parameters affecting the duration and rate of alcoholic fermentation and final wine quality. Many wine makers prefer low-temperature fermentations (10–15°C) for the production of white and "rosé". Wines produced at low temperatures keep volatile aroma compounds more efficiently; therefore, showing better sensory attributes. However, the performance of *S. cerevisiae* at low temperatures decreases, due to growth rate reduction and an increased risk of stuck and sluggish fermentations (López-Malo et al., 2013). Recent studies have confirmed that the cryophilic yeast *S. kudriavzevii* performs better than *S. cerevisiae* at low temperature, thus being an appealing alternative for cold fermentations (Tronchoni et al., 2012). Additionally, *S. kudriavzevii* produces less alcohol than *S. cerevisiae* offering a means to handle the rising sugar content in grape must (Alonso-del Real et al., 2017). Nevertheless, the feasibility of using non-conventional yeasts, such as *S. kudriavzevii*, at the industry, requires an improved understanding of the physiology and metabolism of these cells.

Dynamic modeling brings the potential of exploring the most relevant mechanisms underlying fermentation performance by different species but also the possibility of designing optimal operating conditions more systematically (Banga et al., 2005; Pizarro et al., 2007). The modeling of wine fermentation has received substantial attention. Depending on their aim, available models can be classified into macroscopic kinetic or intracellular metabolic. Macroscopic kinetic models are focused on biomass growth and external metabolites. They require the definition of kinetic rates as functions of the intervening species concentrations. Metabolic models consider cellular metabolic pathways which are defined in terms of fluxes; an optimization based approach is then used to compute metabolic flux profiles compatible with the measured dynamics of biomass growth.

The pioneering works by Boulton (1980) or Caro et al. (1991) adopted the macroscopic scale modeling approach. Subsequently several works focused on the efficiency of *S. cerevisiae* to transform glucose to ethanol within a range of temperatures around that corresponding to the optimal growth (see, for example, the review by Marín, 1999 and the works cited therein). More recently, Cramer et al. (2002), Malherbe et al. (2004), and Coleman et al. (2007) also adopted the macroscopic scale modeling approach to address the role of assimilable nitrogen in ethanol and CO_2 production. Agosin and collaborators considered the cellular metabolism within a dynamic flux balance modeling framework (Sainz et al., 2003; Varela et al., 2004; Pizarro et al., 2007; Vargas et al., 2011). These models reproduced the measured dynamics of biomass growth, substrates uptake as well as ethanol and glycerol production. Alternatively Malherbe et al. (2004) or David et al. (2010) adopted an intermediate strategy that couples the kinetic modeling of external metabolites with some intracellular mechanisms. Their focus is on the role of nitrogen.

In this work we adopt the later strategy to model cold fermentations mediated by non-conventional *Saccharomyces* species. For this purpose we implemented an experimental-modeling pipeline. The experimental pipeline is based in micro-vinifications where small-scale wine fermentations are undertaken at different controlled conditions while monitoring growth rate and a number of critical extracellular metabolites (glucose, fructose, ethanol, glycerol, and acetic acid).

The modeling pipeline is based on model selection, reduction, ensemble modeling and cross-validation. Several candidate models -which account for different biomass growth, transport and inhibitory mechanisms found in the literature- are compared attending to their properties, basically identifiability and robustness in cross-validation. In this respect, we focused on defining a minimal model with the minimum number of parameters to guarantee structural identifiability, i.e. the possibility of uniquely reconciling the model with the data while iteratively improving practical identifiability (Chis et al., 2016). For the most successful models we implemented an ensemble modeling strategy so as to maximize their robustness, i.e., to minimize the uncertainty of their predictions. The results from the obtained models are discussed in a quantitative manner and ensemble of models is used to devise robustified predictions for processing conditions (initial inoculation and temperature) so as to achieve a better compromise between alcohol and glycerol production.

The selected model accounts for the transport of hexoses (glucose and fructose) and their transformation into fructose 6-phosphate (F6P); the F6P is then directed to produce both ethanol, acetic acid and glycerol. The model considers the temperature effects in the cells specific growth rate; but also temperature and ethanol as inhibitors of the transport of hexoses. As a result it can be used to design cold wine fermentations to optimize final product quality.

Finally, we show, by means of cross-validation, that using an ensemble approach delivers more robust solutions than using

a single model approach, thus rendering the ensemble models useful to explain the differences in fermentation performance between the species of interest and to design novel wine-making processes.

MATERIALS AND METHODS

Experimental Methods

Strains

We considered two different *Saccharomyces* strains. We chose a commercial strain, T73 (Lalvin T73 from Lallemand Montreal, Canada), as our wine *S. cerevisiae* representative, and *S. kudriavzevii* strain CR85, a natural isolate from oak tree bark in Agudo, Ciudad Real, Spain. Throughout the rest of the this paper these strains will be referred to as SKCR85 and SCT73, respectively.

Synthetic Must Fermentations

All fermentations were performed in 3*x* replicates in 250 mL flasks that contained 200 mL of synthetic must (SM) miming a standard natural must which is frequently used in microvinification experiments (Rossignol et al., 2003). This medium contains 100 g/L glucose and 100 g/L of fructose, mineral salts (750 mg/L KH_2PO_4, 500 mg/L K_2SO_4, 250 mg/L $MgSO_4.7H_2O$, 155 mg/L $CaCl_2.2H_2O$, 200 mg/L NaCl, 4 mg/L $MnSO_4.H_2O$, 4 mg/L $ZnSO_4$, 1 mg/L $CuSO_4.5H_2O$, 1 mg/L KI, 0.4 mg/L $CoCl_2.6H_2O$, 1 mg/L H_3BO_3, 1 mg/L $NaMoO_4.2H_2O$), vitamins (20 mg/L myo-inositol, 2 mg/L nicotinic acid, 1.5 mg/L calcium panthothenate, 0.25 mg/L thiamine HCl, 0.25 mg/L pyridoxine HCl, 0.003 mg/L biotin), 300 mg/L of assimilable nitrogen (ammoniacal nitrogen and α-amino nitrogen) provided by a mixture of 19 amino acids (612.6 mg/L L-proline, 505.3 mg/L L-glutamine, 374.4 mg/L L-arginine, 179.3 mg/L L-tryptophan, 145.3 mg/L L-alanine, 120.4 mg/L L-glutamic acid, 78.5 mg/L L-serine, 759.2 mg/L L-threonine, 48.4 mg/L L-leucine, 44.5 mg/L L-aspartic acid, 44.5 mg/L L-valine, 37.9 mg/L L-phenylalanine, 32.7 mg/L L-isoleucine, 32.7 mg/L L-histidine, 31.4 mg/L L-methionine, 18.3 mg/L L-tyrosine, 18.3 mg/L L-glycine, 17.0 mg/L L-lysine, and 13.1 mg/L L-cysteine) corresponding to 180 mg nitrogen and 460 mg/L ammonium chloride (corresponding to 120 mg nitrogen). The pH was buffered at 3.3 with NaOH.

We monitored the growth of each strain in monocultures under the same conditions. Overnight precultures were grown in YPD medium at 25°C. Afterwards must was inoculated with the corresponding yeast strain to reach an initial concentration of 10^6 cells/mL, and was incubated at a fixed temperature (8, 12, 20, or 25°C) with agitation at 100 RPMs during fermentation.

Cell samples were collected at several time points during fermentation. Growth curves were obtained by considering cell density calculated from cell counting in a Neubauer chamber (Alonso-del Real et al., 2017). Müller valves were used to monitor fermentation stage through weight loss, until it reached a constant weight, when it was considered to be over. At this point, samples of supernatant were kept at −20°C for further analyses.

High Performance Liquid Chromatography

Residual sugars (glucose and fructose), glycerol, ethanol and acetic acid from the fermentation end point samples were determined by HPLC (Thermo Fisher Scientific, Waltham, MA. USA) using a refraction index detector and a HyperREZTM XP Carbohydrate H+ 8μm column (Thermo Fisher Scientific) equipped with a HyperREZTM XP Carbohydrate Guard (Thermo Fisher Scientific). Samples were diluted to maintain our target compounds within the allowed range of detection, filtered through a 0.22 μM nylon filter (Symta, Madrid, Spain) and injected in duplicate. The analysis conditions were: eluent, 1.5 μM of H_2SO_4; 0.6 mL/min flux and a 50°C oven temperature.

Theoretical Methods: The Modeling Pipeline

Modeling was approached from a systems identification perspective including the following steps: formulation of candidate models, multi-experiment parameter estimation, model selection and reduction, ensemble modeling and cross-validation.

Formulation of Candidate Models

We formulated several candidate models which account for the relevant process variables (biomass growth, sugars, ethanol, glycerol, acetate) based on different mechanisms described in literature. All candidate models consist of a set of ordinary differential equations whose solution depends on the given initial conditions, process temperature and the value of a number of unknown parameters.

Parameter Estimation

The aim of parameter estimation is to compute the unknown parameters - growth related constants and kinetic parameters - that minimize the distance among data and model predictions. The maximum-likelihood principle yields an appropriate measure of such distance (Walter and Pronzato, 1997):

$$J_{mc}(\boldsymbol{\theta}) = \sum_{k=1}^{n_{exp}} \sum_{j=1}^{n_{obs}} \sum_{i=1}^{n_{st}} \left(\frac{y_{k,j,i}(\boldsymbol{\theta}) - y^m_{k,j,i}}{\sigma_{k,j,i}} \right)^2, \quad (1)$$

where n_{exp}, n_{obs} and n_{st} are, respectively, the number of experiments, observables, and sampling times while $\sigma_{k,j,i}$ represents the standard deviation of the measured data as obtained from the experimental replicates. y^m_j represents each of the measured quantities, X^m and C^m in our case, and $y_j(\boldsymbol{\theta})$ corresponds to model predicted values, X and C .

Parameters are estimated by solving a nonlinear optimization problem where the aim is to find the unknown parameter values ($\boldsymbol{\theta}$) to minimize $J_{mc}(\boldsymbol{\theta})$, subject to the system dynamics—the model—and parameter bounds (Vilas et al., 2018).

Model Selection and Reduction

Models were compared, first, attending to their capabilities to fit the experimental data. Since models with a larger number of parameters tend to provide better fits, which may lead to over-fitting, we also considered the number of parameters in our comparison. For this purpose we used the Akaike information

criterion (AIC) defined as follows (Burnham and Anderson, 2002):

$$AIC = 2n_p + n_d \cdot ln(J), \tag{2}$$

where n_p is the number of unknown adjustable parameters, n_d the number of data.

We started with most complex candidate models after data fitting less influencing parameters were iteratively removed from the model following an AIC based strategy. Parameters were removed as long as the AIC was reduced, otherwise, the reduced model was rejected. The decision tree used to simplify the models is detailed in the Supplementary Information.

The most promising candidate models were further compared in terms of their associated uncertainty in cross-validation.

Uncertainty Analysis

In practice, the value of the parameters $\boldsymbol{\theta}$ compatible with noisy experimental data is not unique, i.e., parameters are affected by some uncertainty. The consequence of significant parametric uncertainty is that the model may not be able to predict scenarios other than those used in parameter estimation.

To measure the actual model predictive capabilities, the model is usually given a dataset of known data on which training is run (training dataset), and a dataset of unknown data against which the model is tested (testing dataset). The training dataset regards the data used for parameter estimation; while the testing dataset is obtained under untrained experimental conditions (for example, a different process temperature).

To account for model uncertainty we used an **ensemble approach**. To derive the ensemble we apply the bootstrap smoothing technique, also known as bootstrap aggregation (the Bagging method) in the prediction literature (Breiman, 1996; Bühlmann, 2012). The bagging method is a well established and effective ensemble model/model averaging device that reduces variability of unstable estimators or classifiers (Bühlmann and Yu, 2002). The underlying idea is to consider a family of models with different parameter values $\boldsymbol{\Theta} = [\boldsymbol{\theta}_1 \dots \boldsymbol{\theta}_N]^T$ compatible with the training data $\boldsymbol{y}^m$, when using the model to predict untested experimental setups. The matrix of parameter values $\boldsymbol{\Theta}$ consistent with the data is obtained using N realizations of the data obtained by bootstrap (Efron and Tibshirani, 1988). Each data realization has the same size of the complete data-set but it is constructed by sampling uniformly from all replicates (3 biological replicates per sampling time). Therefore at each bootstrap iteration, a given replicate has an approximate chance of 37% from being left out, while others might appear several times (2,3,...) in a given instance of the bootstrap. The family of solutions, $\boldsymbol{\Theta}$, is then used to make N predictions (dynamic simulations) about a given experimental scenario. The median of the simulated trajectories regards the model prediction while the distribution of the individual solutions at a given sampling time provide a measure of the uncertainty of the model.

Cross-validation In order to test the modeling predictions under untested conditions we apply out-of-sample cross-validation (Elsner and Schmertmann, 1994; Tashman, 2000). To compute the ensemble of predictions for each tested temperature for which we have experimental data, i.e., we omitted the experimental data for each temperature and computed an ensemble model for each scenario (Henriques et al., 2017). Finally we used the obtained models to compute a median solution for each temperature and assess the quality of the solutions using the root mean square error metric:

$$\mathrm{RMSE}(\boldsymbol{\theta}) = \sqrt{\frac{\sum_{k=1}^{n_{exp}} \sum_{j=1}^{n_{obs}} \sum_{i=1}^{n_{st}} (y_{k,j,i}(\boldsymbol{\theta}) - y_{k,j,i}^m)^2}{N_{Data}}} \tag{3}$$

where N_{Data} corresponds to the number of data points used for training and testing. The comparison between the root mean square error in training and in testing gives a measure of the capabilities of the model to predict untested conditions. As it is defined, the RMSE is scale dependent. To provide a normalized value (NRMSE) it is possible to divide by the maximum measurement for each species.

Model selection can be done by comparing the NRMSE as obtained for the training and testing conditions. The lower the NRMSE values the better the model.

Numerical Tools

To automatize the modeling pipeline we used the AMIGO2 toolbox (Balsa-Canto et al., 2016). AMIGO2 is a MATLAB based software tool focused on parametric model identification and optimization, including sensitivity and identifiability analyses. It offers a suite of numerical methods for both simulation and optimization. From the available options we selected CVODES (Hindmarsh et al., 2005) to solve the model equations, and *Enhanced Scatter Search* (eSS, Egea et al., 2009), to find the optimal parameter values in reasonable time.

The ensemble model generation and cross-validation procedures are computationally intensive. However, since each parameter estimation instance in the ensemble is a completely independent task, we were able to solve this problem in less than a day using 60 CPU cores on a Linux cluster. These tasks were automated with the help of bash scripts and the Open Grid Scheduler. All the scripts necessary to reproduce the results are distributed as part of the Supplementary Materials.

RESULTS AND DISCUSSION

Formulation of Candidate Models

This work seeks a minimal yet predictive model to describe the fermentation processes mediated by two different *Saccharomyces* species under a range of cold temperature processing conditions.

Previous modeling efforts focused on the efficiency of *S. cerevisiae* to transform glucose to ethanol within a range of temperatures around that corresponding to the optimal growth (see, for example, the review by Marín, 1999 and the works cited therein). Later, Cramer et al. (2002), Malherbe et al. (2004), or David et al. (2010) proposed oenological models which account for the role of nitrogen sources in sluggish or stuck fermentations.

However, the primary motivation to use other yeasts as wine making starters is to improve final product characteristics such as enhanced glycerol content, low temperature fermentation kinetics or novel attractive aroma profiles. Unfortunately, these

previous models do not include glycerol or acetic acid, and many of them do not take into account the role of the temperature, rendering them as non-valid for our purposes.

We put particular emphasis on developing a minimal model, with nice mathematical properties (i.e., identifiable) and yet comprehensive in the sense of the mechanisms involved. With this aim we formulated three candidate models, regarded as nominal models, which describe the accumulation of extracellular ethanol, glycerol, acetic acid and release of $C0_2$. We also included a simplified model of glycolisis that respects mass conservation coupled to alternative growth models and the transport of hexoses. **Figure 1** shows an overview of the relevant species included in the candidate models.

Modeling Growth

We considered two different alternatives to model biomass (X) dynamics. On the one hand, a linear model accounting for substrate inhibition (in nominal models N1 & N2) and on the other, the Verhulst logistic model (in nominal model N3).

Nominal models N1 & N2 assume linear biomass growth being the specific growth rate modulated by glucose ($\upsilon_{tr,G}$) and fructose transport ($\upsilon_{tr,F}$):

$$\dot{X} = \mu(\upsilon_{tr,G}, \upsilon_{tr,F}) \cdot X \tag{4}$$

These models account for the growth inhibition due to limited substrate. The synthetic must used in our experiments contains 300 mg/L of assimilable nitrogen which is enough for the yeast to reach its maximum fermentation rate and for no issues to arise during fermentation. Therefore assimilable nitrogen is not considered as an inhibiting substrate. However, its initial amount was considered in nominal model N1.

The logistic model (in nominal model N3) is the standard in predictive microbiology (Baranyi and Roberts, 1994) and was also used by Malherbe et al. (2004) or David et al. (2010) to model wine fermentation. The model accounts for intra-species competition for the available nutrients in such a way that the specific growth rate (μ) depends on the environmental conditions (temperature, T, in our case) and the maximum biomass (X_{max}), also known as the species carrying capacity,

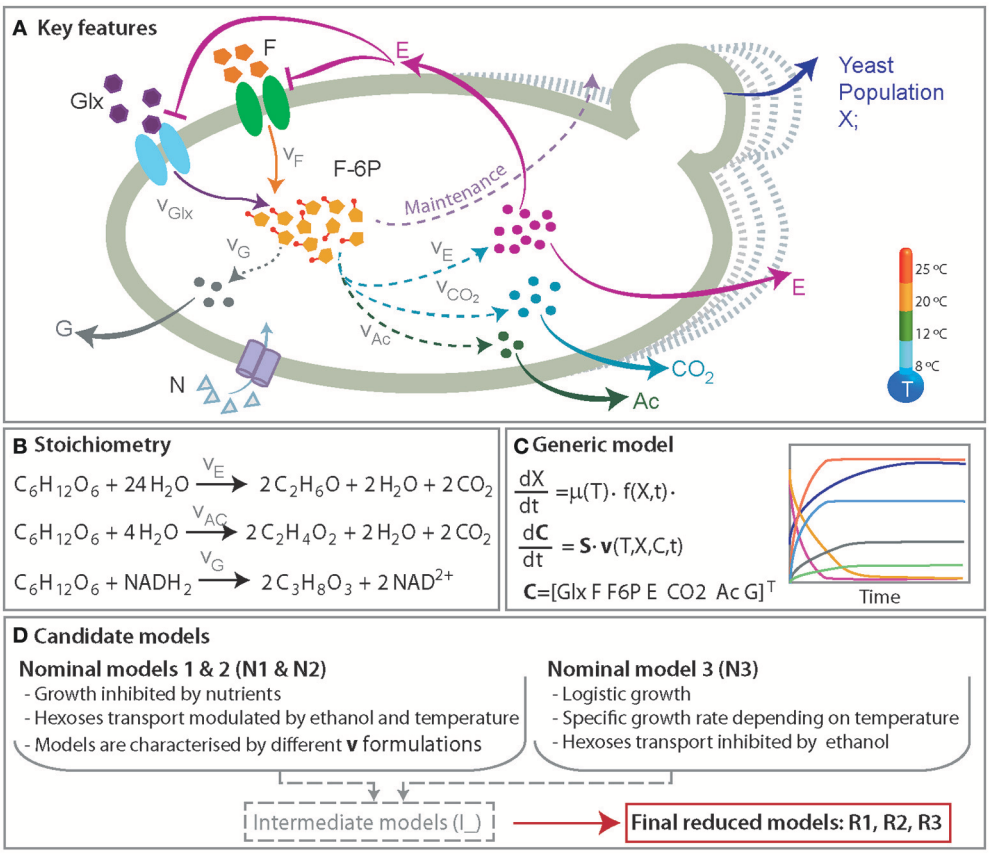

FIGURE 1 | A simplified representation of the anaerobic metabolism of glucose and fructose. **(A)** Presents the key features of the process including yeast population growth and the production of ethanol (E), glycerol (G), acetic acid (Ac), and CO_2. The roles of the temperature and ethanol production in the hexoses transport (glucose, Glx and fructose, F) and growth are also incorporated. **(B)** Presents the set of reactions. **(C)** Shows an overview of the mathematical model consisting of a set of ordinary differential equations describing the dynamics of yeast population and the relevant metabolites concentrations. **(D)** summarizes the major mechanisms included in the candidate models.

depends on the available nutrients. The logistic model is defined as follows:

$$\dot{X} = \mu_T(T) \cdot X \cdot \left(1 - \frac{X}{X_{max}}\right) \quad (5)$$

The specific growth rate depends on the temperature. To include this dependency with the minimum number of parameters we explored previously published data (Arroyo-López et al., 2009). In the range of temperatures of interest, $\mu(T)$ can be well approximated by a quadratic function.

$$\mu_T(T) = k_{T2} \cdot T^2 - k_{T1} \cdot T + k_{T0} \quad (6)$$

Modeling the Transport of Hexoses

Yeasts use several hexose transporters, which transport glucose and fructose amongst other sugars, by facilitated diffusion (Boles and Hollenberg, 1997). Although yeasts show preference for glucose (Berthels et al., 2004), glucose and fructose can be consumed simultaneously.

Hjersted et al. (2007) modeled the transport of hexoses using a Michaelis-Menten (MM) type kinetics as follows:

$$\upsilon_{tr,H} = X \cdot \frac{k_H \cdot H}{k_{sH} + H} \quad (7)$$

where k_H, refers to the transport rate; k_{sH} regards the Michaelis constant; H refers to the relevant hexoses (glucose and fructose) and X is the number of cells.

It should be noted that the transport of hexoses is a very complex process which will be affected by both temperature and ethanol. We took these effects into account by modifying the Equation 7 as follows:

$$\upsilon_{tr,H} = X \cdot \phi_T \cdot \phi_E \frac{k_H \cdot H}{k_{sH} + H} \quad (8)$$

in such a way that we uncouple the effects of temperature (ϕ_T) and ethanol(ϕ_E).

We modeled the effect of temperature with a couple of empirical functions taken from the literature, $\phi_{T,A}$ and $\phi_{T,B}$, defined as follows:

$$\phi_{T,A} = (a/T^2) \cdot e^{-b/T} + c \cdot N_0 \quad (9)$$

This expression, proposed by Pizarro et al. (2007), was considered in nominal model N1 and accounts, not only for the effect of temperature but also for the initial amount of assimilable nitrogen. The expression contains three parameters: a, b and c to be estimated from data; N_0 regards the initial amount of assimilable nitrogen in the medium.

$$\phi_{T,B} = a \cdot e^{-b/T} \quad (10)$$

where a regards the intensity of the temperature effect and b is the rate of the exponential function and T is the temperature. This expression, proposed by Malherbe et al. (2004) and later used by Charnomordic et al. (2010), indicates that transport increases with temperature. This increase is an overall effect resulting from the contribution of different processes: the production of different transporters with different transport affinities which may depend on temperature (Tai et al., 2007; Postmus et al., 2008) and the effect of the amount of intracellular hexoses (Teusink et al., 1998) being directed to glycolysis. $\phi_{T,B}$ was incorporated in the nominal model N2.

Finally, ethanol has been reported as a non-competitive inhibitor (Leão and Van Uden, 1982) of glucose transport. We modeled its effect as follows (Hjersted et al., 2007):

$$\phi_E = \frac{1}{1 + E/K_{Ei}} \quad (11)$$

where K_{Ei} defines the strength of the inhibitory effect.

It should be noted that, to guarantee structural identifiability, k_H and a can not be simultaneously estimated from experimental data, but only their product, $v_G = k_H \cdot a$.

Metabolic Model

In order to provide a simple representation of the metabolism while avoiding over-parameterization and lack of identifiability, we assume that upon transport, glucose and fructose are rapidly metabolized into Fructose 6-Phosphate (F6P).

During alcoholic fermentation, $\mathrm{F6P}_{in}$ (which regards the concentration of F6P per cell) is metabolized to pyruvate, through a number of steps, via the glycolytic pathway. Pyruvate is then decarboxylated into acetaldehyde and finally reduced to ethanol or acetate. All these intermediate steps are lumped into the rates $\upsilon_{F6p \to E}$ and $\upsilon_{\mathrm{F6P} \to \mathrm{Ace}}$ first is described using irreversible Michaelis-Menten type kinetics while the later is described using mass action law. Additionally, part of the carbon flux is redirected to the glycerol pathway. Glycerol production is described with a mass action type equation. Moreover, the rate function, $\upsilon_{manteinance}$, explaining the conversion of F6P into biomass or other maintenance costs is added to account for what was not converted into Glx, G or ACE. From the former rates we are able to derive the following set of ordinary differential equations (ODEs) describing the molar concentration of the different metabolites considered:

$$\mathrm{F6P}_{in} = \frac{\mathrm{F6P}}{X} \quad (12)$$

$$\upsilon_{Maintenance} = X \cdot k_{Maintenance} \cdot \mathrm{F6P}_{in} \quad (13)$$

$$\upsilon_{\mathrm{F6P} \to \mathrm{E}} = X \cdot k_E \cdot \frac{\mathrm{F6P}_{in}}{k_{s,E} + \mathrm{F6P}_{in}} \quad (14)$$

$$\upsilon_{\mathrm{F6P} \to \mathrm{Ace}} = X \cdot k_{Ace} \cdot \mathrm{F6P}_{\mathrm{in}} \quad (15)$$

$$\upsilon_{\mathrm{F6P} \to \mathrm{G}} = X \cdot k_G \cdot \mathrm{F6P}_{\mathrm{in}} \quad (16)$$

$$\dot{\mathrm{F6P}} = \upsilon_{tr,Glx} + \upsilon_{tr,F} - \upsilon_{\mathrm{F6P} \to \mathrm{E}} - \upsilon_{\mathrm{F6P} \to \mathrm{G}} - \upsilon_{\mathrm{F6P} \to \mathrm{Ace}} - \upsilon_{Maintenance} \quad (17)$$

$$\dot{\mathrm{E}} = 2 \cdot \upsilon_{\mathrm{F6P} \to \mathrm{E}} \quad (18)$$

$$\dot{\mathrm{G}} = 2 \cdot \upsilon_{\mathrm{F6P} \to \mathrm{G}} \quad (19)$$

$$\dot{\mathrm{Ace}} = 2 \cdot \upsilon_{\mathrm{F6P} \to \mathrm{Ace}} \quad (20)$$

$$\dot{\mathrm{CO2}} = 2 \cdot \upsilon_{\mathrm{F6P} \to \mathrm{Ace}} + 2 \cdot \upsilon_{\mathrm{F6P} \to \mathrm{E}} \quad (21)$$

where $F6P_{in}$ corresponds to the concentration of F6P per cell; $k_{Maintenance}$, k_{Ace}, k_G correspond to reaction rates for biomass maintenance and the production acetate and glycerol respectively; $k_{s,E}$ is the FP6 per cell concentration at which the reaction rate is half of its maximum, $k_{s,E}$; CO_2 represents the concentration of carbon dioxide released when ACE and E are produced. The coefficient (2) included in Equations 19–21 accounts for the stoichiometry of the reaction as described in **Figure 1**.

Model Selection and Reduction

All nominal candidate models consist of 7 ordinary differential equations. However, they differ in the number of adjustable unknown parameters. The parameter estimation for each model was performed by using the total of 329 data points for both species. It should be noted that a limited number of sampling times is available for the experiments performed at 20°C. The parameter estimation of the nominal models revealed several non influencing parameters which called for model reduction. Details on the various intermediate reduced models can be found in the Supplementary Information. **Table 1** presents the major characteristics of and the best fit statistics for the nominal models plus the final reduced models.

Reduced models are better in terms of the Akaike criterion as compared to their nominal counterparts. The best model in terms of quality of fit is the nominal model N3; while models N1 and N2 based on linear growth with growth rate depending on the substrates where less successful. Note, however, that the reduced model R3 is indeed better than N3 in terms of the Akaike criterion. In R3 the Michaelis-Menten (MM) type kinetics explaining hexoses transport (Hjersted et al., 2007) was reduced to mass action kinetics. Remarkably this reduction was also needed for nominal models 1 and 2, indicating that data coming from fermentations occurring at different initial amounts of glucose and fructose are required to identify Michaelis-Menten kinetics. Similarly, in N1 the term corresponding to the initial amount of assimilable nitrogen was reduced due to lack of identifiability.

TABLE 1 | Major characteristics and best fist statistics for nominal models and final reduced models.

Model	Model characteristics	Best fit, J	AIC	#Pars
N1	- Linear growth - Growth rate depending on the transport of hexoses - Michaelis-Menten transport of hexoses - $\phi_{T,A}$ - ϕ_E	6.78	335.63	31
N2	- Linear growth - Growth rate depending on the transport of hexoses - Michaelis-Menten transport of hexoses - $\phi_{T,B}$ - ϕ_E	5.51	313.95	35
N3	- Logistic growth - Quadratic growth rate - Michaelis-Menten transport of hexoses - $\phi_{T,B}$ - ϕ_E	4.68	286.65	33
R1	- Linear growth - Michaelis-Menten transport of hexoses - $\phi_{T,A}$ - ϕ_E	6.92	326.52	25
R2	- Linear growth - Linear transport of hexoses - $\phi_{T,B}$ - ϕ_E	5.73	291.58	21
R3	- Logistic growth - Quadratic growth rate - Linear transport of hexoses - $\phi_{T,B}$ - ϕ_E	4.87	276.26	25

Model Ensemble and Cross-Validation for Reduced Models

To further compare the most successful reduced models we performed $N = 100$ independent parameter estimations from different bootstrapped realizations of the available data to obtain the ensemble of the reduced models. Besides, to test whether reduced models can predict the process out of the training data set we performed a cross-validation analysis. **Figures 2A–C** show the normalized root mean square error obtained with the training data set vs. the prediction data sets for each reduced model and the corresponding ensemble model (marked with a triangle).

Results demonstrate that the training error is low for all models in all scenarios, between $0.08 - 0.11$. The prediction error increases for all models; to a maximum of 0.28 for the second reduced model. As expected, the maximum discrepancy in cross-validation corresponds to extrapolation scenarios for all models.

Remarkably, for model R3 the ensemble solutions, marked with triangles, are more robust than the individual solutions. In fact, in many cases, it is observed that an individual model with a low RMSE value for the training data set does not necessarily perform well in cross-validation. On the contrary, the ensemble is consistent, providing a good compromise between both training and prediction errors.

Figure 2D presents a comparison of the ensembles of all models. R3 model is more robust than the others with the assembles clustered together in the lower error area, NRMSE lower than 0.086 in training and 0.139 in prediction. It should be noted that, despite having less data for the experiments at 20°C, the training NRMSE for the ensemble in cross-validation is only a 7% higher than that obtained for the best case. This result emphasizes the benefits of using multi-experiment data fitting for parameter estimation and cross-validation.

Its consistency, and the associated lower error values, render the ensemble model R3 the best model of those tested to explain and predict cold fermentations by the two species under the specified wine model.

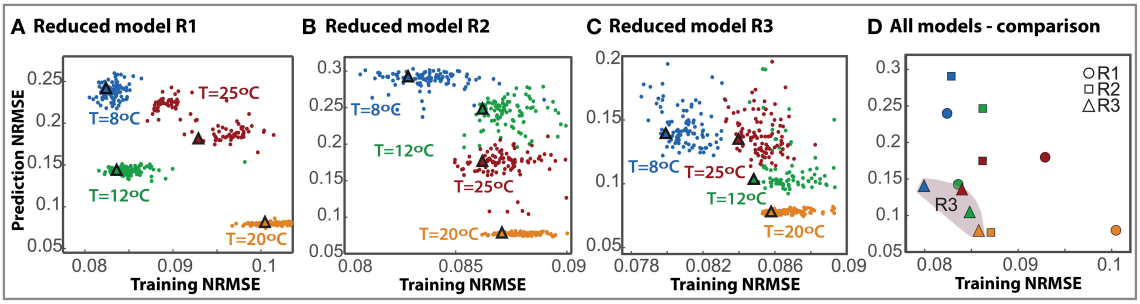

FIGURE 2 | Cross-validation for the selected reduced models. The prediction RMSE is plotted here against the training RMSE for each individual model (dots) and the ensemble (triangle) with different colors owing to different cross-validation scenarios. **(A–C)** Correspond to models R1, R2, and R3 respectively. **(D)** Presents the comparison of all three model ensembles.

The Best Model

Figure 3 shows the experimental data and the ensemble of time course model predictions for both species as obtained for the best ensemble model R3.

The model adequately explains the measurements and the corresponding error bars for both species at all tested temperatures. Temperature affects the duration and rate of alcoholic fermentation as well as final wine quality. At 8°C the system evolves slowly for both species, taking more than 16 days to achieve the maximum biomass. In fact, at 8°C, and after 600 hours the glucose and fructose have not been entirely consumed. In consequence, the production of ethanol and glycerol is significantly lower than the production at higher temperatures. The model fits the glucose and fructose satisfactorily, with the maximum deviations found at the lowest temperature. Both species prefer glucose to fructose, being glucose the first to be consumed in all experimental temperatures. Remarkably, SKCR85 produces less ethanol than SCT73, while producing more glycerol. On the contrary, at 8°C, the production of ethanol is similar in both species, while SKCR85 produces significantly more glycerol, confirming that this species is particularly suited for cold fermentations (Tronchoni et al., 2012).

Ensemble of Parameters for the Selected Model

The ensemble of parameters allows gaining further insights into the mechanisms contributing to the differences observed in the performance of the fermentations mediated by SCT73 and SKCR85. **Figure 4** presents the parameter distributions, while **Table 2** reports the mean values and the corresponding confidence intervals.

Results reveal that, except for ν_{Glx} and ν_F for SCT73, parameters are computed with high reliability. The mean relative standard deviation corresponds to a 13.35% for those parameters related to SKCR85 and a 14.85% for SCT73. The case of ν_{Glx} and ν_F in SCT73 is particular, since those parameters are highly correlated (See Figure 2 in Supplemental Data) and some outliers appear in the ensemble bootstrap approach due to the large bounds used in parameter estimation.

Parameter values differ substantially for SCT73 and SKCR85, indicating distinct behaviors concerning growth, hexoses transport, and metabolism.

The maximum carrying capacity (X_{max}) is 3% higher for SCT73, meaning that the intra-specific competence is lower for SCT73 than for SKCR85. Temperature and ethanol content strongly affect the specific growth rate. Despite OD_{600} data does not suffice to distinguish temperature and ethanol effects in biomass growth, we can draw some conclusions from the comparative analysis of the specific growth rate for both species (**Figure 5A**). The cryotolerant SKCR85 and SCT73 grow at similar rates at lower temperatures, between 8 and 12°C; cases in which the maximum ethanol would barely exceed 50 g/L. At higher temperatures, closer to the optimal growth temperature, SCT73 grows around a 40% faster than SKCR85. The fact that at those temperatures ethanol production is high would indicate that SKCR85 is more susceptible to ethanol, which is in agreement with previously published results (Arroyo-López et al., 2010).

Nevertheless, the differences in growth between both species do not explain their distinct fermentation performance. In fact, the differences found in the transport of hexoses play a crucial role (see **Figures 5B–D**).

In our model, both the temperature and the ethanol affect the transport of hexoses. As mentioned above, both species prefer glucose to fructose ($\nu_{Glx} > \nu_F$), being glucose the first to be consumed in all experimental temperatures. However, the transport rates vary significantly between species.

Figures 5B,C present the ensemble solutions and the associated uncertainty for the glucose and fructose transport. The uncertainty on the associated parameters explains the uncertainty of the transport activity for SCT73. However, since there is no overlap between the uncertainty intervals between species and the ensemble solutions are clearly distinguishable we are able to perform a fair comparison of the transport activities between species.

The rates of transport of glucose and fructose for SCT73 are around 3.7 the value obtained for SKCR85. This result would confirm that the fitness advantage of *S. cerevisiae* species in fermentation is related to a quicker sugar uptake (Piškur et al., 2006; López-Malo et al., 2013).

Hexoses are carried via facilitated diffusion mediated by the HXT gene family. Different genes show distinct capacities and affinities toward hexoses (see the recent review by Bisson and

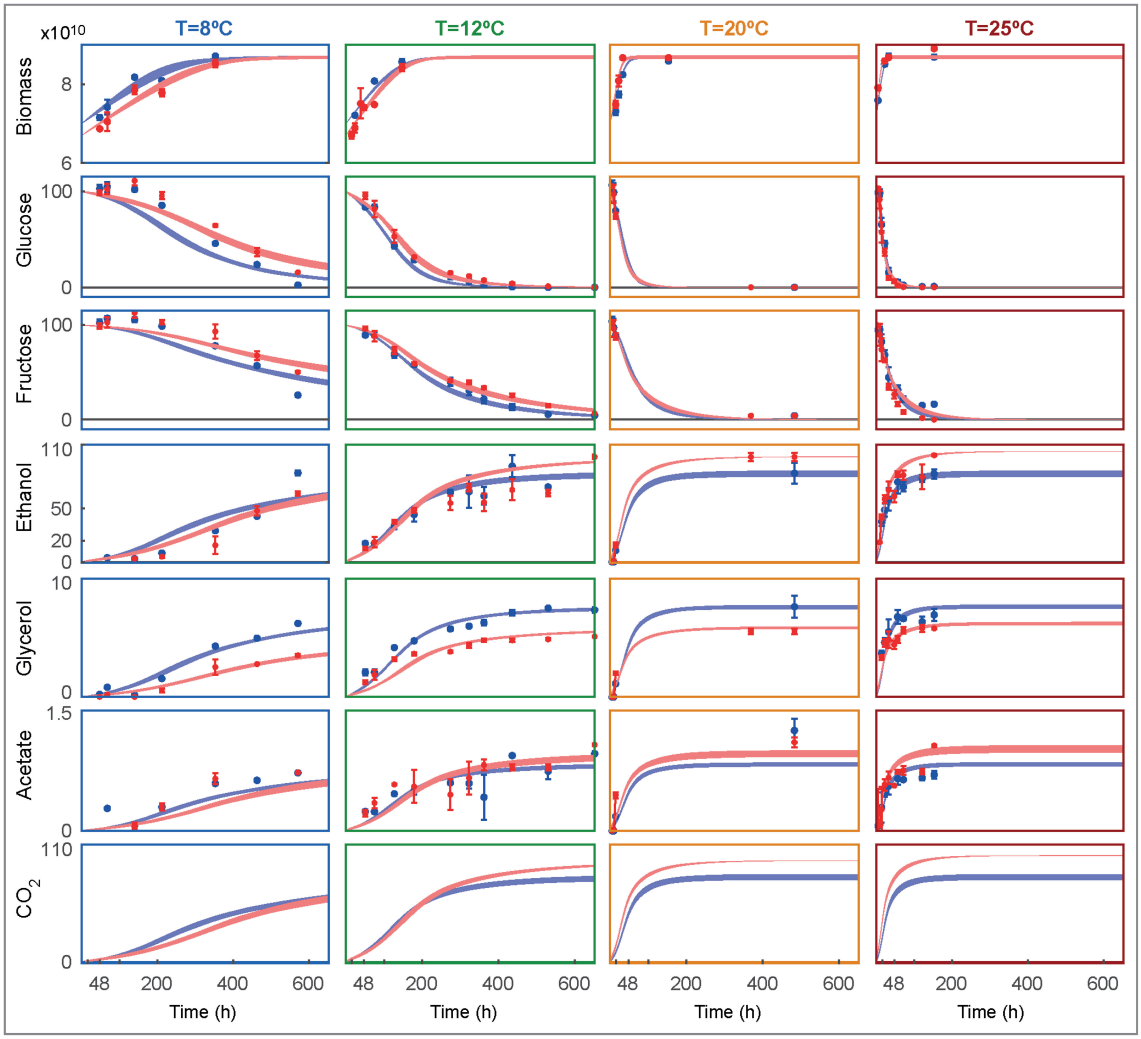

FIGURE 3 | Ensemble of time course predictions for both strains (rows) under different temperatures (columns) as compared to experimental data. The shaded bands depict the predicted non-symmetric 95% confidence interval for SKCR85 (blue) and SCT73 (red). Biomass is shown in decimal logarithm scale (cells/L) while the metabolites are shown in g/L.

Walker, 2016). In general, carriers display lower affinities for fructose as compared to glucose (Boles and Hollenberg, 1997), which would explain that v_{Glx} is greater than v_F in both species.

Remarkably, Karpel et al. (2008) showed that hexose transporters are distinctly tuned and specialized in *S. cerevisiae* laboratory and wine strains. As for SKCR85, the genetic sequences identities are much lower than between different *S. cerevisiae* strains (data not shown). Our hypothesis is that these lower identities may eventually mean differences in transporters affinity, level and moment of expression during fermentation which would explain the disparity in transport found by the modeling approach.

On the other hand, transport is affected by temperature and ethanol. The intensity of the temperature effect as measured by the parameter b differs a 10% between species. These differences have a clear impact on the initial transport of hexoses (when $E \approx 0$) as illustrated in **Figures 5B,C**. The Figures show that specially at higher temperatures, SCT73 presents a greater hexose transport per cell. Remark that this is still true despite the variability associated with the transport parameters for SCT73.

The transport of hexoses will vary with time, i.e., as soon as the cells start producing ethanol. **Figure 5D** shows the inhibition of the transport of hexoses due to the production of ethanol ($\phi_E(E)$). The ethanol inhibition is driven by the value of k_{Ei} which is around three times higher in SKCR85 than in SCT73. This difference between the parameter values leads to greater inhibition of the transport in SCT73 than in SKCR85 and the inhibitory effect increases with the amount of ethanol. Our results indicate that transport would be reduced to up to a 20% for SCT73 and 40% for SCR85.

Santos et al. (2008) analyzed how the individual glucose transporters respond to the presence of ethanol, and how the

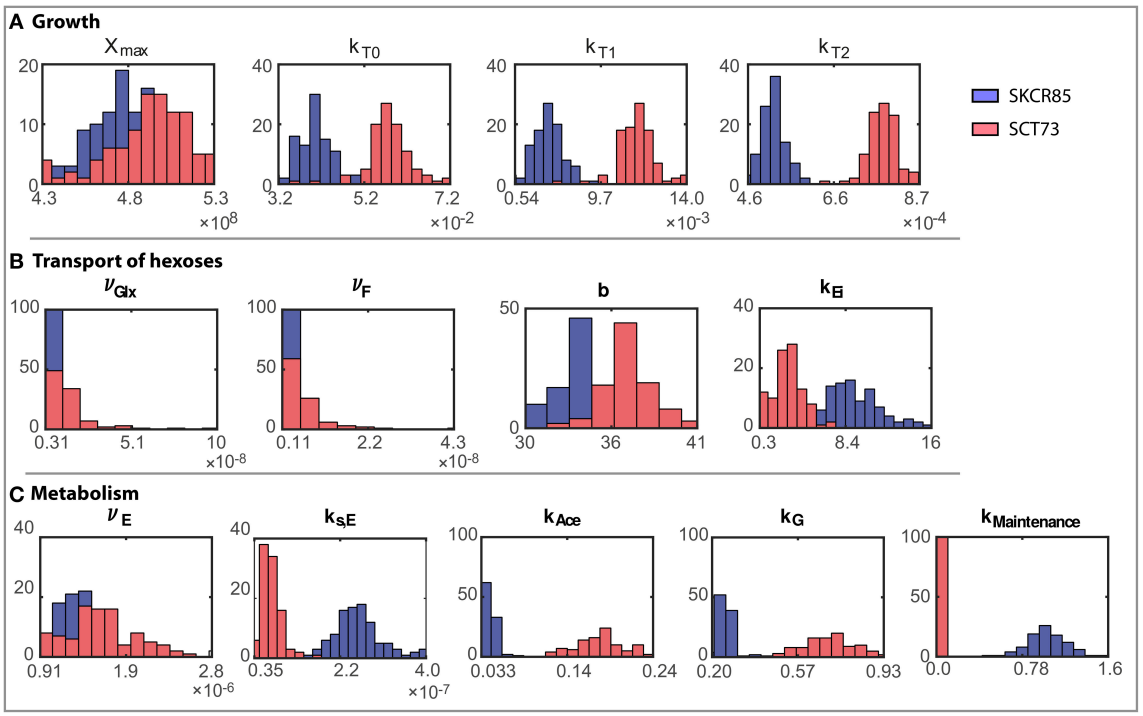

FIGURE 4 | Ensemble of parameter solutions resulting for the multi-experiment data fitting for both strains (model R3). Figures present a comparative analysis of the distributions of parameter values obtained for both species: **(A)** Parameters related to growth, **(B)** Parameters related to transport of hexoses, and **(C)** Parameters related to metabolism. Blue distributions correspond to SKCR85 and red distributions correspond to SCT73.

TABLE 2 | Mean values of the parameters (θ^*) obtained for each strain and the corresponding standard deviation (σ) across the bootstrap estimations.

	SKCR85		SCT73	
Parameter name	θ^*	σ(%)	θ^*	σ(%)
X_{max}	4.75×10^{8}	3.70	4.90×10^{8}	4.39
k_{T0}	4.05×10^{-2}	11.10	5.76×10^{-2}	7.30
k_{T1}	7.14×10^{-3}	9.56	1.16×10^{-2}	6.30
k_{T2}	5.14×10^{-4}	5.13	7.81×10^{-4}	4.01
b	33.5	4.15	36.6	3.81
ν_{Glx}	4.64×10^{-9}	17.90	1.70e-08	80.33
ν_F	1.78×10^{-9}	18.76	6.90×10^{-9}	80.32
K_{E_i}	9.28	25.85	2.93	42.49
k_{Ace}	4.27×10^{-2}	11.41	1.77×10^{-1}	15.29
k_E	1.41×10^{-6}	18.32	1.63×10^{-6}	25.08
$k_{s,E}$	2.52×10^{-7}	17.85	6.89e-08	24.99
k_G	2.57×10^{-1}	11.43	7.01×10^{-1}	14.97
$k_{Maintenance}$	1.01	17.17	0.00	–

growth phase influenced that response. Their results revealed that all the relevant transporters (HXT1-HXT7), except for HXT2, showed different sensitivities to ethanol as a function of the growth stage. For some strains, they demonstrated that the transporters HXT1 and HXT3 were less sensitive to ethanol in exponential-phase cells than in stationary-phase cells. In contrast, the intermediate- and high-affinity transporters HXT4-HXT7 exhibited a higher inhibition of glucose transport by ethanol in exponential-phase cells than in stationary-phase cells while HXT2 transporter was strongly inhibited in both growth phases. Taking into consideration their results it is plausible that the inhibitory effect gradually increases to achieve its maximum at later stages of the fermentation (stationary phase) when more ethanol is present. Our results indicate that transport would be reduced up to 20% for SCT73 and a 40% for SCR85.

The values of the metabolism-related parameters suggest that SCT73 metabolism is faster than SCR85. While SKCR85 requires directing some hexoses to cellular maintenance, it seems that SCT73 heads practically all hexoses to fermentation products, i.e., contributing to its enhanced fermentative performance. As a consequence, the process characteristic times (for example the time to consume the 90% of hexoses, t_{90}) are longer for SKCR85.

Besides, there are substantial differences in the final production of ethanol. SCT73 produces more ethanol than SKCR85, particularly at higher temperatures. This fact may be explained taking into account that SKCR85 directs greater part of the FP6 to produce glycerol as already discussed in the literature (Oliveira et al., 2014) and predicted by the model.

Optimization of Fermentation Parameters

The design of novel wine making processes must take into account the final composition of wine as well as the ability for yeast to consume the hexoses present in the must. We now use

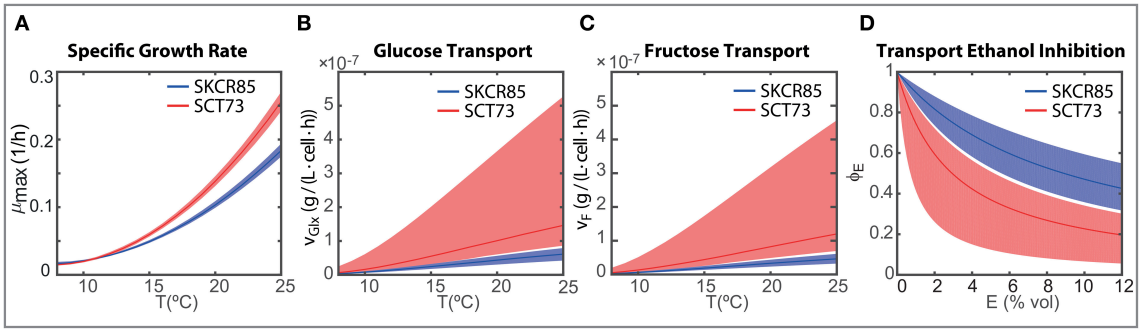

FIGURE 5 | Strain dependent ensemble predictions for **(A)** maximum specific growth rate depending on the temperature; **(B)** temperature dependent glucose transport per cell for initial concentration 100 g/L glucose and 0 g/L ethanol; **(C)** temperature dependent fructose transport per cell for initial concentration 100 g/L fructose and 0 g/L ethanol; **(D)** inhibitory effect of ethanol on the transport of glucose and fructose.

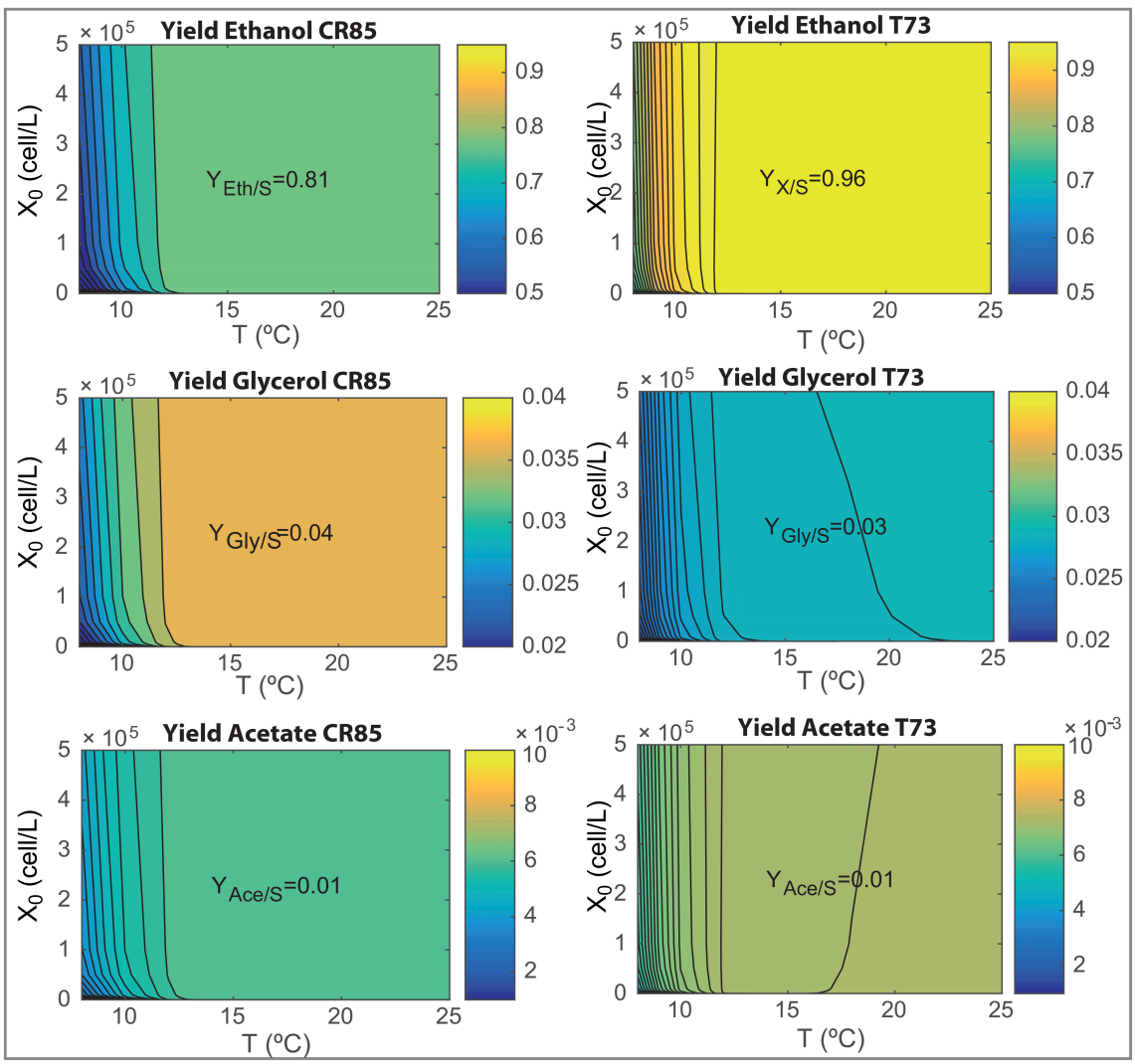

FIGURE 6 | Yields of ethanol, glycerol and acetate as functions of the initial inoculation and the fermentation temperatures for both species as obtained with the ensemble model R3.

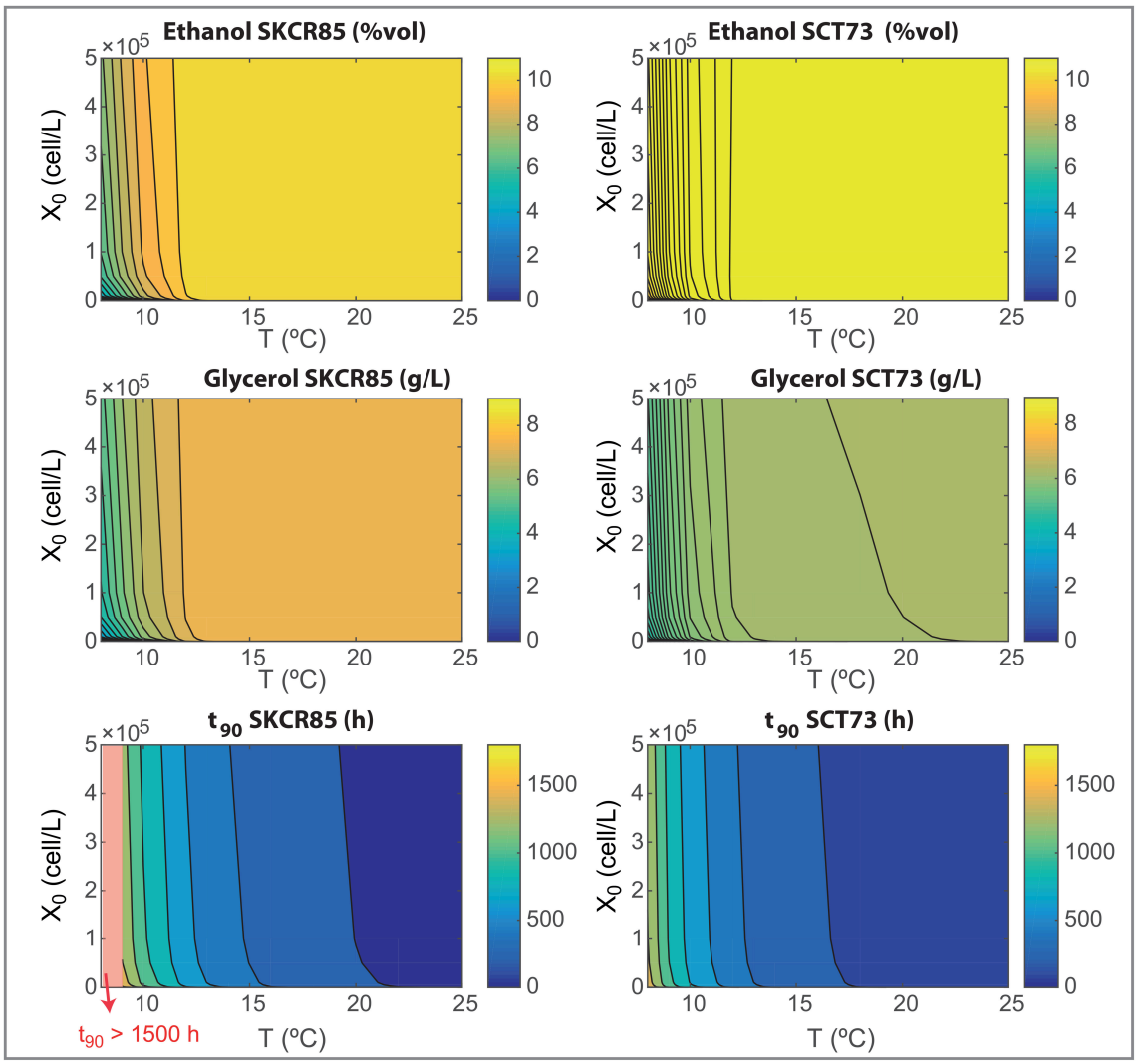

FIGURE 7 | Wine final composition and t_{90} as functions of the initial inoculation and the fermentation temperatures for both species as obtained with the ensemble model R3.

the model to analyze the effects on processing temperature (in the range, 8–25°C) and initial inoculation (in the range, $0-5\cdot 10^5$) on the most relevant fermentation parameters: process yields, final ethanol and glycerol content and the time required to consume the 90% of the initial glucose and fructose content (t_{90}). Results are shown in **Figures 6**, **7**.

Figure 6 show how SCT73 is substantially more effective in transforming hexoses in ethanol for all tested conditions. The maximum yield corresponds to a 0.96 for SCT73 and 0.81 for SKCR85. Only at very low temperatures the yield for SCT73 reduces to a value similar to the maximum achieved by SKCR85. SKCR85 is more effective than SCT73 yielding glycerol for all conditions tested. SCT73 achieves the maximum glycerol yield at higher temperatures (T>22.5°C) for all inoculations. Similar values can be achieved at around 17°C by increasing the initial inoculation. The yield of acetate is quite insensitive to temperature and initial inoculation, only at very low temperatures (T<10°C) a slight reduction in yield is observed for both species.

Differences in yields explain the results shown in **Figure 7**. SKCR85 will produce wines with less ethanol but with higher amounts of glycerol than SCT73 in all tested conditions. Remarkably the production of glycerol is distinctive in SKCR85, it was not possible to achieve the same production of glycerol with SCT73 in any of the conditions tested.

SKCR85 performs similarly, in the sense of final ethanol and glycerol production, in a wide range of temperatures 12.5 – 25°C. Of course, process duration and energy consumption would be different. In contrast, to maximize glycerol content in fermentations driven by SCT73 we would need higher temperatures in the range 18 – 22°C depending on the initial inoculation.

Summing up, the use of SKCR85 will lead to lower ethanol and higher glycerol wines no matter the temperature or the initial inoculation; the best compromise will come from the ethanol/glycerol sought and energy-processing time considerations.

CONCLUSIONS

This work approached the modeling of wine fermentation by two *Saccharomyces* yeast species under different low processing temperatures. We paid major emphasis on achieving a minimal yet robust model. For this purpose we implemented a modeling pipeline which involved the formulation of several candidate models whose parameters were computed by multi-experiment data fitting; models were subsequently reduced and selected attending to the compromise between the quality of fit and the number of parameters (Akaike criterion) as well as their cross-validation properties.

The best model is based on the logistic growth model. The more usual models incorporating the role of substrates inhibition in growth resulted in less robust alternatives due to the poor identifiability of the corresponding parameters. Also, the usual Michaelis-Menten transport formulation could be reduced to a generalized mass action model (linear model) without impacting the quality of the fit and predictive capabilities.

Model predictions were robustified by an ensemble modeling approach. The ensemble satisfactorily predicts process performance thus being suitable for exploring alternative fermentation conditions to optimize final product quality.

We have explored some possibilities by modifying the temperature and initial inoculation. However, more flexibility could be achieved if we also design the feed of hexoses and assimilable nitrogen. This flexibility could be attained by training the models with additional data obtained under various initial hexoses and nitrogen contents. This would allow to either identify an explicit dependency of N_{max} on substrates or to improve identifiability of other candidate models N1 or N2.

In addition, the somehow complementary performance observed between the two species: higher ethanol production by SCT73 and higher glycerol production by SKCR85, offer even further possibilities to improve the feasibility of low-temperature wine fermentations. Here we explored mono-culture cold fermentations. However, we envision that the optimal design of co-culture based processes may have a tremendous potential for the wine-making industry.

AUTHOR CONTRIBUTIONS

EB-C and AQ designed the work; DH and EB-C formulated the model; DH performed the optimizations; AQ and JA-d-R conceived and designed the experiments; JA-d-R performed the experiments; all authors analyzed the results and drafted the manuscript.

FUNDING

This project has received funding from the Spanish government (MINECO) and the European Regional Development Fund (ERDF) through the projects IMPROWINE (grant numbers AGL2015-67504-C3-1-R; AGL2015-67504-C3-2-R) to AQ and EB-C and the PROMETEO grant (ref. PROMETEOII/2014/042) from Generalitat Valenciana, Spain, to AQ. JA-d-R was supported by a FPI grant from the Ministerio de Economa y Competitividad, Spain (ref. BES-2013-066434).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.00088/full#supplementary-material

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

frontiers in Microbiology

ORIGINAL RESEARCH
published: 25 October 2017
doi: 10.3389/fmicb.2017.02087

The Use of Mixed Populations of *Saccharomyces cerevisiae* and *S. kudriavzevii* to Reduce Ethanol Content in Wine: Limited Aeration, Inoculum Proportions, and Sequential Inoculation

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OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: *08 September 2017*
Accepted: *11 October 2017*
Published: *25 October 2017*

Citation:
Alonso-del-Real J, Contreras-Ruiz A, Castiglioni GL, Barrio E and Querol A (2017) The Use of Mixed Populations of Saccharomyces cerevisiae and S. kudriavzevii to Reduce Ethanol Content in Wine: Limited Aeration, Inoculum Proportions, and Sequential Inoculation. Front. Microbiol. 8:2087. doi: 10.3389/fmicb.2017.02087

Saccharomyces cerevisiae is the most widespread microorganism responsible for wine alcoholic fermentation. Nevertheless, the wine industry is currently facing new challenges, some of them associate with climate change, which have a negative effect on ethanol content and wine quality. Numerous and varied strategies have been carried out to overcome these concerns. From a biotechnological point of view, the use of alternative non-*Saccharomyces* yeasts, yielding lower ethanol concentrations and sometimes giving rise to new and interesting aroma, is one of the trendiest approaches. However, *S. cerevisiae* usually outcompetes other *Saccharomyces* species due to its better adaptation to the fermentative environment. For this reason, we studied for the first time the use of a *Saccharomyces kudriavzevii* strain, CR85, for co-inoculations at increasing proportions and sequential inoculations, as well as the effect of aeration, to improve its fermentation performance in order to obtain wines with an ethanol yield reduction. An enhanced competitive performance of *S. kudriavzevii* CR85 was observed when it represented 90% of the cells present in the inoculum. Furthermore, airflow supply of 20 VVH to the fermentation synergistically improved CR85 endurance and, interestingly, a significant ethanol concentration reduction was achieved.

Keywords: *Saccharomyces* yeast, wine fermentation, ethanol reduction, fermentation oxygenation, starter cultures

INTRODUCTION

Wine composition is the product of complex interactions among yeast and bacteria that take place in vineyards and wineries, although one yeast species, *Saccharomyces cerevisiae*, is generally the main microorganism responsible for winemaking process (Pretorius, 2000). Its vigorous fermentative capacity, even in the presence of oxygen (Crabtree effect), makes *S. cerevisiae* a very efficient ethanol producer, strategy that allows its imposition over the rest of the microbiota during fermentation due to the toxicity of this compound (Thomson et al., 2005; Piškur et al., 2006).

However, this high ethanol production capability may be disadvantageous taking into account the challenges currently faced by the wine industry. In the first place, global warming provokes a gap during grape ripening between phenolic maturity and sugar content. If grapes are harvested when the sugar content is appropriate but the phenolic maturity has not been reached, wines can show altered aroma, flavor, mouth feel, and astringency. On the contrary, if grapes are harvested when their phenolic maturity is the appropriate, their sugar contents are higher, giving rise to wines with increasing ethanol concentrations (Jones et al., 2005). This higher ethanol content is undesirable according to consumers' new demands, because affects flavor complexity sensing (Goldner et al., 2009), and its excessive consumption is harmful for health and road safety.

A variety of measures are taken at the different winemaking stages to overcome the problem of the higher ethanol levels in wines. These include new agronomical methods for grape cultivation (Intrigliolo and Castel, 2009), the use of mixed musts from grapes at different ripening stages (Kontoudakis et al., 2011), the use of engineered yeasts producing lower ethanol yields (Varela et al., 2012), or the partial dealcoholisation of wines by chemical or physical procedures (Gómez-Plaza et al., 1999; Pilipovik and Riverol, 2005; Diban et al., 2008; Hernández et al., 2010; Offeman et al., 2010; Belisario-Sánchez et al., 2012). However, some of these approaches have little impact on ethanol contents, negatively affect the quality of wine, are highly expensive industrial processes, or contravene the current regulations about the use of GMO.

In addition, a wide range of different biological strategies have been proposed to reduce alcohol contents in wines (Kutyna et al., 2010). The use of non-conventional yeast strains in winemaking stands out for its potential. Several non-*Saccharomyces* yeasts, usually in combination with *S. cerevisiae*, have been tested to reduce ethanol yields during wine fermentation (Comitini et al., 2011; Sadoudi et al., 2012; Contreras et al., 2014, 2015; Quirós et al., 2014; Ciani et al., 2016). Different strategies have been carried out to improve the fermentation performance of these non-*Saccharomyces* yeasts, such as, sequential inoculation or co-inoculation at increased proportions with *S. cerevisiae*, to provide new characteristics to the final wines (Andorrà et al., 2012; Gobbi et al., 2013; Izquierdo Cañas et al., 2014; Jolly et al., 2014; Loira et al., 2014; Canonico et al., 2016). Another approach to reduce alcohol content in wines is the supply of oxygen to the fermenters, under a controlled flowrate, to promote the respiratory consumption of sugars by these non-*Saccharomyces* yeasts (Gonzalez et al., 2013; Rodrigues et al., 2016). However, temperature under industrial winemaking conditions is generally close to 25°C, which does not allow for any of these alternative yeasts to survive the first hours of the process (Nissen and Arneborg, 2003; Torija, 2003; Pérez-Nevado et al., 2006; Williams et al., 2015).

Alternative *Saccharomyces* yeasts, such as, *Saccharomyces kudriavzevii* or *S. uvarum*, can help to solve some of the new challenges of the wine industry. These species exhibit physiological properties that are especially relevant during the winemaking process, such as, their good fermentative capabilities at low temperatures, resulting in wines with lower alcohol and higher glycerol amounts (Varela et al., 2016; Pérez-Torrado et al., 2017a). In the case of *S. kudriavzevii*, this species displays a different metabolic regulation concerning ethanol and glycerol syntheses (Arroyo-López et al., 2010; Oliveira et al., 2014; Pérez-Torrado et al., 2016). Moreover, it recently showed an ethanol reducing capability in mixed fermentation with *S. cerevisiae* at low temperatures (Alonso-del-Real et al., 2017). Again, temperature appears as the most important factor to determine the preponderance of *S. cerevisiae* during wine fermentation (Nissen and Arneborg, 2003; Torija, 2003; Pérez-Nevado et al., 2006; Arroyo-López et al., 2011; Salvado et al., 2011; Williams et al., 2015; Alonso-del-Real et al., 2017).

However, none of the techniques used to favor the growth of non-*Saccharomyces* yeasts, such as, co-inoculation, sequential inoculation, or microoxigenation, have been applied to *S. kudriavzevii* species to favor their presence during wine fermentation. In this work, we first analyzed the presence of *S. kudriavzevii* during co-fermentation with a *S. cerevisiae* wine strain under different aeration conditions to select the most suitable one. Next, we studied the effect of *S. kudriavzevii* enrichment in the inoculum with and without external oxygen supply, and finally the effect of sequential inoculation of the strains.

MATERIALS AND METHODS

Yeast and Growth Media

The commercial *S. cerevisiae* strain T73 (Lalvin T73 from Lallemand Monteral, Canada), was used as a conventional wine strain. *S. kudriavzevii* CR85, a natural isolate from oak tree bark in Agudo, Ciudad Real province, Spain, was selected as the non-conventional, quality enhancer candidate yeast according to its physiological properties. In a recent study, CR85 was shown to be the *S. kudriavzevii* strain with better fermentation kinetics, despite the high genomic homogeneity among that species (Peris et al., 2016).

Synthetic must (SM, Rossignol et al., 2003) was used in microvinification experiments, with 100 g/L glucose and 100 g/L fructose. YPD medium (2% glucose, 2% peptone, 1% yeast extract) was used for overnight growth of precultures.

Synthetic Must Fermentations

First, in order to determine the best aeration condition, fermentations of 200 mL SM were carried out by a *S. cerevisiae* and *S. kudriavzevii* co-inoculum (ratio 1:1) at four different aeration conditions throughout the process: 1 VVH, 5 VVH, 10 VVH, and 20 VVH taking in account the previous data from non-conventional yeasts (Morales et al., 2015). Secondly, different ratios *S. cerevisiae*/*S. kudriavzevii* (1:1, 3:7, and 1:9) were used in further 200 mL SM fermentations, both in anaerobiosis and with an air flow rate of 20 VVH during the first 48 h. Also, a condition in which *S. cerevisiae* was inoculated after 24 h in a proportion of 1% with respect to *S. kudriavzevii* was also considered. Single cultures of *S. cerevisiae* and *S. kudriavzevii* were taken as control for fermentation. In addition, a bottle containing distilled water and another one with water and 5% (v/v) ethanol were set as control for evaporation and ethanol loss due to aeration.

Aeration system is composed of a compressed air generator, 3.1 mm internal diameter silicon tubes, 0.2 μm pore-size filters, a flow meter and a set of flow regulators (one for each bottle) as depicted in Supplementary Figure 1. All the experiments were conducted in triplicate at 25°C with gentle shaking (100 rpm) and an initial inoculation with an OD_{600} of 0.2. The fermentation process was monitored through weight loss. Yeast cells were collected at different moments during fermentation and kept at −20°C to determine the proportion of both yeast species by QPCR, according to Alonso-del-Real et al. (2017). Supernatants of the samples were also stored at −20°C for the analysis of wine composition by HPLC.

HPLC Analysis and Data Treatment

Sugars (glucose and fructose), glycerol, ethanol, and acetic acid from the fermentation at different time point samples were determined by HPLC (Thermo Fisher Scientific, Waltham, MA, USA) using a refraction index detector and a HyperREZTM XP Carbohydrate H + 8 μm column (Thermo Fisher Scientific) equipped with a HyperREZTM XP Carbohydrate Guard (Thermo Fisher Scientific). Samples were 3-fold diluted, filtered through a 0.22-μm nylon filter (Symta, Madrid, Spain) and injected in duplicate. The analysis conditions were: eluent, 1.5 mM of H_2SO_4; 0.6 ml min-1 flux and a 50°C oven temperature.

Water and ethanol losses were considered as lineal with respect to time. Deviation factors were dimensioned in bottles with 5% (w/v) ethanol in 400 mL water, and bottles with 400 mL of water, all them with air supply (20 VVH). Water mass loss followed a lineal equation ($R^2 = 0.99569$):

$$y = 0.1684t \tag{1}$$

where y refers to weight loss due to H_2O evaporation in bottles with only water and t refers to time.

$$y = 0.2532t \tag{2}$$

where y refers to weight loss due to H_2O and ethanol evaporation in bottles with 5% (w/v) ethanol and t refers to time. HPLC measures of the last were taken at different time points. We observed that ethanol loss followed a lineal function, and that a subtraction of the equation for ethanol bottle minus the one for water bottle, very precisely predicted HPLC results. The calculation was done following Equations (3–5):

$$F_1 = \frac{((a_1 - a_2) \times 100)}{20} \tag{3}$$

where F_1 is factor 1 for ethanol correction (% h^{-1}), a_1 is the slope of Equation (1), a_2 is the slope of Equation (2), and 20 is the value for the total mass of ethanol weighted for 400 mL of solution.

$$F_2 = \frac{(F_1 \times t) \times E_{HPLC}}{20} \tag{4}$$

where F_2 is factor 2 for ethanol correction (%), t is the time corresponding to an assessed value and E_{HPLC} is the HPLC measure for ethanol concentration.

$$E_C = \frac{(F\_2 + E\_HPLC).[V\_T - (a2 \times t)]}{V_T} \tag{5}$$

where E_C is corrected ethanol concentration (%).

The rest of compounds in our system were assumed as nonvolatile, however, their concentration values were considered as affected by water and ethanol volume losses. To calculate this concentration factor, the density of must was considered to be equal to the density of water. HPLC values for glucose, fructose, glycerol and acetic acid were corrected using the following equation:

$$C_C = \frac{C_{HPLC} \times 1000}{(1000 + (a_2 \times t)) + \left[(E_C - E_{HPLC}) \times 10\right]} \tag{6}$$

where C_C is the corrected concentration for the compound.

Fermentations were tested for the significant differences among them with an ANOVA using the one-way ANOVA module of the Statistica 7.0 software. The concentrations of glucose, fructose, glycerol, ethanol, and acetic acid obtained by HPLC were introduced as the dependent variables. Means were grouped using the Tukey HSD test ($\alpha = 0.05$).

RESULTS

Determining the Air Flow Conditions Favoring *S. kudriavzevii* Presence in Mixed Fermentations with *S. cerevisiae*

A controlled aeration system feeding a set of fermentations co-inoculated with *S. cerevisiae* and *S. kudriavzevii* in a ratio 1:1 with 4 different air flow rates: 1, 5, 10, and 20 VVH was installed. **Figure 1** shows a clear disadvantage of *S. kudriavzevii* even in the presence of an external oxygen input. However air flow rate seems to have an influence on the time that *S. kudriavzevii* can remain in the culture in substantial proportions, and thus, can have a more relevant role during fermentation. The percentage of *S. kudriavzevii* was higher than 30% during the first 48 h in fermentations performed with air flows of 10 and 20 VVH. However, after 48 h of fermentation a faster decline of the *S. kudriavzevii* population is observed, which suggests that aeration only favors *S. kudriavzevii* growth at the beginning of the fermentations.

Assaying Different *S. cerevisiae*/*S. kudriavzevii* Inoculation Proportions in Fermentations with and without Air Supply

According to these previous data, aeration was applied only for short periods (48 h) for subsequent fermentations because longer aeration time does not favor growth of *S. kudriavzevii*, and also could increase the final acetic acid concentrations in wines, due to respiration (Salmon, 2006). To test whether a higher inoculation from the beginning of the fermentation, in combination with aeration, could improve *S. kudriavzevii*'s competitive

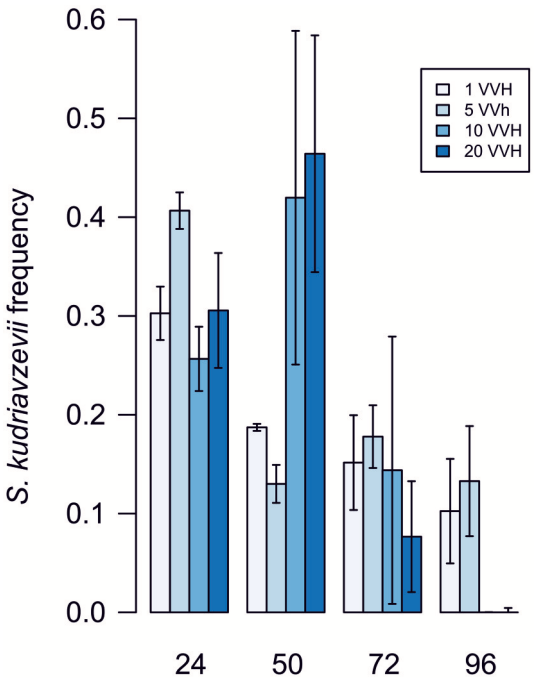

FIGURE 1 | *S. kudriavzevii* frequency under different aeration conditions. Values are mean of three replicates. Error lines represent standard deviations.

performance, starters composed by *S. cerevisiae*/*S. kudriavzevii* proportions of 1:3 and 1:9 in were inoculated into fermentations supplied with an air flow rate of 20 VVH during the first 48 h. Fermentations in the same conditions without aeration were also included to analyze the effect of the yeast species proportions alone.

There were significant differences between aerated and non-aerated fermentations. First, there is a considerable reduction of the fermentation time at which all sugars were totally consumed. Whereas unaerated fermentations took 10 days to finish, aerated fermentations took only 7 days. Second, a clear effect on the maximum cell density was observed, thus, single cultures of *S. cerevisiae* and *S. kudriavzevii* with air supply reached OD_{600} values around 25, however OD_{600} values for single cultures without aeration were around 20 and 15, respectively.

Regarding yeast proportion changes during fermentations, the initial inoculum proportion of 1:3 shows a slight increase of the frequency of *S. kudriavzevii* at the final fermentation stage due to limited air supply (**Figures 2A**,**B**). However, this inoculation ratio does not provide, with respect to the 1:1 proportion a clear competition advantage for *S. kudriavzevii*. However, when the inoculation proportion was 1:9 and without aeration (**Figure 2C**), *S. kudriavzevii* is able to remain at frequencies higher than 40% for 4 days, although at the end, is outcompeted by *S. cerevisiae*. Strikingly, the addition of the oxygen supply to inoculation proportions of 1:9 seems to provide a favorable environment for *S. kudriavzevii* imposition (**Figure 2D**).

Sequential inoculation is one of the most common strategies proposed for the preservation of non-dominant microorganisms during food fermentations (Gobbi et al., 2013; Contreras et al., 2014; Loira et al., 2014). In the present study, this strategy was also applied by inoculating a set of bottles only with *S. kudriavzevii* at the beginning, and adding *S. cerevisiae* after 24 h in a proportion of 1%. In this case, *S. cerevisiae* was able to increase its frequency to 40% at the end of the fermentations (**Figure 2E**).

As a summary of these results, the use of aeration has a slight impact on the relative competitive fitness of *S. kudriavzevii* when inoculated at equal proportions with *S. cerevisiae*. However, highly biased proportions of *S. kudriavzevii*, as well as sequential inoculations, can extend the presence of this less competitive species of interest to promote its impact in the fermentation process. Nevertheless, the combination of aeration and biased inoculation synergistically improves *S. kudriavzevii* presence during fermentation.

Effect of the Different Inoculation-Aeration Strategies on the Final Fermentation Product

To determine if these strategies really improve wine fermentations, the final wine composition was evaluated by HPLC analysis. First, it is important to remark that in all assayed conditions fermentations were finished with the consumption of all sugars present in the original must, except for fermentations performed only with single cultures of *S. kudriavzevii* (**Table 1**), and under aeration, fructose was totally consumed.

Glycerol concentrations were clearly higher in all conditions in which *S. kudriavzevii* is present, compared to fermentations performed only with the reference *S. cerevisiae* wine strain, except for the 1:1 proportion with aeration. This glycerol production increase was especially relevant in fermentations with sequential inoculation (**Table 1**).

Ethanol reduction was accomplished in fermentations with microaeration (up to 1.9% v/v less) and with sequential inoculation (**Table 1**). However, the ethanol reduction achieved by increasing respiration rate had the counterpart of an acetic acid content increase between 0.5 and 0.7 g/L in bottles under limited aeration, which was not observed in non-aerated fermentations.

DISCUSSION

In the last century, alcohol abuse became considered as one of the most important health problems in the world, and promoted new behavioral strategies against alcohol consumption. In addition, because of global warming, in wine-growing regions with a Mediterranean climate there is excessive ripening of the grape, which produces musts with a higher concentration of sugars (Jones et al., 2005), and hence, higher alcohol yields, implying a higher tax burden, which makes wines less competitive, and a rejection by the consumer for health reasons, road safety, etc.

Therefore, wine industry must respond to these challenges posed both by new consumer demands and by changes in the composition and properties of the grape must due to climate change. These demands have a significant impact on the quality

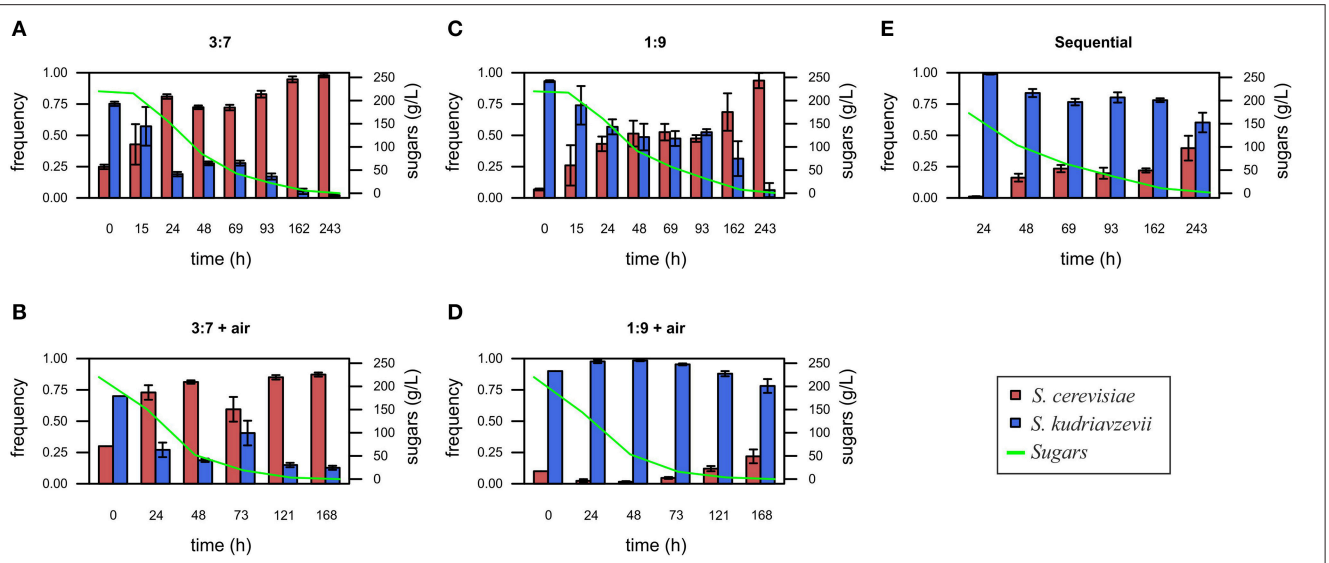

FIGURE 2 | *Saccharomyces cerevisiae* and *S. kudriavzevii* frequency during fermentation under different conditions: inoculum proportion 3:7 without air **(A)**, inoculum proportion 3:7 with aeration during the first 48 h **(B)**, inoculum proportion 1:9 without air **(C)**, inoculum proportion 1:9 with aeration during the first 48 h **(D)**, and sequential inoculation **(E)**. Values are mean for 3 replicates. Error bars represent standard deviations. The sum of glucose and fructose concentrations in the must at every time point was also shown.

TABLE 1 | Chemical composition of the fermented SM obtained through HPLC.

Sce: *Sku* proportion	Aeration (VVH)	Glucose (g/L)	Fructose (g/L)	Glycerol (g/L)	Ethanol (%)	Acetic acid (g/L)
1:0	0	0.00 ± 0.00^{a}	0.12 ± 0.03^{a}	$5.86 \pm 0.11^{a,b}$	$13.13 \pm 0.09^{a,c}$	1.05 ± 0.01^{a}
0:1	0	0.02 ± 0.03^{a}	4.11 ± 2.34^{b}	7.73 ± 0.46^{d}	$12.50 \pm 0.26^{a,b}$	1.27 ± 0.03^{a}
1:1	0	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	6.24 ± 0.29^{a}	13.27 ± 0.50^{c}	1.16 ± 0.13^{a}
3:7	0	0.00 ± 0.00^{a}	0.15 ± 0.10^{a}	$6.13 \pm 0.09^{a,b}$	$13.04 \pm 0.05^{a,c}$	1.15 ± 0.03^{a}
1:9	0	0.00 ± 0.00^{a}	0.78 ± 0.75^{a}	6.53 ± 0.12^{a}	$13.00 \pm 0.16^{a,c}$	1.22 ± 0.01^{a}
Sequential	0	0.00 ± 0.00^{a}	1.63 ± 0.18^{a}	$7.47 \pm 0.21^{c,d}$	$12.46 \pm 0.08^{a,b}$	1.13 ± 0.05^{a}
1:1	20	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	5.36 ± 0.40^{b}	12.12 ± 0.33^{b}	1.57 ± 0.10^{b}
3:7	20	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	6.24 ± 0.55^{a}	12.09 ± 0.18^{b}	1.61 ± 0.23^{b}
1:9	20	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	$6.61 \pm 0.07^{a,c}$	11.26 ± 0.19^{d}	1.79 ± 0.02^{b}

Values are given as mean ± standard deviation of three biological replicates and two HPLC detection runs. An ANOVA analysis was carried out. The values followed by different superindexes in the same column are significantly different according to the Tukey HSD test ($\alpha = 0.05$).

and acceptance of the final wines and require improvements in the enological practices, among which the development of new yeast starters exhibiting lower ethanol yields during wine fermentation is of chief importance.

Different approaches in the use of yeast starters have been proposed to reduce alcohol contents in wines (Schmidtke et al., 2012; Varela et al., 2015). They include controlled aeration, starter strain proportion adjustment, or inoculation of dominant yeast species after a non-*Saccharomyces* yeast of interest (Comitini et al., 2011; Sadoudi et al., 2012; Contreras et al., 2014, 2015; Quirós et al., 2014; Ciani et al., 2016). In the present study, we adapted these strategies to foster a *Saccharomyces* non-*cerevisiae* strain (*S. kudriavzevii* CR85) presence in synthetic must fermentation. This yeast had been proved to foster decreased ethanol content, and also to increase fermentation kinetics and glycerol concentration in a 1:1 inoculum proportion with *S. cerevisiae* under low temperatures conditions. In contrast, this effect was not found under regular red winemaking temperatures (Alonso-del-Real et al., 2017), probably due to some of the already proposed competition mechanisms, such as, antimicrobial GAPDH-derived peptides produced by *S. cerevisiae* (Branco et al., 2016), lower sulfite tolerance and efflux capacity (Pérez-Torrado et al., 2017b), or early nutrient depletion by *S. cerevisiae* (Fleet, 2003). However, the results reported in the present work show that *S. kudriavzevii* presence during an important period of the fermentation was achieved at regular industrial temperatures.

Although *S. kudriavzevii* and *S. cerevisiae* show long-term Crabtree effect, the carbon flux ratio between respiration and fermentation under aerobic conditions seem to be slightly higher in *S. kudriavzevii* CR85 compared to *S. cerevisiae* T73 (our unpublished data). Thus, an external oxygen supply to a fermentation co-inoculated with these two yeast species may benefit *S. kudriavzevii* growth. Nevertheless, high oxygen levels

can deteriorate important compounds of must, originating undesired metabolites correlated to respiration such as acetic acid (Salmon, 2006). Therefore, a fine tuning of the amount of oxygen introduced into the system seems to be critical for the final wine quality. A wide range of airflow rates, from 2.4 to 60 VVH have been used at laboratory scale (Vilanova et al., 2007; Shekhawat et al., 2016). Nevertheless, an air flow rate of 20 VVH has been showed to be on the top limit for acetic acid production when applied to *S. cerevisiae* microvinification (Morales et al., 2015), therefore the screening for the most suitable condition was performed always below this value.

S. kudriavzevii performance under air supply conditions was observed to improve its competitive fitness against *S. cerevisiae* (Arroyo-López et al., 2011; Alonso-del-Real et al., 2017). Our results suggest, though, that despite maintaining an air supply during the whole fermentation, after 48 h, *S. kudriavzevii* was outcompeted by *S. cerevisiae*. This, together with the fact that an aerobic environment produces a higher acetic acid accumulation up to 70%, led us to reduce aeration just for the first 48 h of fermentation for the successive experiments. Nevertheless, it is noteworthy that, as observed by Moruno et al. (1993) and later confirmed by Beltrán et al. (2008), synthetic and natural musts have different impact on the final product composition, acetic acid levels are much higher for synthetic must, as can also be observed for our aerated conditions. Thus, due to laboratory experimental conditions, acetic acid values obtained in the present work are high even for non-aerated synthetic must fermentations performed with the *S. cerevisiae* wine strain, compared to natural must fermentation under industrial conditions (0.35 g/L). Therefore, acetic acid levels produced during fermentations with air supply could still be under the limits of regulation (∼1 g/L) and consumers' acceptance when tested at industrial scale.

Despite the acetic acid increase, ethanol reduction is notable for the aerated fermentations, in concordance with previous studies (Morales et al., 2015; Shekhawat et al., 2016), and similar to ethanol reductions obtained in other works in which similar co-inoculation strategies with non-*Saccharomyces* yeasts have been followed (Contreras et al., 2015; Ciani et al., 2016; Englezos et al., 2016). However, this is the first study in which *S. kudriavzevii* was used to reduce ethanol yields, which, together with a recent study on the sequential inoculation of *S. uvarum* and *S. cerevisiae* (Varela et al., 2016), opens new approaches to the use of other *Saccharomyces* species. These species, in addition to their ethanol metabolic characteristics, also provide richer aroma profiles to wine (Stribny et al., 2015).

The analysis of the non-aerated fermentations also showed a slight ethanol yield reductions clearly correlated with the *S. kudriavzevii* proportions during the fermentation process under the different assayed conditions. Moreover, there also is a clear direct correlation between *S. kudriavzevii* proportions and glycerol production, another desirable enological characteristic of importance for wine quality because it contributes to wine body and astringency masking (Jolly et al., 2014). Glycerol and ethanol metabolism has been proven to differ in *S. kudriavzevii* with respect to *S. cerevisiae* (Arroyo-López et al., 2010; Pérez-Torrado et al., 2016). In fact, cryotolerant *Saccharomyces* species, such as, *S. kudriavzevii* and *S. uvarum*, have been proven to produce wines and ciders with higher glycerol contents than *S. cerevisiae* (Bertolini et al., 1996; Masneuf-Pomarède et al., 2010; Peris et al., 2016; González Flores et al., 2017), so their use could be of great interest for wine industry.

Among the strategies followed to favor *S. kudriavzevii* growth against *S. cerevisiae*, the co-inoculation with a proportion of *S. cerevisiae* lower than 10% and the sequential inoculation showed the more promising results. Air supply showed a synergistic effect in proportion *S. cerevisiae*/*S. kudriavzevii* 1:9, whereas it did not have a significant impact on the rest of the assayed inoculum proportions. These results agree with the fact that *S. cerevisiae* is better adapted to anaerobic conditions such as, wine fermentation, and air supply produces an imbalance in this environment, which promotes *S. kudriavzevii* survival. According to our results, it also seems feasible that a certain threshold in *S. cerevisiae* cell density is necessary to trigger *S. kudriavzevii* lack of viability. This also agrees with the previous observations indicating that the viability of a competitor strain is affected by its interaction with *S. cerevisiae* due to cell-to-cell contacts (Nissen et al., 2003; Arneborg et al., 2005; Branco et al., 2016; Pérez-Torrado et al., 2017b), or by microenvironment modifications produced by *S. cerevisiae* (Goddard, 2008). A rise in temperature due to the higher fermentative rate of *S. cerevisiae* (Goddard, 2008) can affect *S. kudriavzevii* viability (Arroyo-López et al., 2011).

In summary, the most promising results were obtained from the combination of different strategies for promoting *S. kudriavzevii* prevalence during wine fermentation, such as, co-inoculation with a low proportion of *S. cerevisiae* (<10%) or sequential inoculation together with limited aeration, resulting in an ethanol yield reduction as well as a higher glycerol production. Aeration requires costly additional technology, but it is already implemented in the wine industry (Vivas and Glories, 1996; Vidal and Aagaard, 2008) to improve wine quality by accelerating the transformations of phenols reducing the astringency.

Finally, these results have to be confirmed in real grape must to evaluate not only the effect of aeration on yeast physiology but also a potential effect on sensory profile. In addition, lower aeration rates can also be tested at industrial scale, particularly for *S. cerevisiae*/*S. kudriavzevii* proportions lower than 1:9. In addition a deeper understanding of the interactions among *Saccharomyces* yeasts, are also needed in order to finely tune the optimal use of these tools to reduce ethanol contents in wine.

AUTHOR CONTRIBUTIONS

JA, GC, EB, and AQ conceived and designed the experiments. JA, AC, and GC performed the experiments. JA, EB, and AQ analyzed the data and wrote the paper.

ACKNOWLEDGMENTS

JA was supported by a FPI grant from the Ministerio de Economía y Competitividad, Spain (ref. BES-2013-066434) and GC was supported by National Council for Scientific and Technological Development (CNPq), Brazil. This work

was funded by grants AGL2015-67504-C3-1-R and AGL2015-67504-C3-3-R from the Spanish Government and FEDER to AQ and EB, respectively, and by a PROMETEO grant (ref. PROMETEOII/2014/042) from Generalitat Valenciana, Spain to AQ.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2017.02087/full#supplementary-material

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

frontiers in Microbiology

ORIGINAL RESEARCH
published: 19 June 2018
doi: 10.3389/fmicb.2018.01320

Differences Between Indigenous Yeast Populations in Spontaneously Fermenting Musts From *V. vinifera* L. and *V. labrusca* L. Grapes Harvested in the Same Geographic Location

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OPEN ACCESS

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

Received: *27 March 2018*
Accepted: *30 May 2018*
Published: *19 June 2018*

Citation:
Raymond Eder ML, Conti F and Rosa AL (2018) Differences Between Indigenous Yeast Populations in Spontaneously Fermenting Musts From V. vinifera L. and V. labrusca L. Grapes Harvested in the Same Geographic Location. Front. Microbiol. 9:1320. doi: 10.3389/fmicb.2018.01320

Yeast communities associated with *Vitis vinifera* L. ecosystems have been widely characterized. Less is known, however, about yeast communities present in grapes and fermenting musts from *Vitis* non-*vinifera* ecosystems. Moreover, there are no comparative studies concerning yeast communities in grapes from *V. vinifera* L. and non-*vinifera Vitis* species in vineyards from a shared *terroir*. In this work, we have used a culture-dependent strategy, phenotypic analyses, and molecular genotyping, to study the most representative yeast species present in spontaneously fermenting musts of grapes harvested from neighboring *V. vinifera* L. (cv. Malbec) and *V. labrusca* L. (cv. Isabella) vineyards. Phenotypic analyses of H_2S production, ethanol tolerance and carbon utilization, on randomly selected strains of each *Hanseniaspora uvarum*, *Starmerella bacillaris* and *Saccharomyces cerevisiae* strains, as well as microsatellite genotyping of *S. cerevisiae* isolates from each the Malbec and Isabella grape musts, suggest that *V. vinifera* L. and *V. labrusca* L. ecosystems could harbor different yeast strain populations. Thus, microbial communities in exotic *Vitis* species may offer opportunities to look for unique yeast strains that could not be present in conventional *V. vinifera* L. ecosystems.

Keywords: *Vitis*, *V. vinifera* L., *V. labrusca* L., grapes, indigenous yeast, fermentation

INTRODUCTION

During alcoholic fermentation, a dynamic metabolic interaction between grape musts and their associated microbial communities shapes the final sensory and organoleptic character of wines (Fleet, 2008). Because of its scientific and industrial relevance, the study of the indigenous microbial communities in grapes and spontaneously fermenting grape must constitutes a major research area in oenology (Fleet, 2003; Jolly et al., 2014; Padilla et al., 2016; Varela, 2016; Varela and Borneman, 2017; Morgan et al., 2017). Culture-dependent and/or metagenomics approaches and DNA-based

strategies have been used to characterize the complex and dynamic population of microorganisms in oenological ecosystems (Barata et al., 2012; Masneuf-Pomarede et al., 2016; Morgan et al., 2017). In these studies, a direct relationship has been recognized between grape microbiomas and *terroirs*, with the resulting specific microbial populations being a determining factor in the regional identity of vineyards, grapes, musts, and wines (Bokulich et al., 2014; Knight et al., 2015; Capece et al., 2016). A common pattern of development of yeast species, however, has been recognized in spontaneously fermenting musts from *Vitis vinifera* L. grapes, with non-*Saccharomyces* being the most common species at initial stages and *Saccharomyces cerevisiae* the dominant species at the middle and final stages of fermentation (Jolly et al., 2014). The rich diversity of non-*Saccharomyces* species, during the initial stages of fermentation, producing a variety of secondary metabolites, strongly contributes to the organoleptic signatures of wines (Jolly et al., 2006; Medina et al., 2013; Padilla et al., 2016; Varela, 2016).

While extensive research has been conducted on the complexity and dynamics of the yeast microbiota in the *V. vinifera* L. ecosystem (Varela and Borneman, 2017), fewer studies have examined the yeast communities in non-*vinifera Vitis* ecosystems. These non-conventional *Vitis* ecosystems may harbor a rich diversity of yeast species and strains (Raymond Eder et al., 2017). Recently, the diversity of yeasts in *V. labrusca* L. grapes and hybrids has been studied in vineyards from Brazil (Bezerra-Bussoli et al., 2013; Filho et al., 2017), the Azores Archipelago (Portugal) (Drumonde-Neves et al., 2016) and Argentina (Raymond Eder et al., 2017). These studies highlighted the remarkable diversity of non-*Saccharomyces* yeast species in a non-conventional *Vitis* ecosystem, and suggested the existence of specific *Vitis*-yeast species associations (Raymond Eder et al., 2017).

In this work, we report the identification and characterization of the main indigenous yeast species present during spontaneous fermentation of Malbec (*V. vinifera* L.) and Isabella (*V. labrusca* L.) grapes harvested from neighboring vineyards in Colonia Caroya (Córdoba, Argentina). Genetic and phenotypic characterization of a small number of isolates, representative of three relevant yeast species found in Malbec and Isabella ecosystems from this geographic region (i.e., *Hanseniaspora uvarum*, *Starmerella bacillaris,* and *S. cerevisiae*), suggest that spontaneously fermenting grape musts from different *Vitis* species could harbor different *Vitis*-specific yeast strain populations.

MATERIALS AND METHODS

Spontaneous Fermentation of Malbec and Isabella Grape Musts

Malbec (*V. vinifera* L.) and Isabella (*V. labrusca* L.) grapes were harvested at their optimal ripeness stages from vineyards in Colonia Caroya (vintage of March, 2017), located at 31°02′00″S / 64°05′36″O and 491 meters above sea level, in the province of Córdoba, Argentina. The region has an annual rainfall of 765 mm and a mean temperature of 15.8°C. Separate spontaneous fermentations of a pool of destemmed and partially crushed Malbec and Isabella grapes were performed in a local cooperative cellar. Grapes from ∼80% of the Colonia Caroya's Malbec and Isabella vineyards (i.e., 18–20 Ha each) are processed at this cellar. About 20% of these closely located, small vineyards (i.e., ∼1.5 Ha each), have intermixed rows of Malbec and Isabella plants. Must samples (70 liters) were fermented at 25–28°C in stainless steel tanks located in a room of the winery not previously used for winemaking. Musts were punched down twice a day and aliquots were taken daily for ten (i.e., 0–240 h) or five (i.e., 0–120 h) days from Malbec and Isabella musts, respectively, and stored in 30% (v/v) glycerol at −70°C.

Isolation of Yeast Strains From Malbec and Isabella Ecosystems

Appropriate dilutions of fermenting Malbec and Isabella grape must samples were plated in duplicate on YPD-Cm agar [yeast extract 1.0% (w/v), peptone 2.0% (w/v), glucose 2.0% (w/v), agar 2.0% (w/v), chloramphenicol 10 μg/ml] and incubated for 5 days at 25°C. Colony counts on YPD-Cm plates were used to estimate the total number of yeast during fermentation. To identify the most predominant yeast species present at the initial stages of fermentation (i.e., 0, 24, and 48 h), 20 yeast colonies were randomly isolated from each sampling time from YPD-Cm agar plates having 30–50 independent colonies. These high dilution plates give a high probability of isolating strains belonging to dominant yeast species (Osorio-Cadavid et al., 2008; Raymond Eder et al., 2017). Additional colonies were randomly isolated from Malbec and Isabella musts at advanced stages of fermentation (i.e., 120 and 96 h, for Malbec and Isabella, respectively) and isolates identified as *S. cerevisiae* (i.e., 43 from Malbec and 32 from Isabella) were chosen for further analyses. Must samples from Malbec and Isabella, from early stages of fermentation (i.e., 0, 24 and 48 h), were also plated in duplicate on WL-Cm agar [WL Nutrient agar medium (Oxoid) 7.5% (w/v), chloramphenicol 10 μg/ml] and incubated for 5 days at 25°C. Ten yeast colonies from each of the Malbec and Isabella must samples analyzed (i.e., 0, 24, and 48 h) showing distinctive phenotypes (i.e., morphology and/or color), were isolated from these plates. These colonies could correspond to rare yeast species present at each sampling point (Raymond Eder et al., 2017). A total of 255 yeast isolates were obtained from the Malbec and Isabella ecosystems. All isolated yeasts were streaked on YPD agar, grown for 48 h at 25°C in YPD, and stored at −70°C in YPD broth with 30% (v/v) glycerol added.

TABLE 1 | Physicochemical analyses of spontaneously fermenting Malbec and Isabella grape musts.

Parameter	Malbec (days)		Isabella (days)	
	0	10	0	5
Reducing sugars (g/l)	226.0	2.20	169.5	1.8
Ethanol (%)	0	13.3	0	8.9
Acidity (tartaric acid) (g/l)	5.40	5.25	6.90	6.97
PH	3.90	3.94	3.42	3.43

Molecular Identification of Yeast Species

Isolated yeasts were identified by PCR-RFLP and/or DNA sequencing of their 5.8-ITS (*Internal Transcribed Spacer*) rDNA regions (Esteve-Zarzoso et al., 1999). Total genomic DNA was extracted according to Raymond Eder et al. (2017). PCR was carried out using ITS1 and ITS4 primers (White et al., 1990). For PCR-RFLP, 10 μl of each of the PCR products were digested for 3 h at 37°C with the restriction enzymes *Hinf* I (New England BioLabs, United States) and/or *Cfo* I (Promega, United States) and the resulting DNA fragments were characterized by agarose [3.0% (w/v)] gel electrophoresis and analyzed using data from www.yeast-id.org. In most of the cases, yeast species identification was confirmed by Sanger sequencing of their 5.8-ITS rDNA regions and analysis using the BLASTN software NCBI[1]. Species identification was considered valid when the identity of a 5.8-ITS sequence and a reference sequence was 99–100%. Sequences from representative *H. uvarum*, *S. bacillaris,* and *S. cerevisiae* isolates were deposited in the NCBI GeneBank database (**Table 2**).

[1]https://blast.ncbi.nlm.nih.gov/Blast.cgi

TABLE 2 | Phenotypic analyses of *H. uvarum*, *S. bacillaris,* and *S. cerevisiae* isolates from neighboring Malbec and Isabella ecosystems.

Species	Isolate	Strain[1]	H_2S[2]	Ethanol (%)[3]	Genbank
H. uvarum	1	MT017-035	2	2.5	MG734841
	2	MT117-032	2	2.5	MG734842
	3	MT217-024	1	2.5	MG734843
	4	MT217-031	3	2.5	MG734844
	1	IT017-034	2	2.5	MG734838
	2	IT117-025	1	2.5	MG734837
	3	IT117-013	2	2.5	MG734839
	4	IT217-014	2	2.5	MG734840
S. bacillaris	1	MT017-001	2	2.5	MG734849
	2	MT017-005	3	2.5	MG734850
	3	MT117-001	3	2.5	MG734851
	4	MT217-002	4	2.5	MG734852
	1	IT017-025	4	5.0	MG734845
	2	IT017-033	2	2.5	MG734846
	3	IT017-051	4	2.5	MG734847
	4	IT217-001	3	2.5	MG734848
S. cerevisiae	1	MT217-023	3	10.0	MG734853
	2	MT317-003	3	10.0	MG734854
	3	MT417-002	2	10.0	MG734855
	4	MT517-001	3	10.0	MG734856
	1	IT217-022	2	12.5	MG734858
	2	IT217-029	1	10.0	MG734857
	3	IT317-004	1	12.5	MG734859
	4	IT517-004	2	12.5	MG734860
Pm		RG02	5	12.5	Ref[4]
Td		RG07	4	10.0	Ref[4]
Mp		RG01	3	5.0	Ref[4]
Hu		RG06	2	2.5	Ref[4]
Sc		EC1118	3	12.5	Ref[4]

[1]M (Malbec) and I (Isabella) strains; [2]H_2S production was evaluated in Biggy medium and scored as indicated in the Material and Methods section; [3]Tolerance to ethanol is indicated as the maximal ethanol concentration [i.e., % (v/v)] in solid media where strain growth was observed. [4]Raymond Eder et al. (2017). Pm (P. membranifaciens), Td (T. delbrueckii), Mp (M. pulcherrima), Hu (H. uvarum), and Sc (S. cerevisiae).

Phenotypic Analyses of *H. uvarum*, *S. bacillaris,* and *S. cerevisiae* Isolates

Four random isolates of each *H. uvarum*, *S. bacillaris,* and *S. cerevisiae*, from each of the spontaneously fermenting Malbec and Isabella grape musts, were analyzed for production of H_2S, ethanol tolerance, and fermentation ability in media containing either glucose or fructose as carbon sources. Control yeast strains used in these studies have been reported (Raymond Eder et al., 2017). H_2S production was tested on Biggy-agar (*Bismuth Sulfite Glucose Glycine Yeast*; Oxoid). In these studies, 3×10^4 cells (3 μl) were spotted on Biggy agar, incubated at 25°C for 3 days, and graded using the following visual color scale: 1 (white), 2 (cream), 3 (light brown), 4 (brown), and 5 (dark brown) (Sipiczki et al., 2001). Ethanol tolerance analyses were performed according to Belloch et al. (2008) with some modifications. Cells (3×10^4 cells; 3 μl) were spotted on low dextrose [i.e., glucose 0.5% (w/v)] YP agar supplemented with either 0, 2.5, 5.0, 7.5, 10.0, 12.5, or 15.0% (v/v) of ethanol and incubated at 22°C. Growth was considered positive when colony development was recognized with the naked eye.

A simple weight loss microassay, dependent on CO_2 release (Quirós et al., 2010), was designed to characterize glucose and fructose fermentation profiles of the *H. uvarum*, *S. bacillaris,* and *S. cerevisiae* isolates. Similar small scale fermentation assays have been recently published (Liccioli et al., 2011; Peltier et al., 2018). In our studies, strains were grown during ∼15 h at 25°C without agitation in 15 ml Falcon tubes containing 5 ml of YP medium supplemented with either glucose 10.0% (w/v) (YPD-10) or fructose 10.0% (w/v) (YPF-10). Duplicated 1.5 ml

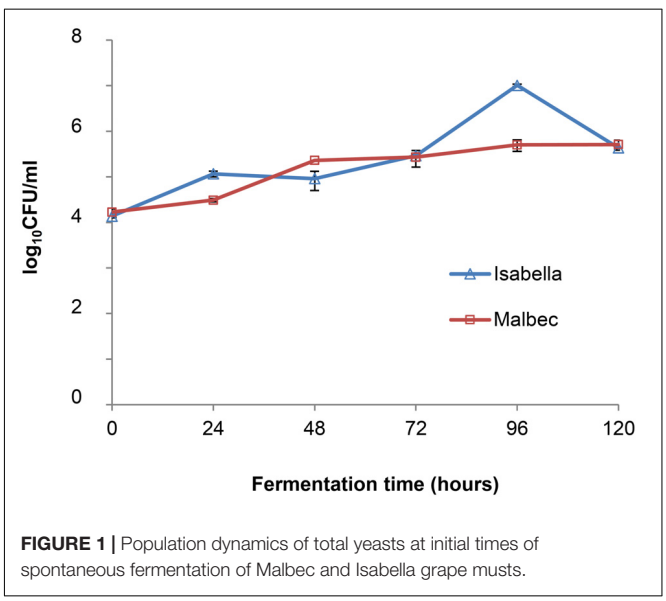

FIGURE 1 | Population dynamics of total yeasts at initial times of spontaneous fermentation of Malbec and Isabella grape musts.

Eppendorf tubes, containing 1.0 ml liquid of YPD-10 or YPF-10, were inoculated with cells (10^7/ml) from the YPD-10 and YPF-10 cultures, respectively, and maintained at 25°C without agitation. Microtubes contained a 0.8 mm perforation on its cap, covered with a small piece of cotton, to allow CO_2 efflux. Tubes were weighed immediately after inoculation and every 24 h for 4 days, using non-inoculated tubes as control of weight loss via evaporation. Fermentation rates were expressed as weight loss (i.e., CO_2 release) in function of time (i.e., $g.l^{-1}.h^{-1}$).

S. cerevisiae Microsatellite Genotyping

Saccharomyces cerevisiae isolates from Malbec and Isabella musts were genotyped using seven microsatellite loci (i.e., SCAAT1, SCAAT2, SCAAT3, C3, C6, YPL009c, and SCYOR267c)

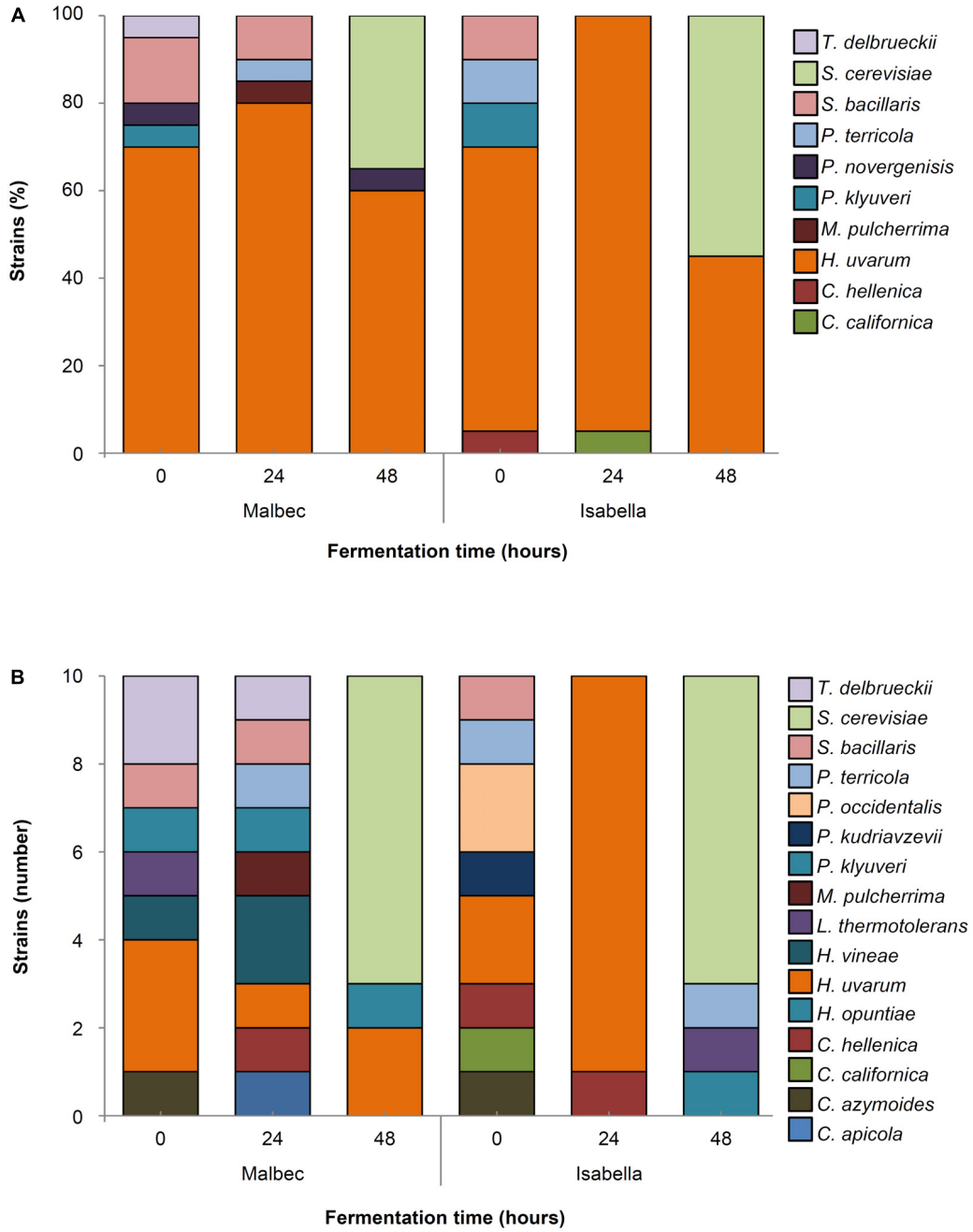

FIGURE 2 | Main contributing yeast species during spontaneous fermentation of Malbec and Isabella grape musts. Percentages represent the relative contribution of the indicated yeast species among 120 randomly selected colonies (20 isolates/sampling time; 60 isolates from each Malbec and Isabella) obtained at the indicated times of fermentation **(A)**. Yeast species identified among 60 rare colonies (10 isolates/sampling time; 30 isolates from each Malbec and Isabella) isolated from WL-Cm Nutrient agar plates at the indicated times of fermentation **(B)**.

(Legras et al., 2005). PCR reactions contained 100 ng of genomic DNA, 1.5 mM $MgCl_2$, *Taq* polymerase buffer 1X (Invitrogen, United States), 200 μM dNTPs, 10 pmol of each primer and 1.25 units of *Taq* polymerase (Invitrogen, United States). Amplification reactions were performed in a MJ Mini Bio-Rad thermocycler (Bio-Rad, United States) using an initial denaturation step at 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, annealing at 57°C for 45 s, extension at 72°C for 1 min followed by a final extension at 72°C for 10 min. PCR products were separated in 8.0% polyacrylamide gels using TBE as the running buffer. Gels were stained with ethidium bromide, photographed under UV light and allele sizes were determined using the *100-bp-DNA-ladder* (Inbio Highway, Argentina) as a reference molecular size standard.

RESULTS

Malbec (*V. vinifera* L.) and Isabella (*V. labrusca* L.) Spontaneously Fermenting Grape Musts

Standard oenological analyses of Malbec and Isabella grape musts were performed at the beginning and the end of fermentation (**Table 1**). As expected, from its low initial levels of total reducing sugars, Isabella grape must fermentation was completed in 5 days, while spontaneous fermentation of Malbec grape must took 10 days. Ethanol concentration in completely fermented Isabella grape must was 8.9% (v/v), which is ∼1% (v/v) lower than expected from its initial concentrations of reducing sugars (169.5 g/l) (**Table 1**).

Population Dynamics and Main Cultivable Yeasts in Spontaneously Fermenting Malbec and Isabella Grape Musts

The population dynamics of cultivable yeast species in the Malbec and Isabella ecosystems were analyzed from time *t0* to *t120*, corresponding to the initial stages of fermentation of Malbec and the entire fermentation period of Isabella (**Figure 1**). The total yeast populations in both ecosystems started with similar counts, and increased similarly as fermentation progressed (**Figure 1**). The highest total yeast count in fermenting Isabella grape must was observed at *t96* while fermenting Malbec must reached its highest yeast count at *t120* (**Figure 1**). As expected, *S. cerevisiae* was the most predominant yeast species recognized among 75 isolates obtained at the middle/advanced stages of fermenting Malbec and Isabella musts (i.e., *t72–t120*) (not shown). Based on this observation, our analyses of the predominant non-*Saccharomyces* species in the Malbec and Isabella ecosystems were limited to the early stages of spontaneous fermentation (i.e., *t0*, *t24,* and *t48*), at which a total of 180 isolates were identified by PCR-RFLP and/or DNA sequencing of their 5.8-ITS (*Internal Transcribed Spacer*) rDNA regions (**Figure 2**).

A great diversity of non-*Saccharomyces* species was evidenced among the yeast isolated from both Malbec and Isabella ecosystems (**Figure 2**). *H. uvarum* was the most common species isolated at early stages of fermentation of Malbec (*t0*, *t24,* and *t48*) and Isabella (*t0* and *t24*) grape musts (**Figure 2A**). Other non-*Saccharomyces* yeast species identified in both Malbec and Isabella musts were *Candida azymoides*, *Candida hellenica*, *Lachancea thermotolerans*, *Pichia klyuveri*, *Pichia terricola,* and

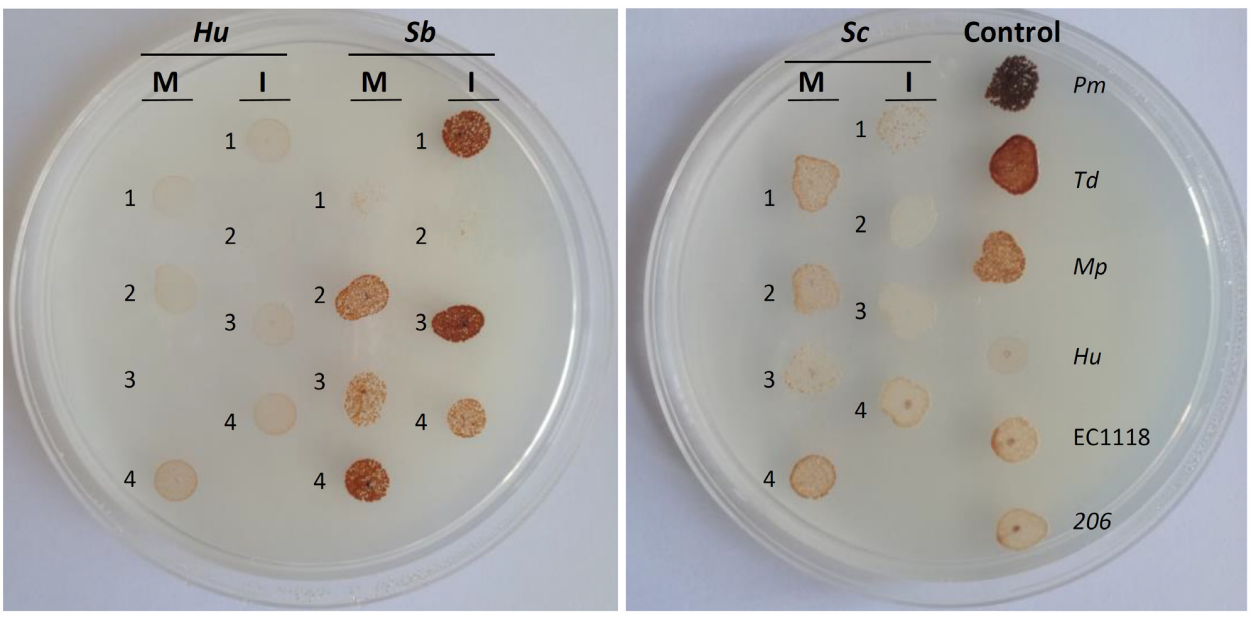

FIGURE 3 | H_2S production. Isolates 1 to 4 from each *H. uvarum* (*Hu*), *S. bacillaris* (*Sb*), and *S. cerevisiae* (*Sc*) (**Table 3**), from the Malbec (*M*) and Isabella (*I*) ecosystems, as well as control strains *P. membranifaciens* (*Pm*), *T. delbrueckii* (*Td*), *M. pulcherrima* (Mp), *H. uvarum* (*Hu*), and *S. cerevisiae* (*EC1118* and *206*), were grown in Biggy medium and H_2S production was scored (see **Table 2**) as indicated in the Material and Methods section.

Starmerella bacillaris. Interestingly, *Torulaspora delbrueckii*, *Hanseniaspora vineae,* and *Metschnikowia pulcherrima*, were not among the yeast species identified in Isabella (**Figure 2**). *Candida californica*, previously recognized in Isabella fermenting must (Raymond Eder et al., 2017), was also isolated from Isabella in this work (i.e., at *t0* and *t24*). *P. occidentalis* and *P. kudriavzevii* were isolated only from fermenting Isabella must (**Figure 2B**) while *P. norvegensis* was isolated only from fermenting Malbec must. *S. cerevisiae,* not isolated at initial stages of spontaneous fermentation, was the predominant yeast species in Isabella must at *t48*, and started to become dominant at the same fermentation time in Malbec must.

H_2S Production by *H. uvarum*, *S. bacillaris,* and *S. cerevisiae* Isolates From the Malbec and Isabella Ecosystems

In order to explore possible phenotypic differences among *S. bacillaris*, *H. uvarum,* and *S. cerevisiae* isolates from the Malbec and Isabella ecosystems, we analyzed the production of H_2S in four randomly selected isolates from each of these species. H_2S production varied greatly among yeast species, as well as between isolates of the same species from the same ecosystem (**Figure 3** and **Table 2**). Interestingly, however, most of the *S. cerevisiae* isolates from Malbec (3 out of 4) showed higher production of H2S than their counterparts isolated from Isabella grape must (**Figure 3**). *S. bacillaris*, on the other hand, was the species with the most consistent production of relatively high levels of H_2S, compared to *H. uvarum* and *S. cerevisiae* (**Figure 3** and **Table 2**).

Ethanol Tolerance of *H. uvarum*, *S. bacillaris,* and *S. cerevisiae* Isolates From the Malbec and Isabella Ecosystems

Tolerance to ethanol of the 24 randomly selected *H. uvarum*, *S. bacillaris,* and *S. cerevisiae* isolates was determined according to their ability to grow in solid media supplemented with different concentrations of ethanol (i.e., 2.5–15.0%). In these studies, *S. bacillaris* isolates from both Malbec and Isabella ecosystems were able to grow only in media containing relatively low levels of ethanol (i.e., 2.5 to 5.0%) (**Table 2**). Most of the *S. cerevisiae* isolates from Isabella (3 out of 4) showed higher ethanol tolerance (i.e., 12.5%) than the four characterized *S. cerevisiae* isolates from Malbec must (i.e., 10.0%). The relatively low tolerance to ethanol of the Malbec *S. cerevisiae* isolates was also observed in *S. cerevisiae* isolates from more advanced

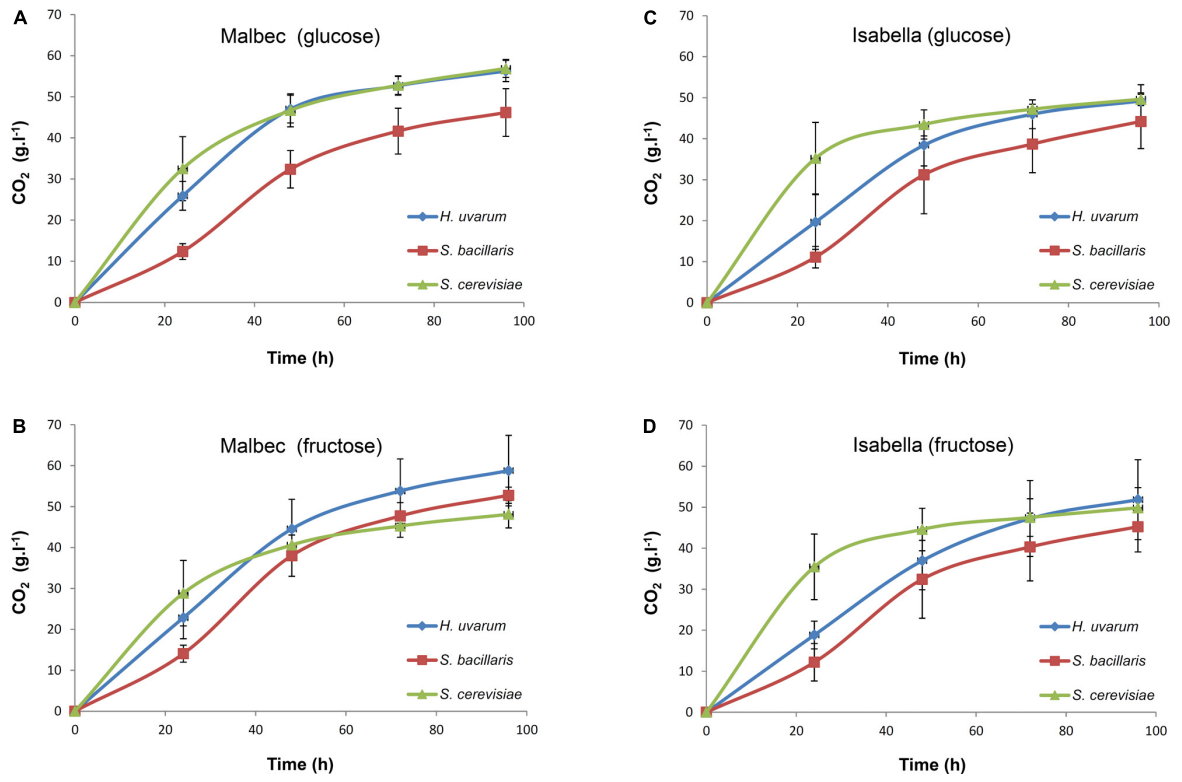

FIGURE 4 | Fermentation profiles of *H. uvarum*, *S. bacillaris,* and *S. cerevisiae* isolated from the Malbec and Isabella ecosystems. Average weight loss (i.e., CO_2 release) of 1.0 ml cultures of the Malbec **(A,B)** and Isabella **(C,D)** *H. uvarum*, *S. bacillaris,* and *S. cerevisiae* isolates, indicated in **Table 2**, grown during 96 h in media containing 10% (w/v) glucose **(A,C)** or 10% (w/v) fructose **(B,D)** as carbon sources. Each point represents the average value (i.e., expressed as $g.l^{-1}.h^{-1}$) of eight independent cultures (i.e., duplicate cultures of the four isolates tested for each species) ± SD.

stages of fermentation of the Malbec must (not shown). Similar results were obtained when ethanol tolerance of the *H. uvarum*, *S. bacillaris,* and *S. cerevisiae* isolates was assayed in liquid media (not shown).

Fermentation Profiles of *H. uvarum*, *S. bacillaris,* and *S. cerevisiae* Isolates From the Malbec and Isabella Ecosystems

A simple microtube assay was designed to explore possible phenotypic differences in glucose versus fructose utilization among the *H. uvarum*, *S. bacillaris,* and *S. cerevisiae* isolates from the Malbec and Isabella ecosystems. Remarkable differences in the fermentation rates between the three analyzed yeast species were observed at initial stages of fermentation (**Figure 4**). Based on this observation, the initial (i.e., 24 h) fermentation rate phenotype was used to compare the *H. uvarum*, *S. bacillaris,* and *S. cerevisiae* isolates. Results from these studies showed a discrete heterogeneity in fermentation rate phenotypes, both in glucose- and fructose-containing media, for the various isolates analyzed (**Figure 5**). *H. uvarum* isolates from Isabella showed slightly higher fermentation ability when grown in YP medium containing fructose versus glucose as the major carbon source (**Figure 5**). Interestingly, the average initial fermentation rate phenotype of *S. bacillaris* isolates from Malbec and Isabella

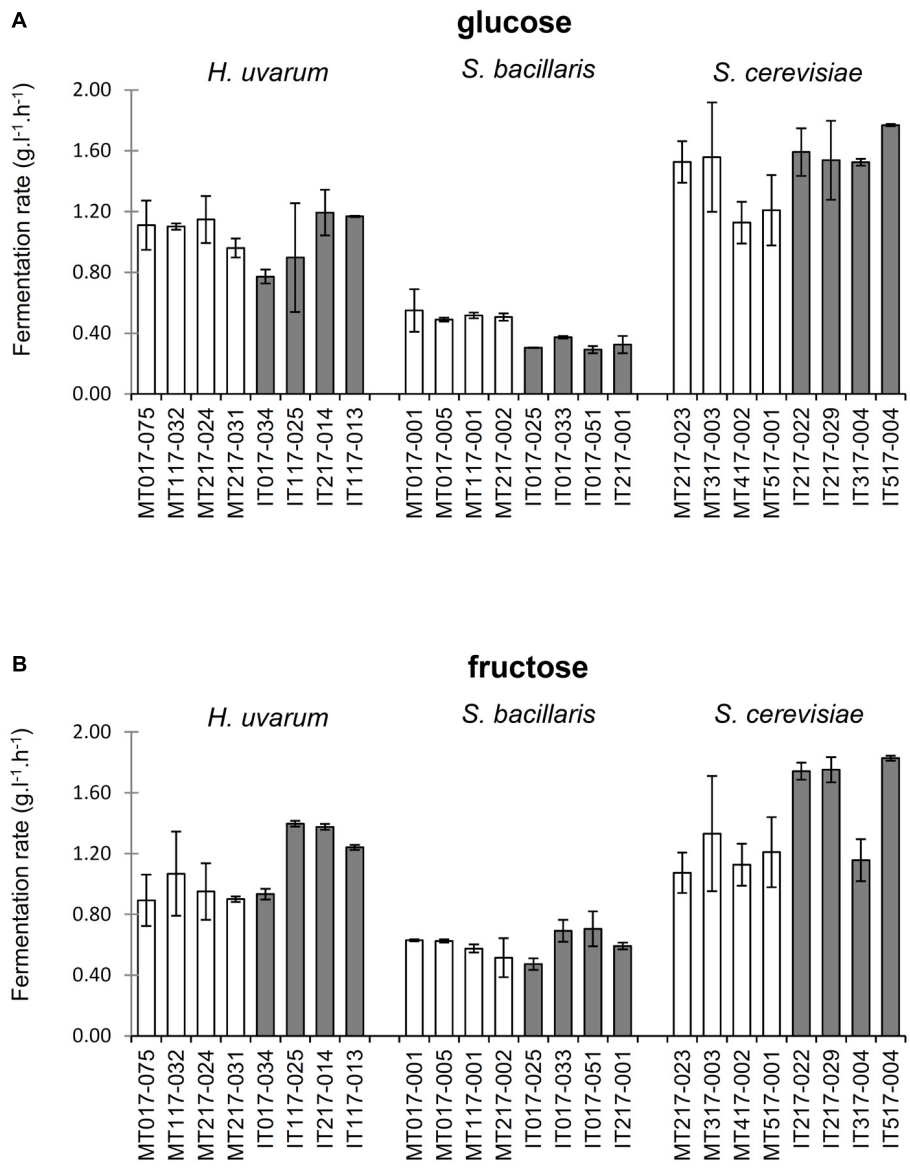

FIGURE 5 | Fermentation rates of *H. uvarum*, *S. bacillaris* and *S. cerevisiae* isolates from Malbec and Isabella ecosystems. Fermentation rate values ($g.l^{-1}.h^{-1}$) were obtained by linear regression of culture weight loss values for the first 24 h of cultures of the indicated isolates in media supplemented with either glucose **(A)** or fructose **(B)**. Experiments were performed in duplicate and bars represent the linear regression error of the 95% confidence band.

TABLE 3 | Genotypes of *S. cerevisiae* isolates from Malbec and Isabella ecosystems.

Isolate[1]	*S. cerevisiae* microsatellite[2]						
	AAT1	AAT2	AAT3	C3	C6	YPL009c	YOR267c
M1	1	1	2	1	1	3	2
M2	1	1	1	1	1	1	1
M3	1	1	2	1	1	2	2
M4	1	1	2	1	1	2	2
I1	4	1	2	1	1	3	2
I2	6	2	ND	1	1	2	ND
I3	5	1	2	1	1	3	3
I4	4	1	4	1	1	2	1

[1]*S. cerevisiae isolates from Malbec (M) and Isabella (I) are: M1 (MT217-023), M2 (MT317-003), M3 (MT417-002), M4 (MT517-001), I1 (IT217-022), I2 (IT217-029), I3 (IT317-004), and I4 (IT517-004) (see* ***Table 2****);* [2]*Microsatellite loci are described in Legras et al. (2005); Numbers identify the different alleles recognized for each microsatellite locus. ND, not determined.*

were ∼1.5- and ∼1.9-fold higher in fructose than in glucose, respectively (**Figure 5**).

Microsatellite Genotyping of *S. cerevisiae* Isolates From Malbec and Isabella Ecosystems

Microsatellite genotyping was used to determine if the *S. cerevisiae* isolates from the Malbec and Isabella ecosystems were genetically related. Results from the analyses of seven highly informative microsatellite loci (Legras et al., 2005) are shown in **Table 3**. Loci C3 and C6 were non discriminant and M3 and M4 isolates could not be differentiated in the analysis. Results from **Table 3** show that Malbec and Isabella fermenting musts harbor a genetically diverse population of *S. cerevisiae* strains.

DISCUSSION

Spontaneously fermenting grape musts constitute rich microbial ecosystems, harboring a remarkable diversity of yeast species. The assembly and evolution of this microbiota, from grape development to the end of must fermentation, is conditioned by the intrinsic biological properties of the grapevine, geographic and climatic conditions at the vineyard, agricultural practices and winemaking procedures (Bokulich et al., 2014; Knight et al., 2015; Jara et al., 2016; Drumonde-Neves et al., 2017).

We have recently proposed that some yeast species may be specifically associated with some *Vitis* species (Raymond Eder et al., 2017). Eventually, different *Vitis* species may harbor specific yeast communities (i.e., yeast species and/or strains of a given yeast species) even in neighboring *Vitis* ecosystems. In this work we explored this hypothesis by studying yeast isolates, from neighboring Malbec (*V. vinifera* L.) and Isabella (*V. labrusca* L.) vineyards, representative of the three major species recognized in Isabella (i.e., *H. uvarum*, *S. bacillaris,* and *S. cerevisiae*) (Raymond Eder et al., 2017). *H. uvarum* was the predominant non-*Saccharomyces* species in the Malbec and Isabella ecosystems, both at early and middle stages of fermentation. In a previous work, we identified *S. bacillaris* as the main yeast species present at early stages of fermentation of Isabella grapes harvested in the same geographic region (i.e., vintage 2015) (Raymond Eder et al., 2017). A similar predominance of either *H. uvarum* or *C. stellata* (reclassified to *S. bacillaris*; Csoma and Sipiczki, 2008; Duarte et al., 2012), in consecutive vintages in the same geographic region, has been reported (Beltran et al., 2002). In addition to *H. uvarum*, a variety of non-*Saccharomyces* species were isolated at early stages of Isabella must fermentation. This diversity quickly decreased between *t0* and *t24* and three main yeast species (i.e., *H. uvarum*, *C. californica,* and *C. hellenica*) were recognized following 1 day of fermentation. In fermenting Malbec grape must, on the other hand, the great diversity of yeast species found at the beginning of fermentation continued at *t24* and *t48*, when *S. cerevisiae* species started to develop.

A total of seventeen different yeast species were isolated from both Malbec and Isabella musts at early stages of fermentation. Although all of these yeast species have previously been described in winemaking environments, their relative contribution to the different neighboring *Vitis* ecosystems analyzed in this work varied. For example, *H. vineae*, *M. pulcherrima,* and *T. delbrueckii*, yeast species commonly found in *V. vinifera* L. grape musts (Jolly et al., 2006), were isolated only from the Malbec ecosystem. The relatively low number of isolates (i.e., 80 isolates from each Malbec and Isabella), however, does not allow to conclude if these yeast species have preferential association with the Malbec versus the Isabella ecosystem. Interestingly, *M. pulcherrima* was not identified in fermenting Isabella must from grapes analyzed in this work nor in grapes harvested from the same vineyards in a previous vintage (Raymond Eder et al., 2017). In addition, *M. pulcherrima* was identified in *V. labrusca* L. grapes from the Azores Archipelago, but only with very low frequency (1.08% of the total isolates) (Drumonde-Neves et al., 2016). On the other hand, the rare yeast species *C. californica*, isolated from Isabella spontaneously fermenting must in the vintage of year 2015 in Colonia Caroya (Raymond Eder et al., 2017), was identified again in the same Isabella ecosystem in this work (i.e., vintage 2017). Moreover, *C. californica* was not found among a total of 150 isolates from the analyzed Malbec ecosystem. Taken together, these observations suggest that *M. pulcherrima* and *C. californica* could have apparent selective and/or preferential association with *V. vinifera* L. and *V. labrusca* L. ecosystems, respectively. However, although *C. azymoides* was originally found associated with fermenting must only from *V. labrusca* L. grapes (Drumonde-Neves et al., 2016; Raymond Eder et al., 2017), this yeast species was also recognized in the Malbec ecosystems studied in this work. Remarkably, *C. azymoides* has not previously been recognized in the extensive worldwide studies performed on the yeast microbiota of *V. vinifera* L. grapes and musts. Therefore, we hypothesize that *C. azymoides* isolates may be limited to some specific *terroirs,* and/or its presence in our Malbec samples may be dependent on the close location of *V. vinifera* L. and *V. labrusca* L. vineyards in Colonia Caroya.

Phenotypic analyses of H_2S production showed a remarkable diversity among the analyzed *S. cerevisiae* isolates from Malbec

and Isabella. Microsatellite genotyping of these *S. cerevisiae* isolates showed that, with the exception of isolates M3 and M4, they correspond to genetically different strains. Interestingly, Isabella's *S. cerevisiae* isolates I2 and I3, which are genetically different, were the lowest producers of H_2S, even when compared with the industrial strain EC1118. Additional characterization of a larger number of *S. cerevisiae* isolates could indicate if medium and low H_2S producer strains are preferentially associated with the Malbec and Isabella ecosystems, respectively. *H. uvarum* and *S. bacillaris* isolates were low and high producers of H_2S, respectively. Although the observed phenotypes suggested genetic heterogeneity among the analyzed *H. uvarum* and *S. bacillaris* isolates, no specific association of the isolates with their Malbec or Isabella ecosystems was observed.

Hanseniaspora uvarum and *S. bacillaris* isolates, from both the Malbec and Isabella ecosystems, showed a relatively low tolerance to ethanol. Although *H. uvarum* and *S. bacillaris* have been found at final stages of spontaneous fermentation of *V. vinifera* L. musts (Combina et al., 2005; Tofalo et al., 2011; Aponte and Blaiotta, 2016; Tristezza et al., 2016), low tolerance to ethanol of *S. bacillaris* from fermenting Isabella grape must has been described (Raymond Eder et al., 2017). On the other hand, *S. cerevisiae* isolates from the Malbec or Isabella musts showed some mild differences in tolerance to ethanol. Ethanol tolerance of the Malbec *S. cerevisiae* isolates was similar among isolates obtained at either medium or advanced stages of fermentation (not shown). Interestingly, ethanol yield in completely fermented Isabella grape must was lower than expected. This phenomenon, which is not observed for Malbec or other *V. vinifera* L. grape musts from Colonia Caroya (Córdoba, Argentina), is typically observed in spontaneously fermented Isabella grape musts from this geographic region, regardless of the vintage (Raymond Eder et al., 2017).

Additional evidence on the phenotypic diversity of yeast species isolated from the Malbec and Isabella ecosystems was obtained from the analysis of their fermentation profiles in media containing either glucose or fructose as the main carbon source. Interestingly, some *S. cerevisiae* isolates appear to have a slightly higher fermentation rate in fructose than in glucose media, which was unexpected given the glucophilic character of this yeast species. Also interestingly, *S. bacillaris* isolates from the Malbec ecosystem showed higher fermentation rates in media containing glucose than *S. bacillaris* isolates from Isabella.

Finally, our results show a remarkable biodiversity among main yeasts isolated from two different neighboring *Vitis* ecosystems and provide preliminary evidence on the potential specific association between *Vitis* species and yeast species and strains. The dynamics of specific yeast populations during spontaneous fermentation could translate into specific organoleptic and sensory characteristics of the final wines, dependent on each *Vitis* species. As shown in this work, Isabella and/or other non-conventional *Vitis* ecosystems may harbor yeast species and/or strains with unique metabolic properties which may not be present in *V. vinifera* L. Thus, non-*vinifera* ecosystems may offer an opportunity to look for valuable *Saccharomyces* and non-*Saccharomyces* strains of potential relevance for the winemaking industry.

AUTHOR CONTRIBUTIONS

MR, FC, and AR made fundamental contributions to the conception and design of the work, contributed to the acquisition, analysis, and interpretation of data, and drafted the work and revised it critically for intellectual content. 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.

FUNDING

This work was supported by PICT 2014-3113 from the Fondo para la Investigación Científica y Tecnológica (FONCYT, Argentina) and a grant from the Catholic University of Córdoba. MR and FC are supported by fellowships from the Argentina National Research Council (CONICET) and FONCYT, respectively. AR is a Principal Investigator of CONICET.

ACKNOWLEDGMENTS

The authors wish to thank C. Reynoso and S. C. Lauret (Bodega La Caroyense, Colonia Caroya) for conducting the fermentations and J. Heywood for English editing.

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

frontiers in Microbiology

ORIGINAL RESEARCH
published: 02 February 2018
doi: 10.3389/fmicb.2018.00059

Impact of Chemical and Biological Fungicides Applied to Grapevine on Grape Biofilm, Must, and Wine Microbial Diversity

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This study was aimed to measure the impact of the application of a bio-fungicide against *Botrytis cinerea* on the microbiota involved in the alcoholic fermentation (AF) of Tempranillo Rioja wines. For this purpose, a bio-fungicide composed of the biological control bacterium *Bacillus subtilis* QST713 was applied to the vineyard. The microbial diversity was analyzed from grape biofilm to wine. Impact on microbial diversity was measured employing indexes assessed with the software PAST 3.10 P.D. Results were compared to non-treated samples and to samples treated with a chemical fungicide mainly composed by fenhexamid. Overall, the impact of the biological-fungicide (bio-fungicide) on the microbial diversity assessed for grape biofilm and for musts was not remarkable. Neither of the tested fungicides enhanced the growth of any species or acted against the development of any microbial groups. The bio-fungicide had no significant impact on the wine microbiota whereas the chemical fungicide caused a reduction of microbial community richness and diversity. Although environmental threats might generate a detriment of the microbial species richness, in this study the tested bio-fungicide did not modify the structure of the microbial community. Indeed, some of the *Bacillus* applied at the grape surface, were detected at the end of the AF showing its resilience to the harsh environment of the winemaking; in contrast, its impact on wine quality during aging is yet unknown.

Keywords: *Botrytis cinerea*, grape biofilm, must, wine, microbiota, biofungicide, diversity, species richness

OPEN ACCESS

Edited by:
Aline Lonvaud,
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Reviewed by:
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Universitat Rovira i Virgili, Spain

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

Received: *10 October 2017*
Accepted: *10 January 2018*
Published: *02 February 2018*

Citation:
Escribano-Viana R, López-Alfaro I, López R, Santamaría P, Gutiérrez AR and González-Arenzana L (2018) Impact of Chemical and Biological Fungicides Applied to Grapevine on Grape Biofilm, Must, and Wine Microbial Diversity. Front. Microbiol. 9:59. doi: 10.3389/fmicb.2018.00059

INTRODUCTION

Traditionally referred to as the "gray mold", *Botrytis cinerea* is a necrotrophic pathogen able to rot grapes that negatively affects must and wine organoleptic quality (Cantoral et al., 2011).

Most grape growers have been fighting against this mould using chemical fungicides. Viticulture is one of the most pesticide consuming crops in spite of its low production rates, what is linked to the sudden resistance that some mould develop to chemical fungicides (Provost and Pedneault, 2016). This situation has encouraged the almost urgent seeking of alternative control tools (Garrido et al., 2017).

Chemical fungicides based on copper molecules have been reported as a drawback for the maintenance of the ecological balance of ecosystems. Consequently, some limits in their use

on crops have been established (Provost and Pedneault, 2016). For this reason, copper free chemical fungicides, which are thought to be harmless to the environment, are currently being commercialized. On another note, consumers are demanding organic products manufactured without chemicals and preservatives (D'Amico et al., 2016). This trend has been extended to oenological industry, what has meant an advance in researching new bio-products to be employed in grapevines as a biocontrol strategy.

As a result, some industries have struggled to apply biological fungicides (bio-fungicides) to the grapevine. This type of commercial products has been inspired in the biological control carried out on other crops affected by similar diseases. The application in the vineyard of some yeasts such as *Candida sake* (Garrido et al., 2017) or of some bacteria such as *Bacillus subtilis* strains (Pertot et al., 2017) has been aimed to reduce some grapevine diseases. Some of these bio-fungicides mean a great advantage compared to chemical products because they can be applied in the grapevine from full bloom to only two or three days before being harvested. Indeed, this organic viticulture tries to reduce the employment of pesticides without altering the production and yields of grapevine.

Oenology is featured for being one of the few food industries in which the raw material and even the elaboration process are not under sterile conditions. For a start, the grapevine ecosystem is the first one involved in the winemaking. In effect, the grapes before the harvest held plenty of microorganisms belonging to yeast and bacteria microbial groups. These yeasts are usually S*accharomyces* and non-*Saccharomyces* genera whereas acetic acid bacteria (AAB), lactic acid bacteria (LAB), and environmental bacteria (EB) represent the bacterial group. Once grapes have been harvested and introduced in the winery, the microbial community established on the biofilm of the grape surface is blended with the own winery microbiota that persists from one vintage to the next one. When grapes are manufactured, usually including destemming and crushing, the microbial community strikes a balance and then the alcoholic fermentation (AF) begins. *Saccharomyces* yeasts that change the must into wine develop this fermentative stage. After AF, the malolactic fermentation could take place. This stage is based on the biological deacidification of malic acid into lactic acid and it is mainly carried out by the LAB *Oenococcus oeni*. Regarding the mentioned above, the winemaking could be one of the most complex microbiological transformation in the food industry.

Furthermore, recent published results have reported the presence of microorganisms during the whole winemaking that are not usually involved in oenological process. Some of these microorganisms have not been deeply analyzed in wines; for instance, some AAB that were thought to be in the early stages of winemaking have been detected in middle AF (Portillo et al., 2016) or some EB genera from open environments that have been also detected in must even after being sulphited (González-Arenzana et al., 2017a).

Overall, microbial ecology studies in wines are usually based on Polymerase Chain Reaction (PCR) of the DNA extracted from colonies (culture-dependent method) and the DNA extracted from Denaturing Gradient Gel Electrophoresis (DGGE) bands, followed by sequencing of amplicons for their later identification. Moreover, the combination of both approaches has been demonstrated to be interesting to tackle ecological studies (González-Arenzana et al., 2017a,b).

As far as it is concerned, this is the first study of the microbial diversity during the whole winemaking in relation to fungicide application in the grapevine. For this reason, this study was aimed to know if the ecological balance of the microbial communities of grape biofilms, musts and wines were altered by the application of a biological fungicide mainly composed by the *Bacillus subtilis* QST713. In order to achieve a general and real approach, results were compared with the impact of a chemical fungicide, based on fenhexamid molecule. Both fungicides were prescribed against the gray mold *Botrytis cinerea.*

MATERIALS AND METHODS

Grapevine Treatments and Sampling

The project was carried out in a *Vitis vinifera* L. cv. Tempranillo vineyard located in the Rioja qualified Designation of Origin (D.O.C. Rioja). The vineyard was managed under conventional soil tillage with approximately 3530 plants per 100 square meters (Ha). An experimental design with randomly established blocks of four replicates per treatment was performed (12 replicates) in the vineyard. Replicates of the same sample were located in the same row; samples were contiguous and separated by an average distance of 2.7 m. Each replicate received the same agronomic management previously to the treatments. The vineyard had not symptoms of being affected by *Botrytis cinerea* at the beginning of the study.

Three treatments were performed in the same vineyard in order to avoid biases caused by the climatic or the agronomic conditions. Treatments were applied with an automatic knapsack sprayer. One was referred to as "C" (control) because no fungicide was applied. Other treatment was applied with a dose of 4 kg/Ha twice, 21 days and 3 days before harvest was referred to as "Bio". This later was based on the application of a wet powder product that was a biological fungicide with 5.3×10^{10} colony forming units (CFU) per millilitre (mL) of the *Bacillus subtilis* strain QST 713 (Serenade® Max, Bayer Crop Bioscience S.L.) The other treatment referred to as "Chem", consisted of the application of a traditional chemical fungicide product based on fenhexamid chemical compound (Teldor®, Bayer Crop Bioscience S.L.) 21 days before harvest (1.7 kg/Ha). The average number of plants per replicate was 25. Control of ripening was performed from veraison stage to the optimal date for harvest. Each replicate was separately harvested and vinified.

Sampling was distributed in three moments. Firstly, the microbiota of grape surfaces was sampled in the vineyard one day before harvest. At this initial stage, 500 g of grapes of each replicate and treatment were randomly selected in the vineyard and incubated in 500 mL of sterile isotonic solution (PT: 0.1% soy peptone and 0.01% Tween 80) (Prakitchaiwattana et al., 2004) in an orbital shaking (80 rpm) for 2 h at room temperature. After

shaking, the biomass was recovered by centrifugation (30 min, 10,000 × *g*, 4°C) and the pellet was then suspended in 15 mL of PT. Oenological parameters of the must such as probable alcohol, pH, and total acidity were analyzed according to ECC official methods (European Community, 1990). Moreover, gluconic acid was determined by an automated enzymatic method (Miura One, TDI, Spain).

The second sampling stage was performed in the experimental winery 48 h after filling tanks of 100 L with crushed, destemmed and sulphited grapes (50 mg/L SO_2). So, that 15 mL of each must was sampled. The AF was spontaneous and the decreasing density was daily controlled (data no shown). When AF was completed, the third sampling took place and 15 mL of each replicate wine were sampled.

Culture Dependent Identification by Ribosomal DNA Sequencing

The viable and cultivable (VC) microbial community of each replicate and treatment was analyzed. For this purpose, several dilutions of the initial samples of PT, must and wines were spread on different culture media plates. The VC yeast community was quantified employing two culture media, GYP for total yeast community incubated at 28°C during 48 h (González-Arenzana et al., 2017a) and DBDM for *Dekkera/Brettanomyces* detection incubated at 25°C for 2 weeks in anaerobic conditions (Gas Pak System, Oxoid Ltd., Basingstoke, England) (Rodrigues et al., 2001; Guzzon et al., 2011). The VC bacteria community was quantified employing two culture media; MRS (De Man et al., 1960) for total LAB, incubated at 28°C for 48 h in anaerobic conditions and Mann (González-Arenzana et al., 2017a), for AAB and EB, at 25°C for 48 h. As soon as had the incubation period finished, the cells growing (CFU/mL) on the different culture media were counted and expressed in logarithmic units (log).

Plates with VC microbial communities between 1 and 2 log units were reserved for randomly isolation of 10 colonies. Genera and species identification of VC microbial community was carried out by ribosomal DNA sequencing. In case of yeasts, partial 26S rRNA genes were amplified using the primers NL1 and NL4 (Cocolin, 2000); for LAB species identification, the PCR was performed with primer pairs WLAB1 and WLAB2 targeted the V4 and V5 16S rDNA regions as López et al. (2003) described. Finally, the AAB and the EB species identification was performed by amplification of the V1 to V6 region of 16S rDNA gene with 8F and 907R primers (Posada et al., 2016). Macrogen Inc. (Seoul, South Korea) sequenced the PCR amplicons. Then, sequences were compared to GenBank nucleotide database using the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990). The identification was considered correct when gene sequences showed identities of at least 98%.

Culture Independent Identification by PCR-DGGE

The biomass obtained from grapes in PT, from musts and from wines was frozen at –80°C in a volume of 10 mL. After this, the DNA was directly extracted from these samples following the protocol described by González-Arenzana et al. (2013).

The DNA extracted from samples was amplified by different PCRs that were run in an Applied Biosystem, GeneAmp® PCR System 2700 thermocycler in a final volume of 50 μL with 2 μL of the extracted DNA (approximately 10 ng) as described González-Arenzana et al. (2017a). For yeasts and moulds, the D1 region of the 26S rRNA gene was amplified using the primers $NL1^{GC}$ and LS2 (Cocolin, 2000). For bacteria, the V7 to V8 region of 16S rDNA gene was amplified with WBAC1 and $WBAC2^{GC}$ primers and with WLAB1 and $WLAB2^{GC}$ (López et al., 2003). The PCR reactions were performed following the indications González-Arenzana et al. (2013). An aliquot (5 μL) of the amplified DNA was analyzed by 1% agarose gel electrophoresis to verify that the PCR worked prior to DGGE.

The DGGE was carried out to separate the respective amplicons with the D-CODE^{TM} universal mutation detection system (Bio-Rad, Hercules, CA, United States). PCR products were run on 8% (wt/vol) polyacrylamide gels in a TAE buffer (2 M Tris, 1 M glacial acetic acid and 50 mM EDTA pH 8) at a constant temperature of 60°C. The urea-formamide content ranged from 35 to 60% for $NL1^{GC}$-LS2 amplicons, and from 35 to 55% for WLAB1-$WLAB2^{GC}$ amplicons and from 35 to 65% for WBAC1-$WBAC2^{GC}$. An initial stage of electrophoresis was performed (10 min at 20 V) and after this, the electrophoresis products run for 18 h with a voltage of 80 V. Then, gels were stained in ethidium bromide and visualized with UV *trans*-illumination (GelDoc, Bio-Rad). Blocks of the polyacrylamide gels with the selected DGGE bands were excised and incubated overnight in 20 μL of sterile, pure water at 4°C to make DNA bands diffuse to the liquid. One microliter of this elution was re-amplified using the PCR conditions described above with primers without the GC clamp. Macrogen Inc. (Seoul, South Korea) purified and sequenced by the PCR amplicons. Sequences were compared to the GenBank nucleotide database with BLAST. The identification was considered correct when gene sequences showed identities of at least 98%.

Measurement of Diversity and Structure Community and Statistical Analysis

Alpha diversity parameters were assessed by the software PAST 3.10 P.D. (Ryan et al., 1995) analyzing the detected species in each of the replicates ($n = 4$) of the three treatment. For each replicate, the average number of detected species (S) and the Margalef index that supposes a functional relation between the number of species and the total number of individuals (Margalef, 1958) were calculated for describing the richness of species of each sample. On another note, the structure of the studied microbial communities was determined by dominance indexes such as Simpson and Berger–Parker, and finally by Shannon–Wiener equity index and by the non-parametric index Chao1. The Simpson index measures the possibility that two randomly chosen individuals belong to the same species (Fedor and Spellerberg, 2013). Opposite in meaning, the Berger–Parker index measures the

dominance in individuals of the dominant taxon (Harper, 1999). Finally, the entropy of the community was measured by the Shannon–Wiener index (H) that takes into account the number of individuals as well as the number of species (Karydis and Tsirtsis, 1996; Death, 2008). The Chao1 is an estimator of the species number based on the odd species (Portillo et al., 2016).

Data of counts and diversity indexes of each replicate ($n = 4$) were processed using the variance analysis (ANOVA) with the Tukey tests (at $p \leq 0.01$) using the software IBM SPSS Statistic 20.0 (Chicago, United States). Hierarchical cluster with all the information of diversity indexes regarding each sample was constructed with the same software.

RESULTS

Initial oenological parameters of grapes from control, bio-fungicide, and chemical fungicide application were quite similar between samples. In effect, the must from control grapes had a probable alcohol degree (% v/v) of 13.5, a pH of 3.38 and a total acidity (g/L tartaric acid) of 6.45. Must from bio-fungicide application had a probable alcohol degree of 13.0% v/v, a pH 3.33, and a total acidity of 6.41 g/L. Eventually, must from chemical fungicide application had a probable alcohol degree of 13.2% v/v, a pH 3.37, and a total acidity of 6.41 g/L. Moreover, gluconic acid was not detected in the three grape samples.

Viable and Cultivable Microbial Community after Fungicide Applications

The culture media employed in this study were selected to make possible the quantification of species usually involved in the vinification process. Therefore, the GYP was employed for quantifying total yeasts, DBDM for *Brettanomyces/Dekkera*, MRS for LAB, and Mann for AAB and EB.

In **Figure 1**, the average VC community (log CFU/mL) found with the different culture media and at the different stages is shown. The VC microbial community of grape biofilm was determined with the culture media GYP, MRS, and Mann. The yeasts growing on GYP plates varied from 0.6 to 1.3 log units without statistical significance. Regarding the VC bacteria growing on MRS plates from grape biofilm, significant differences were established between control sample (1.8 log units) and both fungicide samples, having the bio-fungicide sample the highest VC community (4.3 log units) and the chemical product the lowest one (1.1 log units). The VC bacteria growing on Mann plates from grape biofilms was similar between samples (from 0.5 to 0.8 log units) so that significant differences were not determined.

Data linked to the average VC community (log CFU/mL) at must sampling are also shown in **Figure 1**. In this case, all culture media employed hold colonies growing. The VC yeasts of GYP plates were in the range of 4.5 and 4.9 log units and differences were not significant. Regarding the VC community growing on DBDM plates with the must from grapes treated with the chemical fungicide was significantly lower (1.7 log units) than the other two samples (3 log units). The VC bacteria on MRS plates was not significantly different between samples (from 4.1 to 3.5 log units) although on Mann plates with the control samples was significantly lower (3.8 log unit) than the determined in the other samples (4.4 and 4.6 log units).

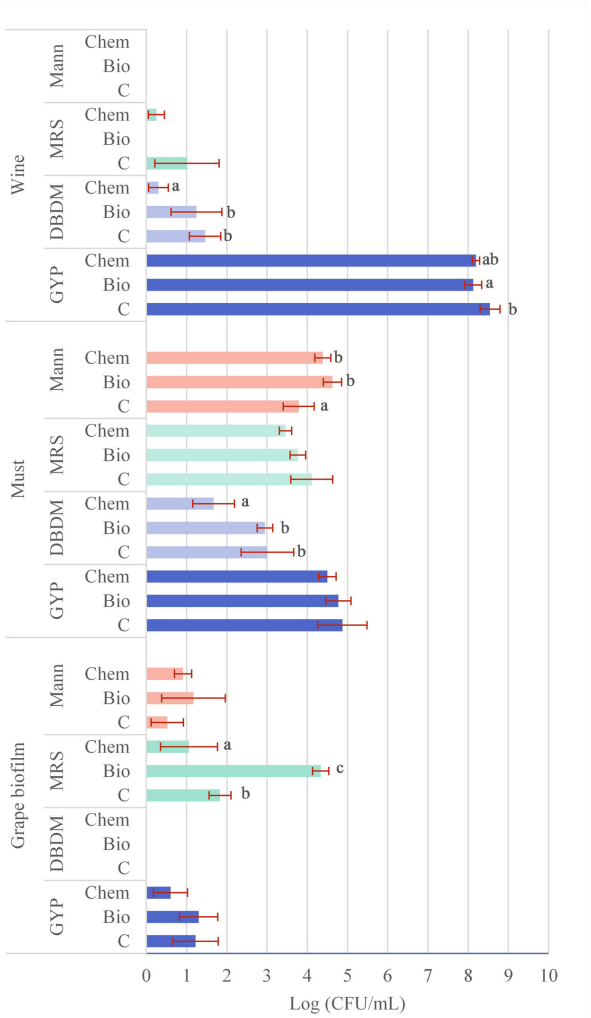

FIGURE 1 | Average viable and cultivable (VC) microbial community in log units (CFU/mL) counted on plates of different samples (C: control, Bio: bio-fungicide, and Chem: chemical fungicide) at different stages (1: grape biofilm; 2: must, 3: wine) and from different culture media (GYP, DBDM, MRS, and Mann), the statistical analysis (different letters mean significant differences $p < 0.05$ between treatments) and error bars. Empty bars mean no colonies detected.

Regarding the VC community (log CFU/mL) quantified at wine sampling (**Figure 1**) results were obtained from GYP, DBDM, and MRS plates. Yeasts growing on GYP plates ranged from 8.2 corresponding to wine from the bio-fungicide application, to 8.5 log units for control samples but without significant differences. Regarding the VC yeasts growing on DBDM plates, VC microbial community from chemical treatment was significant lower (0.3 log units) than in the other two samples (1.2–1.5 log units). Bacteria VC community on MRS plates was not significantly different between samples, being 1 log

unit in control samples and 0.3 log unit in wines from chemical treated grapes and null for bio-fungicide treatment.

Species Composing the Microbial Community

Grape Biofilm

In **Figure 2**, data about species found by culture dependent and independent methods in samples of grape biofilms are shown. On grape biofilm control sample, six yeasts – *Aureobasidium* (*A.*) *pullulans*, *Hanseniaspora* (*H.*) *osmophila*, *Lachancea* (*Lch.*) *thermotolerans*, *Rhodotorula* (*Rh.*) babjevae, *Rh. nothofagi* and *Saccharomyces (S.) cerevisiae*- and six EB -*Bacillus* (*B.*) *amyloliquefaciens*, *B. methylotropicus*, *B. subtilis*, *B. velezensis*, *Enterococcus* (*E.*) *silesiacus*, and *Pantoea sp.*- were identified. The species *B. amyloliquefaciens* and *B. subtilis* were detected with PCR-DGGE and ten species were isolated from GYP, MRS, and Mann plates.

After the application of the bio-fungicide, seven yeasts, one LAB and five EB were detected. Five of them were different regarding grape biofilm control sample: *Hypopichia (Hy.) pseudoburtonii*, *Rh. glutinis*, *Lactococcus (Lc.) lactis, B. axarquiensis, and Pseudomonas (Ps.) rizospherae*-. The species *Lc. lactis, B. axarquiensis*, and *B. subtilis* were detected with PCR- DGGE (**Supplementary Figure S1**) while ten species were isolated from GYP, MRS, and Mann plates.

The species of microorganisms after the application of the chemical fungicide were six yeasts, one LAB and eight EB. Eight of them were different to grape biofilm control sample -*Botrytis* (*Bo.*) *cinerea*, *Pichia* (*Pi.*) *sporocuriosa*, *Trigonopsis* (*Tr.*) *cantarellii*, *Lc. lactis*, *Acinetobacter* (*Ac.*) *bereziniae*, *Pantoea* (*P.*) *dispersa, Ps. putida*, *and Staphylococcus* (*St.*) *capitis*-. The species *Bo. cinerea*, *Lc. lactis, Ac. bereziniae*, *B. amyloliquefaciens*, and *B. subtilis* species were detected with PCR-DGGE (**Supplementary Figure S3**) while ten species were isolated from GYP, Mann, and MRS culture media.

Must

In **Figure 3**, data about species found by culture dependent and independent methods in must samples are shown. In must control sample, five yeasts -*H. osmophila*, *H. uvarum*, *S. cerevisiae*, *S. paradoxus* and *Tr. cantarellii*-, six AAB -*Acetobacter* (*Ace.*) *musti, Gluconobacter* (*G.*) *albidus, G. cerinus, G. japonicus, G. oxydans* and *Kokazia* (*K.*) *baliensis*- and two EB – *St. capitis* and *Tatumella* (*Ta.*) *ptyseos*- were identified. In general, the yeast species detected with PCR-DGGE were also isolated from GYP and DBDM plates while bacteria were isolated from MRS and Mann plates.

The species of musts proceeding from grapes treated with the bio-fungicide were seven yeasts, three AAB and two EB and among them four species were different regarding must control sample -*A. pullulans*, *Lch. thermotolerans, Monilinia*

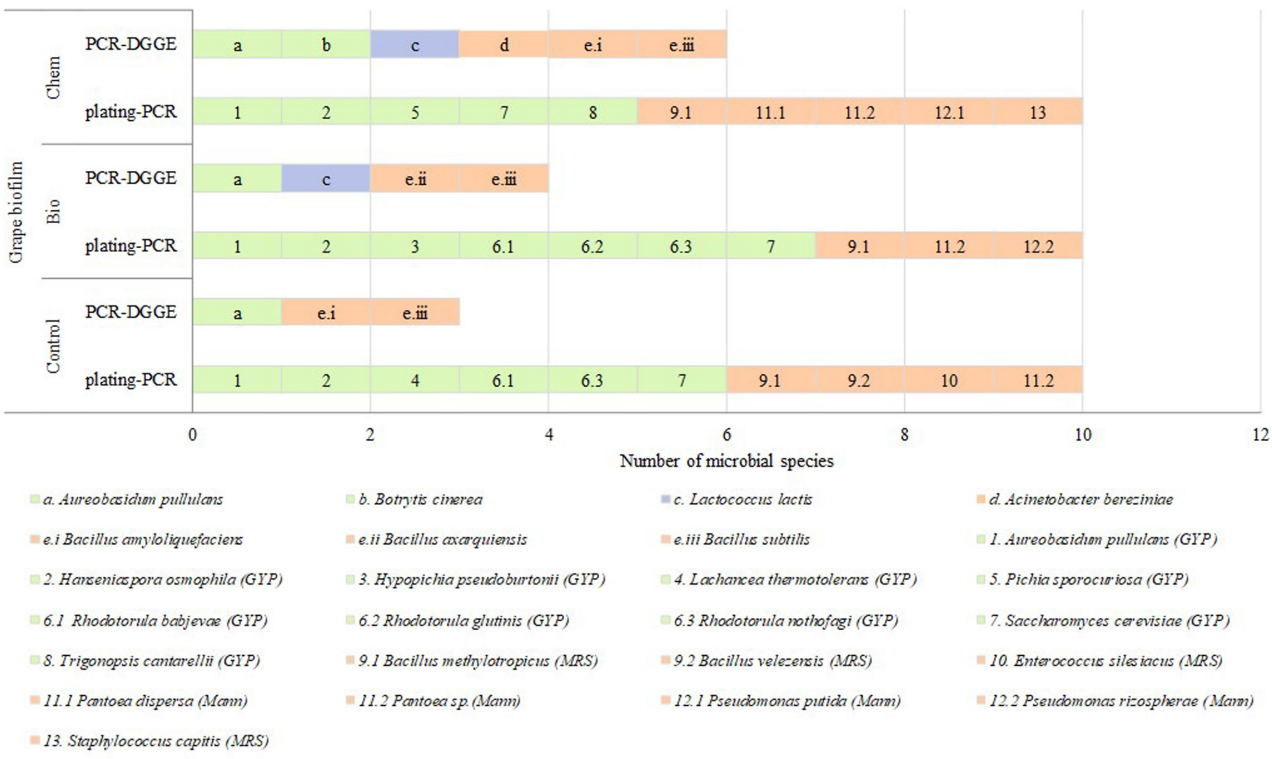

FIGURE 2 | Microbial species detected on grape biofilm samples (C: control, Bio: bio-fungicide, and Chem: chemical fungicide) from GYP, MRS, and Mann plates referred to as letters and by PCR-DGGE referred to as numbers. Green slices identified eukaryotic species; purple slices were LAB; and orange slices EB.

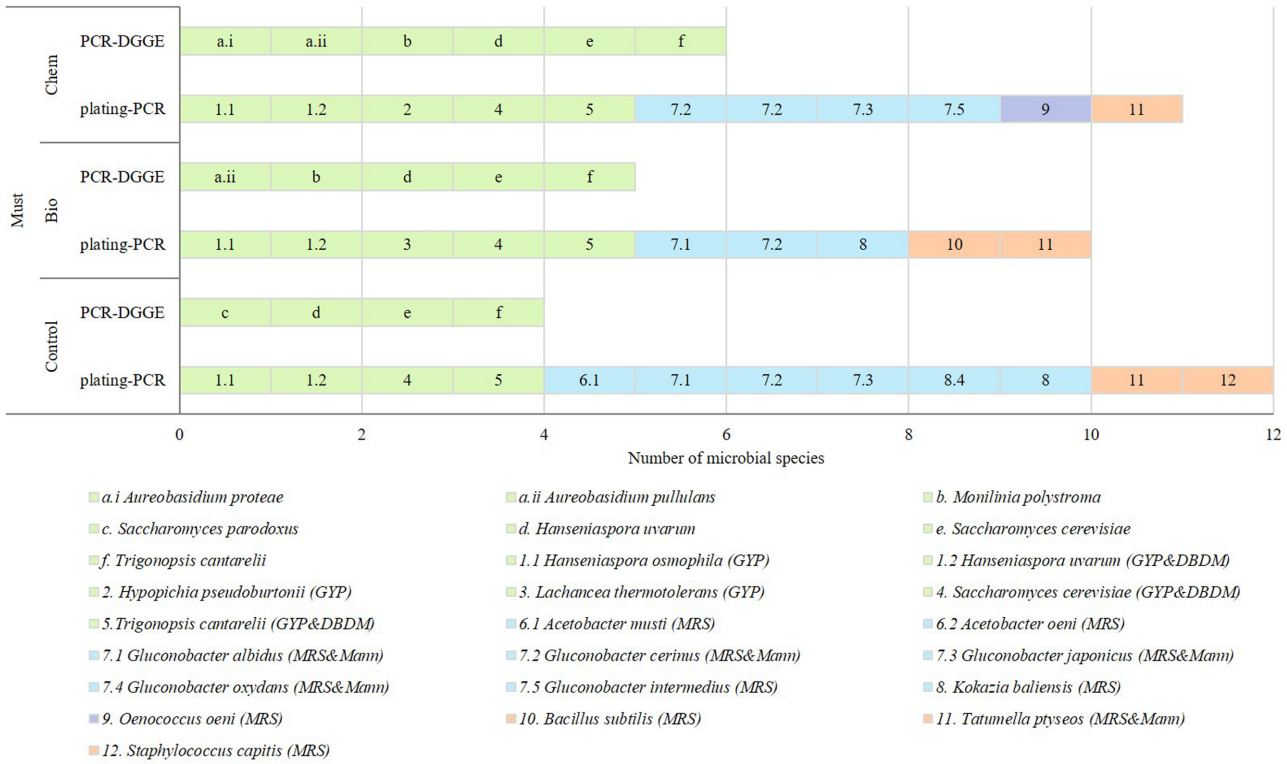

FIGURE 3 | Microbial species detected in must samples (C: control, Bio: bio-fungicide, and Chem: chemical fungicide) from GYP, DBDM, MRS and Mann plates referred to as letters and by PCR-DGGE referred to as numbers. Green slices identified eukaryotic species; blue slices were AAB; purple slices were LAB; and orange slices EB.

*(*Mo.*) polystroma*, and *B. subtilis*-. Most of the yeast species were identified with PCR-DGGE and isolated from GYP and DBDM plates. The bacteria were isolated from MRS and Mann plates.

Musts of grapes treated with the chemical fungicide contained eight yeasts, four AAB, one LAB, and one EB; seven species were different to those of must control sample -*A. proteae*, *A. pullulans*, *Hy. pseudoburtonii, Mo. polystroma*, *Ace. oeni*, *Gl. Intermedius*, and *Oenococcus* (*O.*) *oeni*-. Some of the detected yeasts proceeded from DGGE gels and they were isolated from GYP and DBDM culture media while all the bacteria were isolated from MRS and Mann plates.

Wine

In **Figure 4**, species found by culture dependent and independent methods in wine samples are shown. Nine species were identified in wine control sample, being four yeasts –*H. uvarum*, *S. cerevisiae, S. paradoxus*, and *Tr. cantarellii*-, two AAB –*Gluconoacetobacter* (*Ga.*) *saccharivorans* and *G. albidus*-, one LAB -*Lc. lactis*- and two EB -*Methylobacterium* (*Me.*) *extorquens* and *Ps. putida*-. All the species were found with PCR-DGGE (**Supplementary Figures S1–S3**) and two yeasts were isolated from GYP and DBDM plates.

The wine proceeding from grapes treated with the bio-fungicide had five yeast, one LAB and four EB species, seven out of them were common with the found in wine control samples while three were different -*Torulaspora* (*To.*) *delbrueckii*, *B. amyloliquefaciens,* and *B. subtilis*-. All the species were found with PCR-DGGE and two yeasts were isolated from GYP and DBDM plates.

The wine from grapes treated with the chemical fungicide contained three yeast, one LAB and four EB species, six species appeared either in wine control samples while *B. subtilis* and *Me. extorquens* were different. The bacteria and two of the yeast were detected with PCR-DGGE and four yeasts were also isolated from GYP and DBDM plates.

Microbial Alpha Diversity of the Samples

The average diversity indexes assessed with the data of species identified in each replicate for describing the alpha diversity of the samples are shown in **Table 1**. Significant differences were not established between the diversity indexes of grape biofilm samples. The Margalef index ranged from 2.32 to 3.22, the Simpson index from 0.77 to 0.86, the Berger–Parker index from 0.14 to 0.23, the Shannon–Wiener index (*H*) from 1.50 to 2.00 and the Chao-1 from 13 to 33.

In the diversity indexes of must samples, significant differences were not observed, being results the same with the exception of the Margalef index from 3.07 to 3.08 and the Shannon–Wiener index (*H*) from 1.93 to 1.94.

Diversity indexes at wine stage were in some cases significantly different between treatments. The wine control sample had

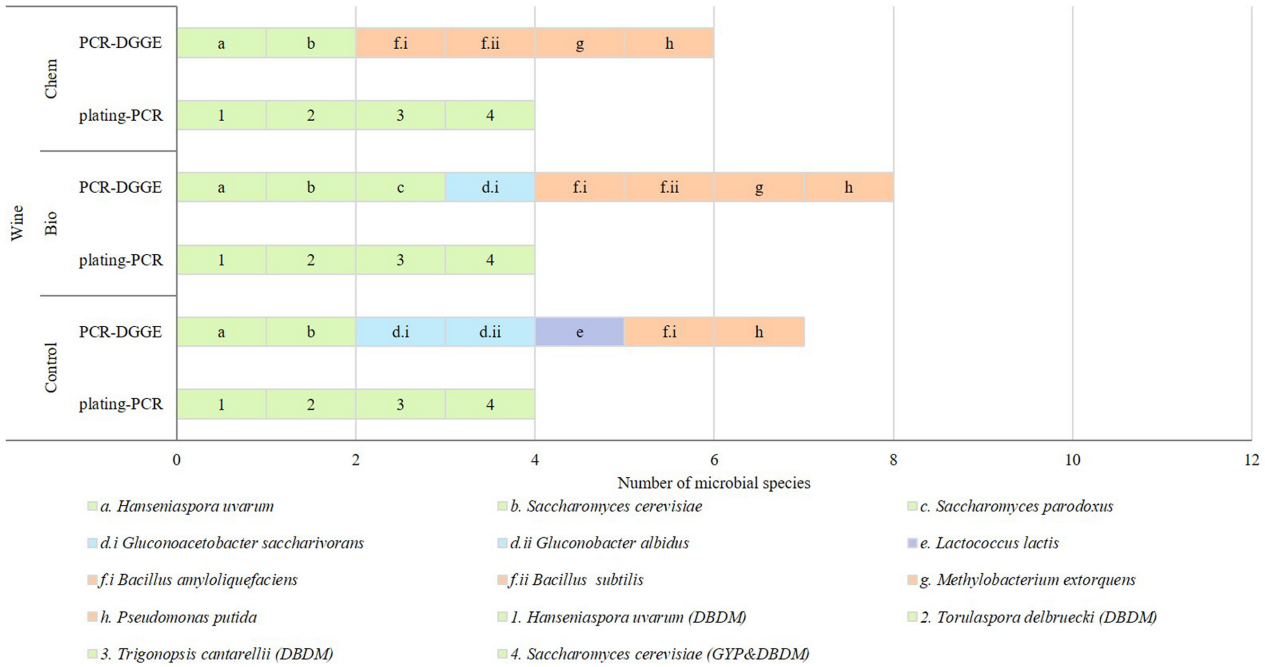

FIGURE 4 | Microbial species detected in wine samples (C: control, Bio: bio-fungicide, and Chem: chemical fungicide) from GYP and DBDM plates referred to as letters and by PCR-DGGE referred to as numbers. Green slices identified eukaryotic species; blue slices were AAB; purple slices were LAB; and orange slices EB.

similar indexes regarding the species richness and the structure of microbial community of the wine from the bio-fungicide application. The Margalef index, the Simpson index, the Shannon–Wiener index (*H*), and the Chao-1 were significantly lower in wine from grapes treated with the chemical fungicide than the determined for the other samples. The Berger–Parker index of wine from grapes treated with the chemical fungicide was significantly higher than the assessed for the other samples.

Hierarchical clusters built with the diversity indexes of samples at each stage are shown in **Figure 5**. With this statistical analysis, it was observed that at grape biofilm stage the samples from fungicide treatments were clustered together. At the other two sampling moments, control samples were clustered together with samples proceeding from grapes treated with the biological fungicide whereas samples from grapes treated with the chemical fungicide stayed separately.

Alcoholic Fermentation

The kinetics of spontaneous AF of all samples were studied by the determination of the daily density and they lasted 13 days. No differences were observed in samples of wines proceeding from grapes treated with fungicides application compared to the control (data no shown).

DISCUSSION

The current research was aimed to determine the impacts of the application of a bio-fungicide on the microbial community of the winemaking.

The bacterium *Bacillus subtilis* is one of the most interesting biological control agent against *B. cinerea*, it might be a tool for developing a more eco-friendly and sustainable viticulture. In effect, it has been described as a natural source of bioactive molecules against several mould diseases of the plants. Furthermore, it has the ability to generate spores, what makes this genus even more adequate to be applied in open and harsh environments having a long shelf-life being even applied along with chemical fungicides (Ongena and Jacques, 2008; Pertot et al., 2017; Reiss and Jørgensen, 2017). Precisely, Reiss and Jørgensen (2017) have recently reported the activity of the strain QST713 of the species *Bacillus subtilis* against the fungi *Puccinia striiformis* in preventive and curative way. This strain QST713 is the most important agent in abundance in the tested bio-fungicide (Serenade® Max, Bayer Crop Bioscience S.L).

Regarding fenhexamid application some authors have described slow AFs probably because an impact on *S. cerevisiae* (Bizaj et al., 2014). However, other authors have not observed this effect (Cabras et al., 2004) and so far, no results have been published on the impact of fungicides on malolactic fermentation or on *O. oeni*.

The current study deals with the effect of *B. subtilis* strain QST713 on the microbiota at three stages of the vinification, grape biofilm, musts and wines. Results were compared to the impact of a traditionally employed chemical fungicide and to a non-treated control sample. Actually, no published research has dealt with this issue despite it could cause a significant impact on the winemaking if the ecological balance of the microbial populations were eventually affected by the biological control bacterium.

TABLE 1 | Microbial alpha diversity indexes (C: control, Bio: bio-fungicide, and Chem: chemical fungicide) assessed for the samples (grape biofilm, must, and wine), and statistical analysis at the same stage.

Samples		Species richness	Structure of community			
		Margalef	Simpson	Berger–Parker	*H*	Chao-1
Grape biofilm:	C	2.48	0.80	0.20	1.60	15
	Bio	3.05	0.83	0.17	1.87	32
	Chem	3.22	0.86	0.14	2.00	33
Must:	C	3.08	0.85	0.15	1.94	29
	Bio	3.08	0.85	0.15	1.94	29
	Chem	3.07	0.85	0.15	1.92	29
Wine	C	3.37b	0.88b	0.13a	2.08b	36ab
	Bio	3.51b	0.89b	0.12a	2.14b	41b
	Chem	2.71a	0.82a	0.18b	1.73a	20a

Different letters mean significant differences ($p < 0.05$) between the diversity indexes of samples at the same stage.

For this purpose, culture-dependent and independent techniques were employed. Culture dependent techniques allowed the detection of the microorganisms VC in culture medium under specific conditions. The PCR-DGGE approach allowed the detection of DNA proceeding from both alive and dead cells. Therefore, both techniques have quite important limitations to take into consideration. Moreover, the two methods of analysis do not generally give the same results for the same sample. The detection limits of PCR-DGGE technique have been usually considered higher than culture-dependent techniques even more with mixed populations (Bester et al., 2010). Nevertheless, in previous studies it was determined that, even with counts lower than 10^1 CFU/mL, this culture-independent method provided interesting results (González-Arenzana et al., 2017b). Consequently, the combination of both approaches could reach a wide insight in the microbial community.

The initial oenological conditions of grapes were similar between samples and significant differences were not found after statistical analysis of analytical data. Furthermore, grape samples had not evidence of being infected by *Botrytis cinerea*, so that the health grape state was adequate.

Impact of Fungicides on Grape Biofilm

In general, focusing on VC microbial community of grape biofilms, small populations of yeasts and bacteria were observed, accordingly to the described by Renouf et al. (2005). Apparently, the employment of both fungicides did not exert a dramatic change either in VC yeasts or in the yeast genera detected by culture-dependent and independent techniques. For example, the species *S. cerevisiae* was found in all samples although its detection in the grapevine environment with so small yeast population is considered really difficult (Renouf et al., 2005). Moreover, the yeast *A. pullulans* that is said to be a natural antagonist of the gray mold was present in all samples (Pertot et al., 2017). Additionally, the yeast *H. osmophila* was also found in all the samples being easily detectable at early stages of the vinification (Garijo et al., 2011). The combination of *Saccharomyces* and non-*Saccharomyces* genera in grape biofilm control sample was similar in grapes with the bio-fungicide. Nevertheless, some differences were observed in comparison to the grape biofilms with the chemical fungicide. For instance, the gray mold or *B. cinerea* was detected by culture-independent methods, not being found either in control sample, or in the grapes with the bio-fungicide.

Regarding the VC bacteria community after the application of the bio-fungicide, it was significantly higher than for control and chemical fungicide samples what would be due to the ability of *Bacillus* for growing on MRS plates (González-Arenzana et al., 2017a). Thus, viability of *Bacillus* cells applied to the grapevine was corroborated in the current study being found in great percentage in samples from bio-fungicide application and being also detected in the other two samples. Precisely, *Bacillus subtilis* is an EB gram-positive ubiquitous bacterium in the nature, similarly to *Pantoea* genus that was also found in some samples of this study. Furthermore, *Pseudomonas* genus was identified in all the samples after the application of both fungicides while in control sample it was not found. This genus is also an EB that has been described for playing an important role in both the grape biofilm formation and in the biological control of some spoilage microorganisms linked to grape surface (Renouf et al., 2005). Overall, genera of bacteria usually involved in winemaking such as AAB and LAB were not abundant on the grape surface. A case in point are the AAB usually linked to grape surface diseases, such as the gray mold (Barata et al., 2012) that were not detected at this first sampling probably because of the good health status of grapes. The only LAB detected was the genus *Lactococcus* recently described also in the grape surface (González-Arenzana et al., 2017a).

Apart from genera and species identified on grape surface, the microbial alpha diversity was numerically assessed. The microbial community of the grape biofilms was not significantly modified after the application of both fungicides. This result is contrary to others reporting significant changes in yeast and bacteria community of grape surfaces after applying copper based fungicides (Martins et al., 2012, 2014). Authors such as Cordero-Bueso et al. (2011) have reported higher diversity of the microbial communities of grapes after the organic management of grapevine, but in the current study this result was not clearly established. In fact, statistical differences in the richness

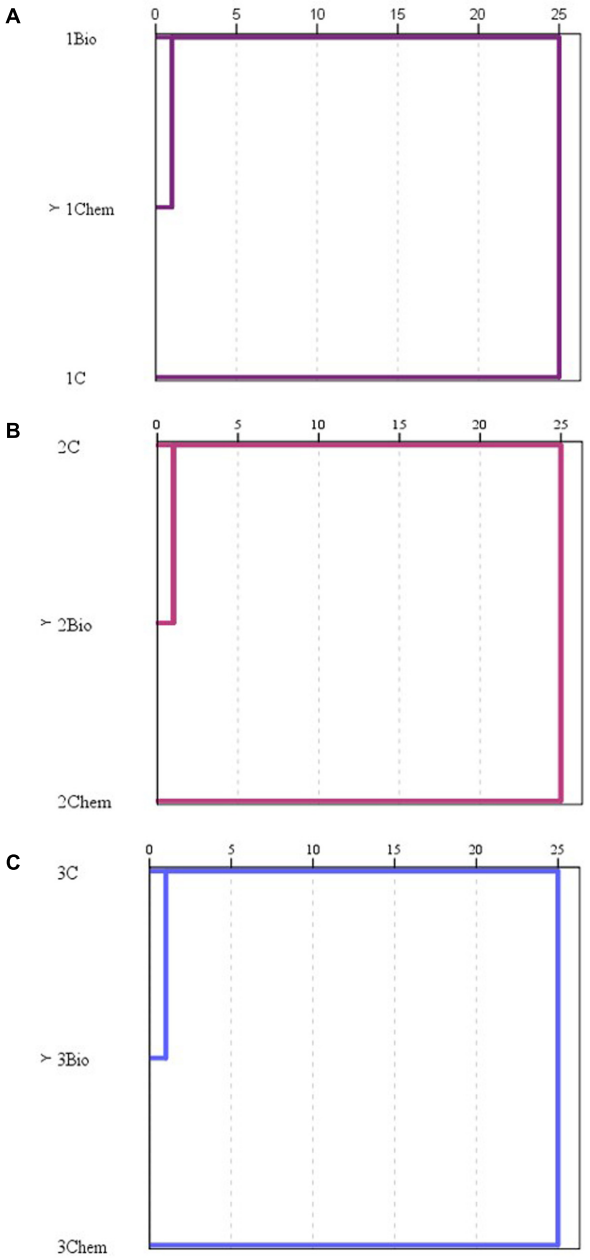

FIGURE 5 | Hierarchical cluster of the diversity indexes of the samples (C: control, Bio: bio-fungicide, and Chem: chemical fungicide) assessed for the grape biofilm **(A)**, for the must **(B)**, and for the wine stage **(C)**.

of species or in the structure of the microbial community of the three different samples were determined neither after the application of the bio-fungicide, nor after the application of the chemical fungicide. Only in the hierarchical cluster, both fungicide treatments were clustered in the same branch.

Impact of Fungicides on Must Microbiota

Must microbial community is a mixture of microorganisms from grape biofilms and of microorganisms from winery facilities. Focusing on must from crushed, destemmed and sulphited grapes, it was observed that VC community experienced two significant changes linked to the fungicide applications. On one hand, yeast community on DBDM plates, proceeding from chemical fungicide, were lower than the one counted on plates from control and bio-fungicide treated samples. This culture media did not provide information about *Dekkera* and *Brettanomyces* genera despite being prescribed for their detection (Rodrigues et al., 2001). On another point, the genera detected by DBDM plates were mostly found by GYP, so significant differences found by DBDM were not representative of the total yeast community. Yeasts on all the GYP plates corresponded with the commonly found in the literature about early stages of spontaneous AF, around 5 log units (Gutiérrez et al., 2001). Yeast genera identified in must from grapes treated with both fungicides were qualitatively similar. The genera *Hanseniaspora*, *Saccharomyces*, and *Trigonopsis* were found in all musts. In must sample control, *S. paradoxus* was detected along with *S. cerevisiae* what might deteriorate the organoleptically features of wines if both acted in the AF, according to the described by Alonso-del-Real et al. (2017). The genera *Aureobasidium* was present in musts after the fungicide treatments what could enhance the effectivity of the treatments, because it is thought to be a natural antagonist of some moulds affecting grapevine health state. Contrary to this, *Mo. polystroma*, which is a cherry pathogen (Poniatowska et al., 2016), was detected only after both fungicide application. In this case, these type of contradictory results made very difficult to establish some clear impact of agronomic treatments on microbial communities of musts.

Most of the bacteria found at must stage grew on MRS and Mann culture media, but AAB were mainly found in Mann with significantly lower populations in control sample. AAB presence in musts could proceed from the winery environment, because in grape biofilms were not detected. Definitely their detection at this stage is considered negative for wine organoleptic characteristics (Guillamón and Mas, 2011). The number of AAB species in must from grapes that were biologically treated were lower than in the other samples what might mean a positive impact of the bio-fungicide. In a similar way, *Oenococcus* was the only LAB in must from grapes treated with the chemical fungicide but it also could come from the winery environment because it was not found in the previous sampling stage. The number of EB species was lower than the observed in grape biofilms being only *Ta. ptyseos* detected in all the must samples. It is a rare food borne pathogen that causes some human infections (Mardaneh et al., 2014) and its origin must be located on the winery. This is the first time that this genus has been reported in must samples being traditionally found in the coffee fermentation (Silva et al., 2008). The presence of *B. subtilis* only in must from grapes treated with the bio-fungicide could be indicating that this genus was able to resist the operations performed in the vinifications process even the sulphiting. These results were even more interesting because these bacteria were isolated in a VC form.

Statistically, the microbial alpha diversity of must samples was equal in most of the assessed indexes, showing only slight differences between samples. In spite of being clearly similar

regarding indexes of microbial alpha diversity, the hierarchical cluster of must samples clustered together the control samples with the must proceeding from the bio-fungicide treatments, staying the chemical fungicide must apart. This could indicate that the chemical fungicide applied to grapes might exert some kind of impact on microbial community at must stage that made this sample slightly different to the other two samples.

Impact of Fungicides on Wine Microbiota

Each must successfully underwent through spontaneous AF regardless the type treatment applied to grapes. Regarding microbial community when AF was completed, great yeast populations on GYP plates were observed in all samples. At this point of winemaking, the yeast community, especially the *S. cerevisiae* population, was so high that the other microorganisms stayed in a secondary place. Again, likewise musts, yeasts on DBDM plates of wine from grapes treated with the chemical fungicide were significantly minor than yeasts of the other samples but their identification provided interesting information because genera different to *Saccharomyces* were detected. For instance, *H. uvarum* was again found by culture dependent and independent methods in all the wine samples. Furthermore, wine proceeding from grapes treated with the bio-fungicide were qualitatively more diverse than the other because *To. delbrueckii* and *Tr. cantarelli* species were also found. *To. delbrueckii* had not been detected in previous stages, so its presence in wines after the AF could be indicating an important population during this harsh stage probably due to its resistance to factors such as sulfur dioxide, as it has been demonstrated in recent studies (González-Arenzana et al., 2017a). Cordero-Bueso et al. (2011) reported this increase in the qualitative diversity on grape biofilm after organic agronomic practices.

In relation to bacteria community growing on MRS and Mann plates, important differences between fungicides and control samples were not noticed, but culture-independent methods provided some important results about bacteria community. An obvious example was the LAB *Lc. lactis* that was detected in all samples with PCR-DGGE. This result was in accordance to the previously described in wines from this same region in which several LAB species, including *Lc. lactis*, were found after the AF in a non-cultivable form (González-Arenzana et al., 2017b). The EB identified in wines were also in a non-cultivable form, thus, for instance, *Me. extorquens* and *Ps. putida* were detected in all samples and *Bacillus* was found in samples proceeding from both fungicide treatments. The presence of *Methylobacterium* has been recently reported by Portillo et al. (2016) in the grape surface of different varieties; and *Ps. putida,* present in grapevine soil, is very important because its capacity of resisting different metal contamination (Chong et al., 2016). Furthermore, the identification of *Bacillus subtilis* applied from grapevine until the AF depletion should be taken into consideration in future works.

At this sampling stage, differences in the microbial alpha diversity of wines were established in samples proceeding from grapes treated with the chemical fungicide. This sample was significantly different from the other two, having lower richness of species (Margalef index), lower diversity (Shannon–Wiener index, H) and lower number of odd species (Chao-1). This would have made these samples more sensitive to external threats than other samples with higher diversity of microorganisms. In contrast, the possibility of finding two randomly selected individuals belonging to the same species was lower in this sample and the equity assessed with the Berger-Parker index was also lower. This means that chemical fungicide samples were balanced in terms of structure of the microbial community in spite of having lower richness of species (Provost and Pedneault, 2016). The hierarchical cluster was approximately the same than the described for musts although the indexes of the alpha diversity were much more differenced at wine stage. Thus, clustering was very useful to corroborate the statistical analysis.

CONCLUSION

On balance, the application of the bio-fungicide in the grapevine caused an increase in viable microbial community growing on the MRS culture media in grape surface; in contrast, it did not affect significantly the microbial alpha diversity of the grape biofilm. Some of the *Bacillus* applied with the bio-fungicide were detected at must stage, and the microbial alpha diversity of this sample was more similar to the determined for control than the determined for must from grapes treated with the chemical fungicide. Despite these slight effects, the spontaneous AF was developed without problems in all the samples. Nevertheless, the microbial alpha diversity was very different for the wine from grapes treated with the chemical fungicide whereas control and wine from grapes treated with *Bacillus* were quite similar with higher species richness and quite similar structure of the microbial community based on a high diversity but also a high dominance of some species.

To sum up, under the conditions of this experiment (no contamination by *B. cinerea*) and with the analysis protocols used, the results showed that the biofungicide had no impact on alpha microbial diversity until the end of fermentation. If confirmed in other environmental and analytical conditions, this biofungicide could be applied to the vine as a biological control of the grey grape rot.

AUTHOR CONTRIBUTIONS

RE-V has been in charge of the acquisition, analysis, or interpretation of data for the work. RL and PS have made substantial contributions to the conception or design of the work. LG-A, AG, RL, and IL-A have revised the work critically for important intellectual content. All the authors have provided the final approval of the version to be published and therefore, are in agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

ACKNOWLEDGMENTS

This work was supported by funding and predoctoral grants of La Rioja Government. The authors thank the Bayer Crop Bioscience S.L. for the funding for application of these fungicides to the grapevine.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.00059/full#supplementary-material

FIGURE S1 | Some of the WLAB1-2 amplicons and species detected by DGGE in control replicates (C1, C2, C3, and C4), in samples form biofungicide treatment (B1, B2, B3, and B4) and in samples from chemical fungicide treatment (Ch1, Ch2, Ch3, and Ch4).

FIGURE S2 | Some of the WBAC 1-2 amplicons species detected by DGGE in control replicates (C1, C2, C3, and C4), in samples form biofungicide treatment (B1, B2, B3, and B4) and in samples from chemical fungicide treatment (Ch1, Ch2, Ch3, and Ch4).

FIGURE S3 | Some of the NL1-LS2 primers amplicons species detected by DGGE in control replicates (C1, C2, C3, and C4), in samples form biofungicide treatment (B1, B2, B3, and B4) and in samples from chemical fungicide treatment (Ch1, Ch2, Ch3, and Ch4).

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

frontiers in Microbiology

ORIGINAL RESEARCH
published: 11 June 2018
doi: 10.3389/fmicb.2018.01260

Molecular Diagnosis of *Brettanomyces bruxellensis*' Sulfur Dioxide Sensitivity Through Genotype Specific Method

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The yeast species *Brettanomyces bruxellensis* is associated with important economic losses due to red wine spoilage. The most common method to prevent and/or control *B. bruxellensis* spoilage in winemaking is the addition of sulfur dioxide into must and wine. However, recently, it was reported that some *B. bruxellensis* strains could be tolerant to commonly used doses of SO_2. In this work, *B. bruxellensis* response to SO_2 was assessed in order to explore the relationship between SO_2 tolerance and genotype. We selected 145 isolates representative of the genetic diversity of the species, and from different fermentation niches (roughly 70% from grape wine fermentation environment, and 30% from beer, ethanol, tequila, kombucha, etc.). These isolates were grown in media harboring increasing sulfite concentrations, from 0 to 0.6 mg.L^{-1} of molecular SO_2. Three behaviors were defined: sensitive strains showed longer lag phase and slower growth rate and/or lower maximum population size in presence of increasing concentrations of SO_2. Tolerant strains displayed increased lag phase, but maximal growth rate and maximal population size remained unchanged. Finally, resistant strains showed no growth variation whatever the SO_2 concentrations. 36% (52/145) of *B. bruxellensis* isolates were resistant or tolerant to sulfite, and up to 43% (46/107) when considering only wine isolates. Moreover, most of the resistant/tolerant strains belonged to two specific genetic groups, allowing the use of microsatellite genotyping to predict the risk of sulfur dioxide resistance/tolerance with high reliability (>90%). Such molecular diagnosis could help the winemakers to adjust antimicrobial techniques and efficient spoilage prevention with minimal intervention.

Keywords: ***Brettanomyces bruxellensis*, resistance, tolerance, sulfur dioxide, wine, spoilage yeast**

OPEN ACCESS

Edited by:
Linda F. Bisson, University of California, Davis, United States

Reviewed by:
Mariagiovanna Fragasso, Consiglio per la Ricerca e la sperimentazione in Agricoltura, Italy
Lucía González-Arenzana, Instituto de Ciencias de la Vid y del Vino (ICVV), Spain

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

Received: *15 March 2018*
Accepted: *24 May 2018*
Published: *11 June 2018*

Citation:
Avramova M, Vallet-Courbin A, Maupeu J, Masneuf-Pomarède I and Albertin W (2018) Molecular Diagnosis of Brettanomyces bruxellensis' Sulfur Dioxide Sensitivity Through Genotype Specific Method. Front. Microbiol. 9:1260. doi: 10.3389/fmicb.2018.01260

INTRODUCTION

Winemakers manage the transformation of must into wine through various processes, aiming to obtain high quality product according to their wishes and the expectations of their customers. However, wine chemical and microbiological properties are in constant evolution throughout the winemaking process, and some parameters are difficult to control. Yeast metabolism is one of the

multiple factors shaping wine aromatic and flavor properties by contributing to its complexity or, in some cases, leading to undesirable aromas (Fleet, 2003). One example of such phenomenon is wine spoilage by *Brettanomyces bruxellensis*, a yeast species related to production of off-aromas perceived as barnyard, horse sweat, or medicinal (Heresztyn, 1986; Chatonnet et al., 1992). Prevention methods against *B. bruxellensis* development include spoilage risk evaluation, SO_2 addition, the use of biocontrol agents, e.g., through the inoculation/co-inoculation of various species and/or strains of yeast and bacteria (Berbegal et al., 2017, 2018), etc. If *B. bruxellensis* is detected, different elimination techniques exist which could be roughly divided in physical (filtering, the use of electric current, pressure, temperature, ultrasonics, etc.) and chemical (SO_2, chitosan, DMDC, yeast-derived killer toxins, etc.), see for details (Delfini et al., 2002; Lustrato et al., 2010; Francesca and Maurizio, 2011; Luo et al., 2012; Umiker et al., 2013; Mehlomakulu et al., 2014; Fabrizio et al., 2015; Taillandier et al., 2015; González-Arenzana et al., 2016, 2018; Petrova et al., 2016; Berbegal et al., 2017). Still, the most common method to prevent and/or control *B. bruxellensis* spoilage remains the addition of sulfur dioxide into must and wine, with regular adjustments if needed. Sulfites are used in winemaking at least since the 18th century and are introduced either through the burning of sulfur tablets in barrels, or in liquid form, mainly through addition of potassium bisulfite solution to must and wine (Ribéreau-Gayon et al., 2006). Sulfur dioxide is broadly used in winemaking not only for its antiseptic action, but also for its antioxidant and antioxidasic properties (Ribéreau-Gayon et al., 2006). Thus, SO_2 addition is the preferred choice when it comes to *B. bruxellensis* spoilage prevention. Unfortunately, over the last years, some *B. bruxellensis* strains were reported to be tolerant to commonly used doses of SO_2, with a high variability amongst isolates (Barata et al., 2008; Curtin et al., 2012; Agnolucci et al., 2014). This variability makes the prediction of *B. bruxellensis* spoilage potential and the choice of adequate antimicrobial agent a challenge for winemakers. Recently, it was shown that *B. bruxellensis* SO_2 sensitivity correlates with genotype defined by both AFLP and microsatellite markers (Curtin et al., 2012; Avramova et al., 2018). The former study analyzed a total of 41 isolates, with a focus on Australian wine strains. The latter study assessed the intraspecific genetic diversity of a larger number of isolates (1488 strains from 29 countries and 5 types of fermentation niches). Microsatellite genotype analysis revealed that the population was structured according to ploidy level (some clusters being mainly composed of diploid isolates, whereas others – of triploid ones). Statistical analysis of the generated data highlighted that both substrate of isolation and geographical origin of the isolates contribute to the observed population structure. The results suggested an anthropic influence on the spatial biodiversity of *B. bruxellensis*. The hypothesis of human-related factors effect on the population was further supported by the correlation between genotypic clustering and tolerance to SO_2, the main antimicrobial agent used by winemakers. In particular, among the six main clusters of *B. bruxellensis* population (Avramova et al., 2018), two genetic clusters (AWRI1499-like and L0308-like) were highlighted to comprise isolates with high SO_2 tolerance (Avramova et al., 2018). However, SO_2 sensitivity was tested on a limited number of isolates (39), particularly for the L0308-like cluster (2 isolates). Thus, the aims of this study were (i) to extend the screening of SO_2 sensitivity to 106 additional isolates and thus confirm/infirm the correlation between genetic clusters and SO_2 sensitivity to a larger collection representative of the global *B. bruxellensis* population and (ii) to validate the applicability of a method allowing the prediction of *B. bruxellensis* SO_2 sensitivity through genetic markers analysis.

MATERIALS AND METHODS

Strains

In this study, 106 strains – in addition to the 39 strains tested previously (Avramova et al., 2018) – from different geographical and industrial fermentation origins were used based on their microsatellite profile (full protocol details and population dendrogram assessment in Avramova et al., 2018). Twelve microsatellite markers were used for genotyping, and a dendrogram was produced using Bruvo's distance and Neighbor Joining (NJ) clustering. Those strains were evaluated for their tolerance to SO_2 using the same protocol as previously described (Avramova et al., 2018) (details in the section "Sulfite Tolerance Assessment") which made possible the combination of both datasets together to give a total of 145 strains (**Table 1** and **Figure 1**).

Sulfite Tolerance Assessment

The assay was performed in liquid medium containing 6.7 $g.L^{-1}$ of YNB (Difco™ Yeast Nitrogen Base, Becton, Dickinson and Company), 2.5 $g.L^{-1}$ D-glucose, 2.5 $g.L^{-1}$ D-Fructose, 5% (v/v) ethanol and increasing concentrations of potassium metabisulfite (PMB, $K_2S_2O_5$, Thermo Fischer Scientific) in order to obtain 0, 0.2, 0.4, and 0.6 $mg.L^{-1}$ mSO_2 final concentrations. For the calculation of mSO_2 it was considered that $K_2S_2O_5$ corresponds to about 50% of total SO_2 (therefore a solution of 10 $g.L^{-1}$ $K_2S_2O_5$ corresponds to approximately 5 $g.L^{-1}$ total SO_2).

TABLE 1 | Summary of the collection of 145 *Brettanomyces bruxellensis* strains used for sulfur dioxide tolerance assay.

Substrate	Beer (13); Cider (1); ethanol (2); Fruit wine (1); Kombucha (6); Tequila (6); Wine (107); NA (9)
Country	Argentina (1); Australia (9); Belgium (6); Brazil (4); Chile (3); Denmark (5); France (60); Germany (1); Italy (27); Mexico (6); Netherlands (1); New Zealand (1); Portugal (4); South Africa (6); Spain (2); Thailand (1); United Kingdom (1); Uruguay (1); United States (5); NA (1)
Vintage	1912 (1); 1926 (1); 1931 (1); 1938 (1); 1941 (1); 1949 (1); 1959 (1); 1990 (4); 1991 (2); 1992 (5); 1993 (1); 1994 (4); 1995 (2); 1998 (1); 2001 (6); 2002 (6); 2003 (6); 2003–2011 (2); 2004 (5); 2005 (2); 2006 (1); 2010 (1); 2011 (1); 2012 (17); 2013 (20); 2014 (19); 2015 (6); NA (27)
Genetic group	AWRI1499-like (32); AWRI1608-like (30); CBS 2499-like (42); CBS 5513-like (11); KOM1449-like (18); L0308-like (12)

Full details available in ***Supplementary Table S1****. NA stands for Not Available.*

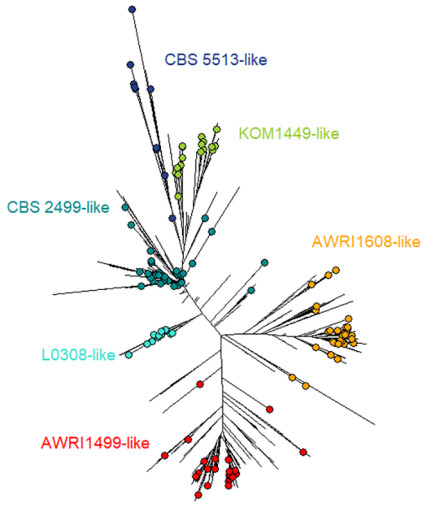


FIGURE 1 | Dendrogram tree showing the 145 phenotyped *B. bruxellensis* isolates. The dendrogram tree includes 1488 isolates, and was built using 12 microsatellite markers, Bruvo's distance, and NJ clustering, as described previously (Avramova et al., 2018). The 145 isolates used in this work are represented by colored circles. The six different colors correspond to the main genetic groups identified and were named from one isolate (e.g., L0308-like means genetic group close to L0308 strain).

In order to deduce the final mSO_2 concentration, the free SO_2 concentration was assessed by aspiration/titration method. Then, the mSO_2 was calculated by using the Henderson–Hasselbalch equation on dissociation constant p*K*1 (Divol et al., 2012). Ethanol concentration (5%) was chosen to allow growth of all strains, isolated from wine as well as from other fermentation niches with lower initial ethanol content. Final pH was adjusted to 3.5 (corresponding to an average value for pH generally encountered in red winemaking conditions) with phosphoric acid (1 M H_3PO_4) and the four media (corresponding to the four different concentrations of SO_2) were filtered separately with 0.22 μm pore filter (Millipore).

Small-scale fermentations were performed in sterile 4 mL spectrophotometer cuvettes containing a sterile magnet stirrer (Dutscher, France). The cells were grown on YPD agar and inoculated into the YNB-based medium without SO_2. After 96 h of pre-culture (the point at which all strains reached stationary phase), the cells were inoculated at OD_{600nm} 0.1 in a final volume of 3 mL. The inoculated medium was then covered with 300 μL of sterile silicone oil (Sigma-Aldrich) to avoid oxidation of the medium which could favor the free SO_2 consumption. Then, the cuvette was capped with a plastic cap (Dutscher) and sealed with parafilm. A sterile needle was added by piercing the cap to allow CO_2 release. These so-called nano-fermenters were then placed in a spectrophotometer cuvettes container box and on a 15 multi-positions magnetic stirrer plate at 25°C (the final temperature in the nano-fermenters was therefore 29°C due to the stirrer heating). Optical density (OD_{600nm}) was measured every 24 h during at least 150 h to follow cell population growth until stationary phase was reached.

Growth Parameter Calculation and Statistical Analyses

For each growth curve, the following three parameters were calculated: OD_{max} was the maximal OD reached at 600 nm and corresponded to the maximal population size, the lag phase (in hours) was the time between inoculation and the beginning of cell growth (5% maximal OD increase), and finally, the maximal growth rate was calculated (maximal number of division per hour based on the OD measurement divided by time).

Non-parametric Kruskal–Wallis tests were performed ($\alpha = 5\%$) to identify the means that were significantly different. All statistical analyses and graphs were produced using R language (R Development Core Team, 2010).

RESULTS

Growth Behavior in Presence of SO_2

The growth behavior of 145 strains of *B. bruxellensis* was evaluated regarding sensitivity to sulfite treatment. The selected strains were distributed amongst the six main genetic groups defined using microsatellite markers and were representative of the genetic diversity of the species (**Figure 1**): CBS 2499-like, KOM1449-like, AWRI1608-like, AWRI1499-like, CBS 5513-like, and L0308-like groups were represented by 42, 18, 30, 32, 11, and 12 strains, respectively (**Table 2**). A total of >2050 small-scale fermentations were performed, corresponding to each strain tested at increasing concentrations of mSO_2 (0, 0.2, 0.4, and 0.6 $mg.L^{-1}$) at least in triplicate. The strains had different response to sulfur dioxide concentrations in means of lag phase, maximal growth rate, and maximum OD. Depending on the growth parameters' variation (**Supplementary Table S1**), three growth behaviors were defined (**Figure 2**). Sensitive strains showed significantly longer lag phase and slower growth rate and/or lower maximum OD in presence of increasing concentrations of SO_2: for example, strain B002-14 T14 7 (**Figure 2**) showed 22.4, 39.7, 99.2, and 173.4 h of lag phase with 0, 0.2, 0.4, and 0.6 $mg.L^{-1}$ mSO_2, respectively. Maximal growth rate decreased along sulfite concentration with 0.09, 0.06, 0.02, and 0.01 division/h, and OD_{max} decreased drastically with 1.42, 1.27, 0.77, and 0.09 OD. The same pattern (increased lag-phase, decreased growth rate, and decreased OD_{max}) was observed for strains 12AVB1 and 2OT14_02 (**Figure 2**). The degree of sensitivity varied depending on the isolates: some strains showed low growth in presence of 0.2 $mg.L^{-1}$ mSO_2 like strain CBS 3025 which OD_{max} drops from 1.92 to 0.13 at 0 and 0.2 $mg.L^{-1}$ mSO_2, respectively, or strain 12AVB1 that shows a twofold decrease of OD_{max} between 0 and 0.2 $mg.L^{-1}$ mSO_2 (1.46 to 0.63, see **Supplementary Table S1**). Other isolates showed close to normal growth at 0.2 $mg.L^{-1}$ mSO_2 ($OD_{max} > 1$), but low/no growth at 0.4 $mg.L^{-1}$ mSO_2 (AWRI1615, L02/E2 AZ, L14160, L14186, YJS5447, etc.). Finally, other strains, although showing a significant growth decrease, were still able to show moderate growth at 0.6 $mg.L^{-1}$ mSO_2: for example, lag-phase of UWOPS 92–297.4 was drastically impacted, from 7 and 10 h (0 and 0.2 $mg.L^{-1}$ mSO_2) to 154 and 171 h (0.4 and 0.6 $mg.L^{-1}$ mSO_2).

Its OD_{max} was also clearly impacted, ranging from 1.29 to 0.54 (at 0 and 0.6 $mg.L^{-1}$ mSO_2, respectively), yet with a residual growth. In conclusion, all strains considered to be sensitive had significantly longer lag phase and slower growth rate and/or lower maximum OD in presence of increasing concentrations of SO_2. However, the sulfite concentration at which growth began to be impacted varied, as well as the level of growth's decrease.

By contrast, tolerant strains displayed increased lag phase with SO_2 increase, while others growth parameters (maximal growth rate and maximal OD) remained statistically unchanged (Kruskal–Wallis test, $\alpha = 0.05$). For example, strain VP1545 (**Figure 2**) showed varying lag phase (36.9, 55.7, 63.4, and 94.4 h at 0, 0.2, 0.4, and 0.6 $mg.L^{-1}$ mSO_2, respectively), but unchanged maximal growth rate (0.07–0.09 division/h) and OD_{max} (1.82–1.85 OD). The same pattern is observed for AWRI 1606 (lag-phase ranging from 27 to 57 h) or AWRI 1605 (lag-phase between 39 and 57 h). Finally, strains for which none parameters were significantly impacted whatever the SO_2 concentrations were considered as resistant: VP1503 (**Figure 2**) had unchanged lag phase of 27.2 to 36.4 h, maximal growth rate of 0.08–0.09 division/h and OD_{max} of 1.11–1.31 OD. Identically, Merlot_329_M_1 and L0615 showed identical growth's kinetics whatever the SO_2 concentrations tested.

Relationship Between SO_2 Sensitivity and Genetic Groups

When analyzed globally, clear differences between the different genetic groups were observed (**Figure 3**): the L0308-like group showed mostly resistant behavior (invariant growth parameters whatever sulfite concentration). The AWRI1499-like group showed mostly unchanged maximal growth rate and OD, and showed either unchanged lag phase (resistant strains) or poorly increased lag phase (tolerant strains). All other groups were mostly sensitive to sulfite treatments, with an important variability amongst strains regarding to their degree of sensitivity.

A more precise analysis, strain by strain, was performed (**Table 2** and **Supplementary Figure S1**). An important proportion of the tested isolates (52/145, 36%) were either tolerant or resistant to sulfite treatments, and this was strongly related to genetic groups. For example, all 12 isolates of the L0308-like group were either resistant (11) or tolerant (1) to sulfite treatments. Similarly, amongst the 32 isolates tested for the AWRI1499-like group, 21 were resistant, 7 tolerant, and only 4 sensitive to sulfite treatments. This confirms that, globally, most isolates from L0308-like and AWRI1499-like groups are resistant/tolerant to sulfite. By contrast, the other groups contained mostly sensitive strains (38/42 for CBS 2499-like; 14/18 for KOM1449-like; 27/30 for AWRI1608-like; 10/11 for CBS 5513-like).

In addition, 46 out of 52 tolerant or resistant strains were isolated from wine (**Supplementary Table S1**). Indeed, the proportion of tolerant/resistant isolates from wine represented 43% (46/107).

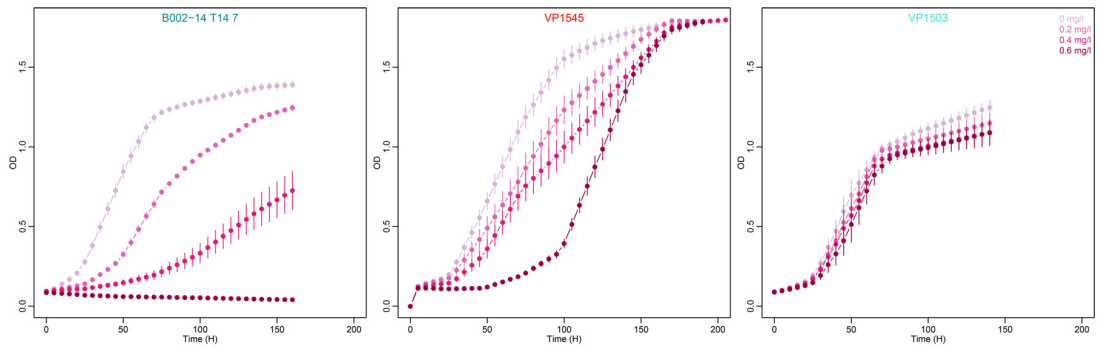
TABLE 2 | Number of isolates by genetic group and phenotype.

Genetic group	Sensitive	Tolerant	Resistant	Total
CBS 2499-like	38	1	3	42
KOM1449-like	14	3	1	18
AWRI1608-like	27	2	1	30
AWRI1499-like	4	7	21	32
CBS 5513-like	10	0	1	11
L0308-like	0	1	11	12
Total	93	14	38	145

DISCUSSION

Sulfur dioxide is usually used by winemakers as preventive or curative treatment for spoilage microorganisms including *B. bruxellensis* contamination. Concentrations of 0.2 to 0.5 $mg.L^{-1}$ molecular SO_2 are typically reported to inhibit growth in wine (Conterno et al., 2006; Barata et al., 2008). However, some *B. bruxellensis* strains were shown to be rather sulfite tolerant (Barata et al., 2008; Vigentini et al., 2008; Curtin et al., 2012; Agnolucci et al., 2014; Avramova et al., 2018) and sulfite efficiency was elucidated as population level dependent (Longin et al., 2016). Previous studies highlighted genotype-dependent tolerance to sulfur dioxide for *B. bruxellensis* among Australian isolates with AFLP markers (Curtin et al., 2012), and this was recently confirmed for 39 isolates analyzed with microsatellite markers (Avramova et al., 2018). Taking into account the high intra-species genetic diversity of *B. bruxellensis*, 106 additional isolates from various origins were included to the previous phenotypic test to confirm the link between genotype and SO_2 tolerance at larger and finer scale. Here, we show that 36% of *B. bruxellensis* isolates are resistant/tolerant to sulfite (up to 43% amongst wine isolates), and we confirm the relationship between genetic groups and survival patterns in presence of sulfite treatments.

In our previous study, it was noticed that representatives of the L0308-like group exhibited a peculiar profile characterized by unmodified growth parameters at all tested SO_2 concentrations. However, these observations were based on only two isolates with similar origin (Avramova et al., 2018). To complete these results, we analyzed 9 additional L0308-like strains from different origins and confirmed their (mostly) resistant phenotype. Here, a resistant phenotype corresponds to behavior for which there were no significant differences for all studied growth parameters at increasing SO_2 concentration. On the other hand, tolerant strains were those for which lag phase was modified with SO_2 increase. Those two terms are used in clinical microbiology, where they serve to describe microbial pathogenicity (Anderson, 2005; Brauner et al., 2016). Often, tolerance is related to the capacity of the organism to survive under inhibition by an agent, whereas resistance is linked to the capacity to actively proliferate in presence of antibiotic, and is measured as minimum inhibitory concentration or fitness (Anderson, 2005). The peculiarity of SO_2 application, however, is that the main active antimicrobial fraction (mSO_2) of this agent depends on



B002-14 T14 7

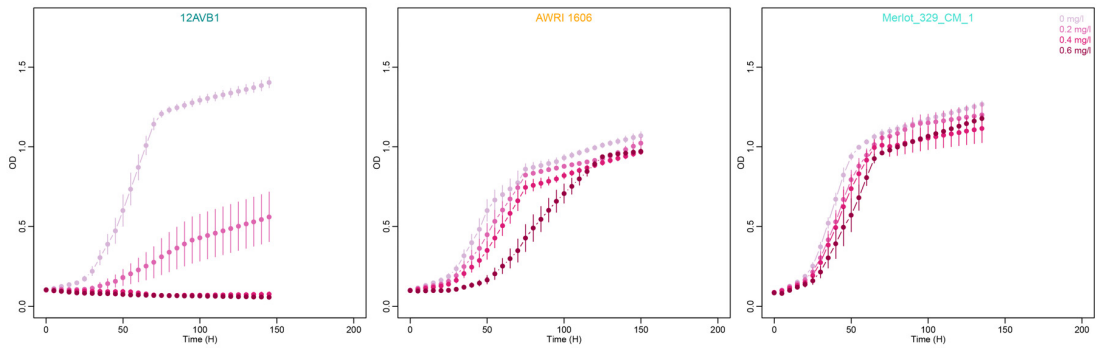
mSO2 (mg/L)	lag phase (h)	max. growth rate (div/h)	ODmax (600nm)
0	22.4 ± 3.2	0.09 ± 0.01	1.42 ± 0.04
0.2	39.7 ± 4.51	0.06 ± 0	1.27 ± 0.06
0.4	99.25 ± 36.59	0.02 ± 0	0.77 ± 0.33
0.6	173.37 ± 8.3	0.01 ± 0	0.09 ± 0.01

VP1545

mSO2 (mg/L)	lag phase (h)	max. growth rate (div/h)	ODmax (600nm)
0	36.91 ± 7.87	0.07 ± 0.01	1.82 ± 0.1
0.2	55.67 ± 12.6	0.06 ± 0.01	1.85 ± 0.04
0.4	63.43 ± 14	0.09 ± 0.02	1.83 ± 0.04
0.6	94.42 ± 11.62	0.06 ± 0.01	1.85 ± 0.08

VP1503

mSO2 (mg/L)	lag phase (h)	max. growth rate (div/h)	ODmax (600nm)
0	27.24 ± 3.46	0.08 ± 0.01	1.31 ± 0.08
0.2	30.59 ± 5.45	0.09 ± 0	1.19 ± 0.12
0.4	32.77 ± 6.68	0.09 ± 0	1.11 ± 0.12
0.6	36.44 ± 6.4	0.09 ± 0.01	1.12 ± 0.14



12AVB1

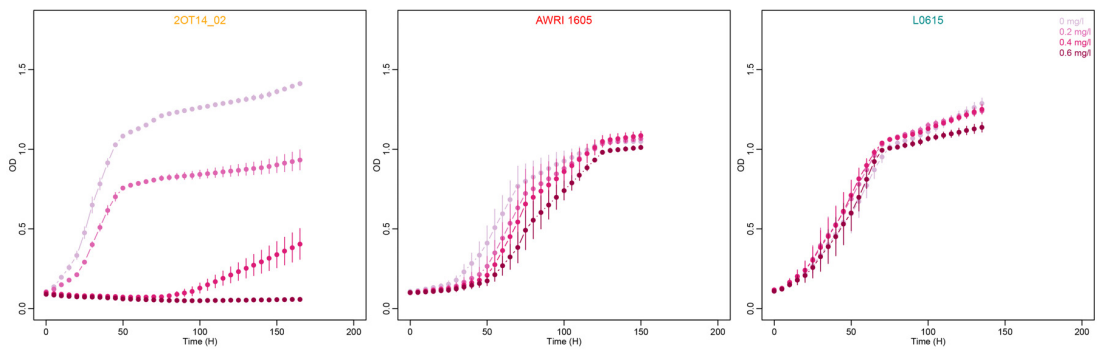
mSO2 (mg/L)	lag phase (h)	max. growth rate (div/h)	ODmax (600nm)
0	32.89 ± 3.38	0.08 ± 0.01	1.46 ± 0.07
0.2	94.36 ± 31.27	0.04 ± 0.01	0.63 ± 0.32
0.4	162.97 ± 13.51	0.01 ± 0	0.12 ± 0.01
0.6	162.97 ± 13.51	0 ± 0	0.1 ± 0

AWRI 1606

mSO2 (mg/L)	lag phase (h)	max. growth rate (div/h)	ODmax (600nm)
0	26.93 ± 4.22	0.09 ± 0.01	1.1 ± 0.06
0.2	33.1 ± 5.29	0.07 ± 0	1.09 ± 0.1
0.4	38.43 ± 6.98	0.07 ± 0	1 ± 0.03
0.6	57.19 ± 9.07	0.07 ± 0	0.98 ± 0.03

Merlot_329_CM_1

mSO2 (mg/L)	lag phase (h)	max. growth rate (div/h)	ODmax (600nm)
0	22.27 ± 1.82	0.1 ± 0	1.29 ± 0.04
0.2	25.42 ± 2.48	0.1 ± 0	1.23 ± 0.13
0.4	27.33 ± 2.78	0.09 ± 0.01	1.15 ± 0.14
0.6	30.32 ± 5.19	0.09 ± 0.01	1.22 ± 0.06



2OT14_02

mSO2 (mg/L)	lag phase (h)	max. growth rate (div/h)	ODmax (600nm)
0	14.68 ± 1.87	0.11 ± 0	1.48 ± 0.03
0.2	22.45 ± 0.84	0.1 ± 0.01	0.99 ± 0.09
0.4	128.02 ± 21.54	0.03 ± 0	0.51 ± 0.21
0.6	186.4 ± 8.9	0.03 ± 0.03	0.2 ± 0.11

AWRI 1605

mSO2 (mg/L)	lag phase (h)	max. growth rate (div/h)	ODmax (600nm)
0	39.07 ± 8.26	0.08 ± 0.01	1.06 ± 0.01
0.2	50.44 ± 12.95	0.08 ± 0.02	1.08 ± 0.01
0.4	52.7 ± 11.93	0.08 ± 0.02	1.1 ± 0.03
0.6	56.56 ± 11.43	0.08 ± 0.01	1.02 ± 0.03

L0615

mSO2 (mg/L)	lag phase (h)	max. growth rate (div/h)	ODmax (600nm)
0	28.81 ± 7.97	0.08 ± 0.01	1.39 ± 0.12
0.2	24.99 ± 7.09	0.09 ± 0.01	1.28 ± 0.04
0.4	25.62 ± 7.14	0.1 ± 0	1.3 ± 0.02
0.6	29.96 ± 8.36	0.09 ± 0.01	1.17 ± 0.04

FIGURE 2 | Examples of *B. bruxellensis* sensitive, tolerant, and resistant behavior at four mSO_2 concentrations. Strains B002-14 T14 7, 12AVB1, and 2OT14_02 represent sensitive strains. VP1545, AWRI 1606, and AWRI 1605 are tolerant isolates and VP1503, Merlot_329_CM_1, and L0615 are examples of resistant strains. Each curve is built using the mean of three to four replicates, and error bars represent standard deviations and curve colors correspond to increasing SO_2 concentration (light pink 0 mg/L mSO_2 to dark pink 0.6 mg/L mSO_2). The estimated growth parameters (lag phase, maximal growth rate, and maximal OD) are shown below each curve, with mean ± standard deviation.

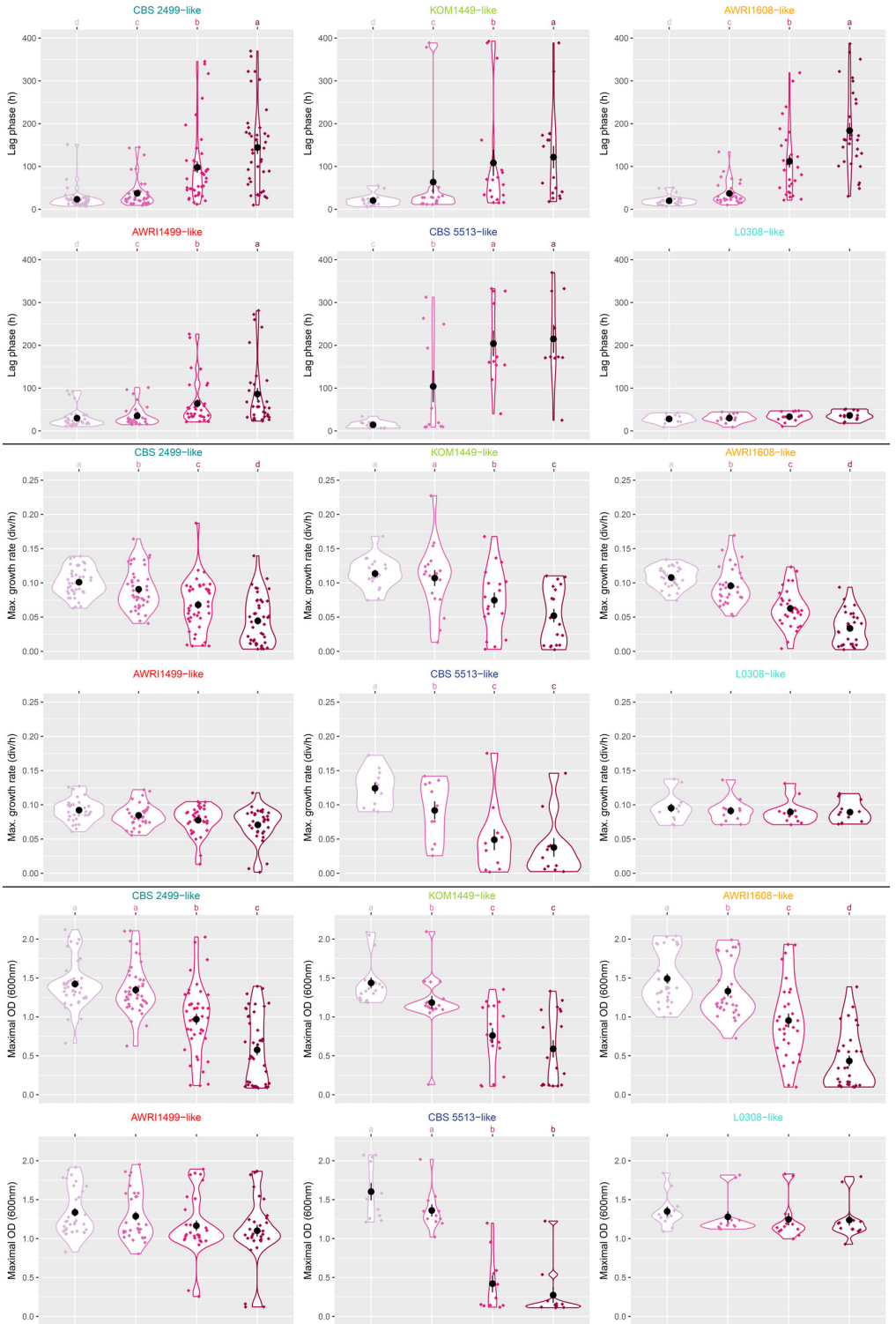


FIGURE 3 | Violin plots for three growth parameters and six genetic groups of *B. bruxellensis*. Three growth parameters were represented: lag phase (h), maximum growth rate (division per hour), and maximum OD (600 nm). For each genetic group, numeric values (corresponding to the different strains) are represented as diamonds, the corresponding probability densities are represented as plain traits, means, and standard errors are represented by black circles and segments, respectively. Increasing SO_2 concentrations are represented by the same coloring (pink shades, light pink corresponding to 0 mg/L and darker color representing increasing SO_2 concentrations) as in **Figure 2**. The plots were obtained using *ggplot2* package (R). Top letters represent significance groups as defined by Kruskal–Wallis test (*agricolae* package, p-value < 0.05). Absence of top letters indicates non-significantly different sulfur conditions.

environmental parameters (such as temperature, alcohol content, and mainly pH) and that the active fraction decreases over time due to free SO_2 combination. Furthermore, *B. bruxellensis* is able to enter a VBNC (viable but not cultivable) state after sulfites addition (du Toit et al., 2005; Agnolucci et al., 2010; Serpaggi et al., 2012; Capozzi et al., 2016; Longin et al., 2016), followed by growth recovery when sulfites decrease over time. In winemaking, sulfite levels are regularly re-adjusted at different time intervals, thus creating seasonality in SO_2 administration during the winemaking process. In these conditions, the actual survival of *B. bruxellensis* in wine could be related to (i) survival and growth besides initial "hit" with SO_2, that could be related to resistant-type mechanism and (ii) survival at the initial SO_2 "hit" and until a stage when mSO_2 concentration is lower in the medium, followed by growth recovery that could be described as tolerance mechanism. Indeed, resistant and tolerant phenotypes are often interconnected and related to different types of metabolism and cell structure differences. In clinical microbiology, it is suggested that tolerant and resistant strains should be treated differently: resistant should be treated with higher doses and shorter treatment, whereas tolerant strains should be treated with lower doses but extended treatment duration (Brauner et al., 2016). The detection of both resistant and tolerant growth profiles in the present dataset suggests that *B. bruxellensis* strains have developed not one, but multiple strategies to cope with SO_2 present in wine.

Here, the majority of tolerant or resistant strains were isolated from wine (46 out of 52). This suggests a strong link between SO_2 exposure related to the winemaking industry and *B. bruxellensis* survival in presence of SO_2 (Curtin et al., 2012). This data highlights the role of SO_2, and therefore human activity, in shaping *B. bruxellensis* population structure, which was also suggested in previous studies (Curtin et al., 2012; Avramova et al., 2018). Sulfur dioxide resistance is broadly studied in *S. cerevisiae* and the main molecular mechanisms explaining this phenotype is efflux through Ssu1p active pump (Park and Bakalinsky, 2000; Perez-Ortin et al., 2002; Nardi et al., 2010). It was demonstrated that SSU1-R allele, which is involved in SO_2 resistance, is the product of reciprocal translocation between chromosomes VII and XVI, thus highlighting the importance of gross chromosomal rearrangements in the adaptive evolution of *S. cerevisiae* (Perez-Ortin et al., 2002). Later, another translocation involved in SO_2 tolerance (XV-t-XVI) was shown to shorten lag phase in presence of SO_2, thus conferring relative selective advantage compared to non-translocated XVI strains (Zimmer et al., 2014). Following those studies, it was suggested that those translocations were empirically selected by humans (Perez-Ortin et al., 2002; Zimmer et al., 2014). The lack of effect of SO_2 on lag phase observed for the resistant *B. bruxellensis* strains could be related to similar mechanisms. Indeed, allele specific expression of efflux pump BbSSU1 was detected by comparative transcriptomics (Curtin et al., 2015). However, the molecular mechanisms underlying resistant phenotype in *B. bruxellensis* remain to be elucidated. As for the tolerant strains, the longer lag phase would reflect the time needed for the adaptation through complex mechanisms or the survival until a lower mSO_2 concentration is attained in the medium. Using staining with propidium iodide detection by flow cytometer analysis, Longin et al. (2016) showed that sulfite induces increased yeast cell permeability, which probably leads to cell death. The ability of cells to restore functional cell permeability could constitute another sulfite adaptation mechanism for *B. bruxellensis*. The SO_2 molecule has various effects on the cell structure, metabolism, and genome (Divol et al., 2012), and the corresponding mechanisms could include synthesis of binding molecules (like acetaldehyde), specific membrane structure, etc (Divol et al., 2012).

The sensitivity/survival phenotype in presence of SO_2 correlates with genotypic profiles defined by microsatellite analysis in a set of 145 representative strains (Avramova et al., 2018). The groups CBS 2499-like, KOM1449-like, AWRI1608-like, and CBS 5513-like are all susceptible to SO_2 presence in synthetic medium. On the contrary, AWRI1499-like and L0308-like survived in presence of high concentrations of mSO_2. This behavior was confirmed by independent study (Longin et al., 2016) performed in wine medium, where the strain L0417 (AWRI1499-like) was demonstrated to be more tolerant than L02E2 (CBS 2499-like). The use of microsatellites as selection markers was previously proposed for *S. cerevisiae* wine strains (Franco-Duarte et al., 2009, 2014). In the latter work, 30 different phenotypes were analyzed, and SO_2 tolerance was one of the factors that correlate the most with microsatellite patterns. In the winemaking context, SO_2 tolerance is a positive trait for the selection of *S. cerevisiae*, whereas it is the opposite for *B. bruxellensis* strains, for which it is directly related to spoilage potential. Defining SO_2 tolerance through genetic markers can therefore be used as an efficient tool to adapt antimicrobial treatment in winery. Similar methods are used for resistance prediction for pathogenic fungi (Park and Perlin, 2005; Irinyi et al., 2015). Namely, in the case of *C. albicans*, PCR-based methods were proposed for the detection of mutations related to fluconazole resistance (Park and Perlin, 2005). This method allows the adoption of alternative techniques to cope with this microorganism. Contrary to fluconazole, SO_2 has a very broad range of actions on the cell at structural, genetic, and metabolic level (White et al., 2002; Divol et al., 2012), and detection method of specific mutation responsible for resistance would be a challenge. Therefore, the strong correlation between genotype and SO_2 tolerance presents a reliable alternative for the prediction of this phenotype through microsatellite analysis. Indeed, resistant/tolerant genotypes can be reliably predicted: 91% (40/44 strains) of the AWRI1499-like and L0308-like isolates are actually tolerant or resistant to sulfite. For comparison, this percentage was 91% for *C. albicans* (based on 32 isolates) when using targeted PCR (Park and Perlin, 2005). Combined with the fact that clonal populations of *B. bruxellensis* strains were isolated over a long period of time in the same winery (Albertin et al., 2014), the use of microsatellite markers is also applicable as a prediction method based on spoilage populations from previous vintages. Hence, the use of microsatellite markers is a reliable method for predicting spoilage potential in means of SO_2 tolerance for *B. bruxellensis* populations, although a bit

expensive and time-consuming for routine analysis. Therefore, we developed an alternative analysis, based on a single duplex PCR and classical gel electrophoresis migration that indicates (i) whether the isolates belong to *B. bruxellensis* species and (ii) their sulfur dioxide sensitivity (Albertin et al., 2017a, 2018). This approach was patented (Albertin et al., 2017b) and is compatible with day-to-day analysis by oenological laboratories. Such diagnosis could allow application of adequate antimicrobial techniques according to the survival mechanism in presence of SO_2 of the contaminating *B. bruxellensis* population, and thus to assure efficient spoilage prevention with minimal intervention.

AUTHOR CONTRIBUTIONS

IM-P and WA conceived the study. MA, AV-C, and JM performed the experiments. All authors analyzed the data and wrote the manuscript.

FUNDING

This work was partly funded by AST (Aquitaine Science Transfert), as 'Typebrett' Project.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.01260/full#supplementary-material

FIGURE S1 | Growth parameters of 145 *B. bruxellensis* isolates grown at different SO_2 concentrations. Growth parameters lag phase (h), maximum growth rate (division per hour), and maximum OD (600 nm) are presented for 145 isolates. Isolates are clustered by genetic group as defined previously (Avramova et al., 2018), in order: CBS 2499-like group (dark cyan), KOM1449-like (light green), AWRI1608-like (orange), AWRI1499-like (red), dark blue (CBS 5513-like), turquoise (L0308-like). Vertical traits present standard deviations.

TABLE S1 | Growth parameters of *B. bruxellensis* strains in different concentrations of sulphur dioxide. [a]AWRI, The Australian Wine Research Institute, Glen Osmond, SA, Australia; CBS, Centraalbureau voor Schimmelcultures, Fungal Biodiversity Centre, Utrecht, Netherlands; CRBO, Centre de Ressources Biologiques Œnologie, Villenave d'Ornon, France; HGU, Hochschule Geisenheim University, Geisenheim, Germany; ICV, Institut coopératif du vin, Lattes, France; Inter-Rhone, Inter Rhône, Avignon, France; ISA, Instituto Superior de Agronomia, Lisbon, Portugal; ISVV, Institut des Sciences de la Vigne et du Vin, Villenave d'Ornon, France; IUVV, Institut Universitaire de la Vigne et du Vin Jules Guyot, Dijon, France; Microflora, Microflora, Villenave d'Ornon, France; UFPE, Federal University of Pernambuco, Recife, Brazil; UNIB, Université de Brest, Brest, France; UNIFG, University of Foggia, Foggia, Italy; UNINA, University of Naples Federico II, Napoli, Italy; UNISTRA, University of Strasbourg, Strasbourg, France; UWOPS, Culture collection of the University of Western Ontario, London, On, Canada; NA, Not Available.

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

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REVIEW
published: 15 February 2018
doi: 10.3389/fmicb.2018.00241

Yeast Immobilization Systems for Alcoholic Wine Fermentations: Actual Trends and Future Perspectives

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Yeast immobilization is defined as the physical confinement of intact cells to a region of space with conservation of biological activity. The use of these methodologies for alcoholic fermentation (AF) offers many advantages over the use of the conventional free yeast cell method and different immobilization systems have been proposed so far for different applications, like winemaking. The most studied methods for yeast immobilization include the use of natural supports (e.g., fruit pieces), organic supports (e.g., alginate), inorganic (e.g., porous ceramics), membrane systems, and multi-functional agents. Some advantages of the yeast-immobilization systems include: high cell densities, product yield improvement, lowered risk of microbial contamination, better control and reproducibility of the processes, as well as reuse of the immobilization system for batch fermentations and continuous fermentation technologies. However, these methods have some consequences on the behavior of the yeasts, affecting the final products of the fermentative metabolism. This review compiles current information about cell immobilizer requirements for winemaking purposes, the immobilization methods applied to the production of fermented beverages to date, and yeast physiological consequences of immobilization strategies. Finally, a recent inter-species immobilization methodology has been revised, where yeast cells are attached to the hyphae of a Generally Recognized As Safe fungus and remain adhered following loss of viability of the fungus. The bio-capsules formed with this method open new and promising strategies for alcoholic beverage production (wine and low ethanol content beverages).

Keywords: yeast immobilization, wine, yeast biocapsules, fermentation, yeast metabolism

OPEN ACCESS

Edited by:
Aline Lonvaud,
Université de Bordeaux, France

Reviewed by:
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Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: *10 December 2017*
Accepted: *31 January 2018*
Published: *15 February 2018*

Citation:
Moreno-García J, García-Martínez T, Mauricio JC and Moreno J (2018) Yeast Immobilization Systems for Alcoholic Wine Fermentations: Actual Trends and Future Perspectives. Front. Microbiol. 9:241. doi: 10.3389/fmicb.2018.00241

INTRODUCTION

Yeast immobilization offers numerous opportunities for industrial fermentation processes such as beer, cider production, or winemaking. This technology aims to confine intact, active yeast cells to a specific region, thus increasing the cell density, permitting the enhancement and prolongation of certain metabolites (e.g., aromatic) production, allowing better control and stability of the yeast strain, providing cell protection against shear forces, and enabling cell recovery/reutilization and

continuous fermentations, among other advantages (Williams and Munnecke, 1981; Groboillot et al., 1994; Sakurai et al., 2000; Kourkoutas et al., 2004b; Baptista et al., 2006; Nedović et al., 2015).

Although this technology reduces process cost and allows customization of wine properties, industrial use of immobilized cells is still limited (Djordjevic et al., 2016; Berbegal et al., 2017). Nedović et al. (2015) proposed that future investigation should approach the storage of immobilized cells long-term and new designs of the processes and bioreactors that are simple, flexible, and non-expensive and can be readily scaled up. Moreover, to accomplish crucial factors in winemaking like consumer acceptance, safety issues, and profitability, Kourkoutas et al. (2004b) recommended supports that are abundant in nature, cost-effective, and of food-grade quality for successful industrial application. Nevertheless, questions such as "what particular immobilization system utilize in what wine elaboration process" or "how immobilization affects cell physiology, flavor formation, and wine stability – including microbial, chemical, and sensorial" still need to be addressed in order to promote yeast immobilization technologies in wine industrial processes.

The overall objective of this review is to compile the most updated information about the requirements of cell immobilizers for winemaking, the immobilization systems applied and proposed to the production of wine (including advantages and drawbacks), and yeast physiological consequences of immobilization strategies. Special attention was placed on inter-species immobilization methodologies, which are considered novel approaches for winemaking and other fields of applications. This is the case for "yeast biocapsules" which consist of yeast cells attached to the hypha of a dead filamentous fungus cataloged as Generally Recognized As Safe (GRAS).

CELL IMMOBILIZER REQUIREMENTS FOR WINEMAKING PURPOSES

Accurate selection of the immobilization method and the carrier material (with consideration of legality and stability, safety, operating costs, and product quality) is essential. Among the production systems that have been the subject of investigations, some seem to fulfill the above prerequisites and lead to promotion of aroma formation during the fermentation process and improvement of the overall sensory characteristics of the final products, e.g., wine, beer, and cider. However, actions should be also focused on economical, abundant, non-damaging, and food-grade immobilization supports, which will ameliorate quality and provide a singular aroma profile and fine taste to the final product. In general, for alcoholic beverage production purposes, the cell carrier has to comply with certain requirements as follows (Martin and Etievant, 1991):

(i) Big surface, with functional properties and/or chemical groups favoring cells to adhere.
(ii) Easy to handle and regenerate.
(iii) High and retained cell viability and operational stability.
(iv) Catalytic activity not affected.
(v) Uniform and controllable porosity to allow free exchange of substrates, products, cofactors, and gases.
(vi) Good mechanical, chemical, thermal, and biological stability.
(vii) Easy, cost-effective, and amenable to scale-up immobilization technique.
(viii) Not affect product quality.

IMMOBILIZATION METHODS DEPENDING ON THE YEAST CELL LOCALIZATION

To date, different methods for yeast immobilization have been developed depending on the mechanism of cell localization (**Figure 1** and **Table 1**).

Auto-Immobilization

Winemakers benefit from the ability of certain yeasts species to auto-immobilize in an innate way. From a biological point of view, immobilization favors yeast cells as it allows cell cooperation to fully utilize available resources and maximize chances of survival through improved resistance to stress (Honigberg, 2011). Microorganisms, notably *Saccharomyces cerevisiae* can perform various multi-cellular manners of immobilization: adhesion, biofilm formation, filament formation, and flocculation. The effect of some of these behaviors on the wine quality is widely known to be beneficial and is already applied industrially. This is the case of yeast biofilm formation for biological aging in the elaboration of Sherry wines and flocculation for the second fermentation of sparkling wines.

Yeast immobilization in biofilms is formed spontaneously in the wine-air interface of wines that are stored in barrels during a

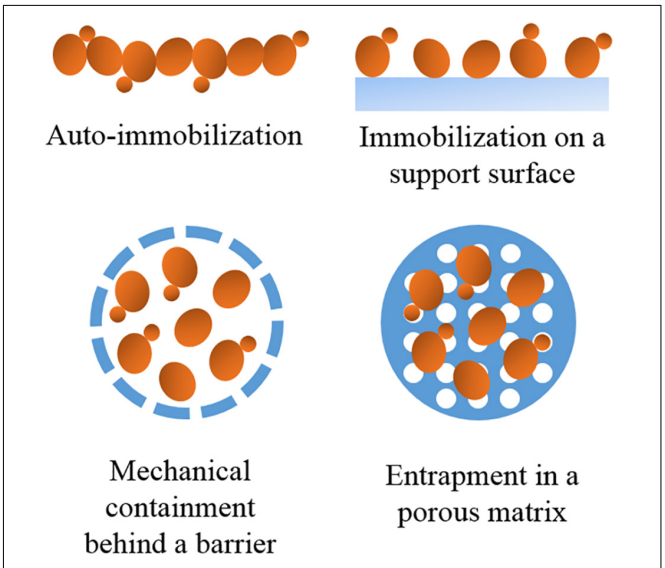

FIGURE 1 | Basic methods of cell immobilization depending on the cell localization.

TABLE 1 | Methods of yeast immobilization: brief description, advantages, disadvantages, and examples of applications in winemaking.

Methods of immobilization	Brief description	Advantages	Disadvantages	Examples in winemaking (proposed or industrially applied)
Auto-immobilization	Innate ability of cells to aggregate (i.e., adhesion, biofilm formation, filament formation, and flocculation).	Beneficial effects on wine quality and industrially used.	Sensitive to factors like pH, medium, composition, O_2 content, etc.	Biofilms of flor yeasts are traditionally used in biological aging for Sherry wine elaboration. Biofilms were also proposed for the reduction of the ethanol content in wines (Moreno et al., 2016). Flocculation is being used for wine clarification in sparkling wine production.
Immobilization on a support surface	Adsorption of cells to a carrier by cell membrane-immobilizer covalent bonding or by electrostatic forces.	Cheap carrier materials and ease of carrying out the process.	Depth and bonding strength of the cells are not determined. Potential detachment of yeast cells.	Cellulose covered with Ca-alginate and DEAE-cellulose covered with an anion-exchange resin were recommended by Lommi and Advenainen (1990) while gluten pellets and delignified cellulosic materials were recommended for room and low-temperature industrial fermentations (Bardi and Koutinas, 1994; Bardi et al., 1996a,b, 1997; Mallouchos et al., 2003). Fruit pieces were investigated for continuous processes (Kourkoutas et al., 2001, 2002, 2003a,b), and combined alcoholic-malolactic fermentations (Mallouchos et al., 2002; Genisheva et al., 2014).
Mechanical containment behind a barrier	Cells are entrapped in microporous or ultraporous membrane filters, microcapsules or on an interaction surface of two immiscible liquids.	Useful when minimal transfer of compounds or cell-free products is needed.	Cell loss during mass transfer and possible membrane biofouling.	Glass pellets coated with a membrane of alginates were proposed for batch and continuous winemaking processes (Ogbonna et al., 1989), "Millispark" cartridge for sparkling wine production (Ramon-Portugal et al., 2003) and a two-vessel bioreactor system (one operated as a membrane bioreactor) employed for continuous dry winemaking (Takaya et al., 2002).
Entrapment in a porous matrix	Cells incorporation to rigid networks.	Prevention of cell diffusion and allowance of transfer of substrates and metabolism products.	High cost, low mechanical, and chemical stability. The biomass entrapped in a gel matrix is critical for usage of biotechnological processes utilizing viable immobilized yeast cells.	Ca-alginate gels were promoted for clarification in sparkling winemaking (Colagrande et al., 1994; Fumi et al., 1988), cell-recycle batch process and optimization of primary must fermentations (Suzzi et al., 1996), increase glycerol of wines (Ciani and Ferraro, 1996; Ferraro et al., 2000), treatment of sluggish and stuck fermentations (Silva et al., 2002), removal of ethanol or toxins (Canonico et al., 2016; Farbo et al., 2016), and simultaneous alcoholic-malolactic wine fermentations (Bleve et al., 2016).
Natural supports	Principle of food-grade purity and used with slightest or no pre-treatment.	High abundance, low cost, and food-grade nature.	Degradation process of the supports not evaluated. Industrial scale-up not described.	Delignified cellulosic material, gluten pellets, grains, and fruit pieces were proved effectively for winemaking. Yeast immobilized in a GRAS fungi (yeast biocapsules) has been tested for white wine, sparkling wine, and natural sweet wine elaboration (Peinado et al., 2004; Puig-Pujol et al., 2013; García-Martínez et al., 2015). Usage of corn grains for ambient/low temperature batch fermentations was found adequate (Kandylis et al., 2012). Delignified cellulosic material was proven for simultaneously alcoholic-malolactic fermentations (Servetas et al., 2013).

(Continued)

TABLE 1 | Continued

Methods of immobilization	Brief description	Advantages	Disadvantages	Examples in winemaking (proposed or industrially applied)
Organic supports	Synthetically made (e.g., plastic) or extracted from natural sources by more complex processes (e.g., polymeric hydrogels) regardless of their food-grade purity.	Ability to gel under mild conditions and form spherical beads that protects against contamination and inhibitory substances while favoring substrate utilization and enhancing stability, flavor productivity and efficiency.	High costs, low mechanical, and chemical stability.	Alginate gels have been commercially applied for sparkling wine production (Fumi et al., 1988; Busova et al., 1994; Colagrande et al., 1994). Organic supports have been also successfully applied to: continuous fermentations (Ogbonna et al., 1989), pomegranate winemaking at ambient temperatures (Sevda and Rodrigues, 2011), wine produced from the tropical fruit cagaita (Oliveira et al., 2011), wine from cabernet sauvignon and pinot noir grape varieties (Andrade Neves et al., 2014).
Inorganic supports	Not organic materials like porous ceramics, porous glass, polyurethane foam, etc.	Usually abundant and can improve fermentation productivity and aroma.	Strong changes in cell metabolism and viability. High concentrations of mineral residues.	Mineral kissiris proposed in low temperature winemaking (Bakoyianis et al., 1992, 1993), γ-alumina and kissiris for continuous or batch alcoholic fermentations (Kana et al., 1992; Loukatos et al., 2000). Gellan particles cross-linked with magnesium acetate were proposed for alcoholic fermentation of grape must (Iurciuc et al., 2016).

process that is known as "biological aging." This type of biofilm is called "flor" or "velum" – formed by special yeast strains known as "flor yeasts" – and protects wine from oxidation and influences the sensory properties of Sherry type wines. The yeast metabolic activity mainly results in a consumption of ethanol and glycerol – the major carbon sources – and production of acetaldehyde – the main metabolite liberated into the aged wine. Additionally, consumption of ethanol raises the contents of acetic acid, acetoin, and 2,3-butanediol and promotes their inclusion as carbohydrates, lipids, and proteins into yeast cells via the Krebs Cycle (Martínez et al., 1998; Zara et al., 2010; Moreno and Peinado, 2012; Moreno-García et al., 2013, 2014, 2015a,b, 2017). The resulting wines are characterized by sensorial characteristics known as flor or velum bouquet (López-Alejandre, 2005).

Cell flocculation consists of non-sexual aggregation of single-celled organisms in suspension to form a larger unit or aggregates of many cells known as flocs (Jin and Speers, 1998). The large size of the flocs makes their potential use in reactors feasible. It is considered the simplest and cheapest immobilization technique although it is easily influenced by several factors like cell wall composition, medium, pH, and dissolved oxygen (Kourkoutas et al., 2004b; Nedović et al., 2005). It is used in the production of sparkling wines, such as Champagne, performed by the "Champenoise" technique, which consists of a second fermentation in a sealed bottle of a base wine previously obtained by fermentation of a grape must. In the last phase of this course, the bottles are turned down and yeast cells deposit on the neck of the bottle. Here, the utilization of flocculent yeast cells is important as it eases the process of removing cell deposit from the bottle, clarifying the wine, and reducing wine losses (a process called dégorgement) (Valles et al., 2008). Simultaneously, yeast immobilization through flocculation reduces the wine production costs as there is less energy expended, thus turning into a 'greener' process that could enhance the quality of final products. It is also used in the brewing industry as packed-bed or fluidized-bed or even continuous stirred-tank reactors (Kourkoutas et al., 2004b) and it affects fermentation productivity and quality, as well as yeast removal and retrieval. Agents or cross-linkers can enhance flocculation of cells that do not spontaneously aggregate.

Immobilization on a Support Surface

Immobilization on a support surface is defined as the binding of yeast cells to a carrier by covalent bonding between the cell and the support, or by adsorption (ionic bonds or electrostatic forces). Examples of known support surfaces are cellulosic materials like diethylaminoethyl-cellulose (DEAE-cellulose), delignified sawdust, sawdust, and wood; or inorganic materials like hydromica, montmorillonite, palygorskite, porous glass, and porous porcelain. This method has been widely applied due to low cost of used immobilization materials, such as cellulosic and inorganic materials, and the simplicity of achieving the process. However, the depth of the cell biofilm and the bonding strength often vary and are not readily determined. As cells are directly exposed to the solution, detachment and relocation are possible while yeast growth.

Among the cellulosic material, fruit pieces, delignified cellulosic materials (DCMs), and gluten pellets (GPs) have been applied in winemaking. Fruit pieces ease the immobilization methods required. Apple and quince constitute abundant and low price supports of food-grade purity of immobilization that were found suitable for continuous processes and lead to production of improved sensory traits (Kourkoutas et al., 2001, 2002, 2003a,b). Further, grape skins were used to immobilize *S. cerevisiae* because of easy application, increased productivity, and positive influence on wine aroma compared to free cells (Mallouchos et al., 2002). This support was established by these authors as suitable for winemaking and proposed for future investigation to their utilization in combined alcoholic fermentation (AF) and malolactic fermentations (MLFs). On the other hand, DCM and GP were considered effective in carrying out fermentations at both room- and low-temperature as well as increasing rates and improving organoleptic quality compared to free cells (Bardi and Koutinas, 1994; Bardi et al., 1996a,b, 1997; Mallouchos et al., 2003). DCM and GP were proposed to use at industrial levels because they are inexpensive and abundant supports of food-grade purity that are easy to produce industrially. In comparison with other natural supports, they lower fermentation rates, present a longer operational stability, are suitable for low-temperature winemaking, and also accepted by consumers. Yeast cells immobilized with DCM and GP were found to fit commercialization objectives through freeze-drying techniques as the freeze-dried immobilized yeasts produced wines of similar quality to those made by fresh immobilized yeast cells and of enhanced properties in comparison with free cells (Iconomopoulou et al., 2000, 2002, 2003; Bekatorou et al., 2001). This last feature makes DCM and GP attractive for industrial use.

Inorganic support surfaces (e.g., palygorskite, hydromica, and porous porcelain) were shown to have mainly few advantages in winemaking (Hamdy et al., 1990; Colagrande et al., 1994). Researchers recommended the utilization of cellulose and DEAE-cellulose (as main carrier) covered with Ca-alginate and an anion-exchange resin, respectively, as immobilization supports for winemaking; the first for continuous winemaking purposes (Lommi and Advenainen, 1990). Increases of calcium ion contents and off-flavors due to the use of alginate or DEAE-cellulose, respectively, must be considered in winemaking processes.

Mechanical Containment behind a Barrier

The most common are the microporous or ultraporous membrane filters and the microcapsules. They are utilized when the minimal transfer of compounds or cell-free products is necessary (Park and Chang, 2000). This cell immobilization type can be attained by three methods: (i) by utilization of microporous membrane filters, (ii) by entrapment of cells in a microcapsule, or (iii) by cell immobilization on to an interaction surface of two immiscible liquids. It has been used in winemaking, and however, its use is limited because of loss during mass transfer (Lebeau et al., 1998) and potential membrane biofouling caused by cell growth (Gryta, 2002). "Millispark" cartridge developed by Millipore is an example utilized for secondary fermentation in a bottle of sparkling winemaking (Ramon-Portugal et al., 2003). *S. cerevisiae* and *Schizosaccharomyces pombe* were co-immobilized on glass pellets coated with a membrane of alginates to further use them for batch and continuous winemaking processes (Ogbonna et al., 1989). Wines with similar features to those produced with free cells were obtained. Takaya et al. (2002) reported that a system consisting of two-vessel bioreactor (one operating as a continuous stirred tank reactor and the other one as the membrane bioreactor), where cells were entrapped by a cross-flow type microfilter, was suitable for continuous dry winemaking and had 28-fold higher production than a batch system. Moreover, microfiltration and ultrafiltration membranes as well as silicon, ceramic, and other membranes have been employed.

Entrapment in a Porous Matrix

Entrapment in a porous matrix is attained when cells are incorporated in a rigid network which prevents them from diffusing into the neighboring medium while still admitting mass transfer of substrates and metabolism products. They are divided in two methods: (i) cells infiltrate into the porous matrix until their motility is interfered by other cells and (ii) the porous material is assembled *in situ* into a culture of cells. Some examples are polysaccharide gels like alginates, *k*-carrageenan, agar, chitosan, and polygalacturonic acid or other polymeric matrixes like gelatine, collagen, and polyvinyl alcohol (Norton and D'Amore, 1994; Park and Chang, 2000). One of the problems of this technique is cell release when located on the outer surface of the matrix. To bypass this possibility, double layer beads have been used (Tanaka et al., 1989; Taillandier et al., 1994; Ramon-Portugal et al., 2003). In general, the use of polysaccharide hydrogels and alginates is not a suitable industrial choice for several reasons: (i) high prices, (ii) low mechanical and chemical stability that causes cell and residues release in wine, and (iii) biomass entrapped in a gel matrix that is critical for utilization of biotechnological processes using viable immobilized cells (Kourkoutas et al., 2004b).

Salts like Na-, Ca-, or Ba-alginate have been extensively used for cell immobilization, and among them, Ca-alginate gels are the most advisable for AF (Colagrande et al., 1994). Notably, Ciani and Ferraro (1996) proposed a system entrapping *Candida stellata* in Ca-alginate gels as an attractive system to increase glycerol content in wine. These authors reported a 30-fold improvement in fermentation rate (g of CO_2/day) in comparison with free cells and twofold production of ethanol and a reduction in acetaldehyde and acetoin production. Moreover, Ferraro et al. (2000) attempted to scale up the immobilization systems to pilot and industrial scales. They revealed an interesting flavor profile of wines produced when co-inoculated with *S. cerevisiae*, and however, the wild wine microbiota was not completely repressed. Ca-alginate beads have also been recommended to entrap highly flocculent *S. cerevisiae* strains to perform cell-recycle batch process and optimize primary must fermentations (Suzzi et al., 1996). Another application of Ca-alginate cell entrapment is the secondary fermentation in sparkling winemaking for easy clarification and removal of cells. *S. cerevisiae* strains are being

immobilized for this purpose and commercially applied in winemaking processes (Fumi et al., 1988; Colagrande et al., 1994). *S. cerevisiae* encapsulated in Ca-alginate were also utilized with success for the treatment of sluggish and stuck fermentations and revealed better results than the traditional free cells method – the system attained a decrease rate of 2.8 g/L × day of reducing sugar with a viable cell concentration of 5×10^6/mL and no increase in off-flavor content or volatile acidity (Silva et al., 2002). According to the winemakers, one of the major drawbacks of calcium salt-based systems is the high Ca^{2+} content provoked by the low solubilization of calcium tartrate in the bottled wine.

IMMOBILIZATION METHODS DEPENDING ON THE CHEMICAL COMPOSITION OF THE CARRIER

The materials used as immobilization supports (carriers), can be divided, based on their origin, into natural materials and artificially treated materials; according to Kourkoutas et al. (2010) and Nedović et al. (2010), they can be categorized as shown in **Table 1**.

Natural Supports

Carrier materials are mainly of food-grade purity and are used with minimal to no pre-treatment; like brewer's spent grains, DCM, GP, pieces of fruit, sawdust, wood, etc. Their abundance, low cost and food-grade composition have made them an interesting way to enhance the aroma character of many products, e.g., wine, beer. The utilization of natural supports such as DCM, GP, grains, and fruit pieces, for immobilization, was proved effective for winemaking as previously mentioned (see the section "Immobilization on a Support Surface"). Natural materials with certain food-grade meet the prerequisites for the selection of the carrier and result in promoting aroma formation and advancement of the sensory features of the final fermented product. *S. cerevisiae* cells immobilized in corn grains were considered a good candidate system because it was efficient for fermentations at ambient and low temperatures during repeated batch fermentations of grape must (Kandylis et al., 2012).

Organic Supports

Organic supports are artificially made (e.g., plastic) or obtained from natural sources by more complicated techniques (e.g., polymeric hydrogels) regardless of their food-grade composition. Natural or synthetic polymers have been widely researched most probably due to their gel-forming ability under gentle conditions and the capacity to form spherical beads that protect yeast cells against contamination and inhibitory substances while favoring substrate utilization and improving stability, flavor production, and efficiency (Nedović et al., 2010). Most used are those comprised of alginates, cellulose, carrageenan, agar, pectic acid, and chitosan. Ca-alginate gels among them are more convenient for AF (Colagrande et al., 1994), and however, the use of alginates and polysaccharide hydrogels generally did not offer a favorable industrial alternative as previously explained (see the section "Entrapment in a Porous Matrix"). Most attempts were made for the utilization of alginate gels for the second fermentation in order to improve the technology of sparkling wine and have been commercially applied (Busova et al., 1994; Colagrande et al., 1994; Fumi et al., 1988). Immobilization of yeasts in organic supports has also been successfully applied to the following: mead production (Pereira et al., 2014), pomegranate winemaking (Sevda and Rodrigues, 2011), wine made from the tropical fruit cagaita (Oliveira et al., 2011), wine from Cabernet Sauvignon or Pinot noir grape varieties (Andrade Neves et al., 2014), green beer production (Wang et al., 1989), stout beer production (Almonacid et al., 2012), lager-beer (Naydenova et al., 2013), and cider (Nedovic et al., 2000).

Inorganic Supports

Several inorganic materials such as porous ceramics, porous glass, polyurethane foam, etc., have been introduced as yeast cell carrier materials for many fermentation processes: beer production (Virkajärvi and Pohjala, 2000; Virkajärvi et al., 2002; Kourkoutas et al., 2004b) and wines (Ogbonna et al., 1989; Bakoyianis et al., 1992, 1993; Kana et al., 1992; Loukatos et al., 2000; Bonin and Skwira, 2008). However, even though they are usually abundant and can improve fermentation productivity and aroma, they can experience strong changes in metabolism and viability as the cells used in artificial immobilization methods are not in their natural form. Also, they are usually considered undesirable for winemaking due to high concentrations of mineral residues found in the product. Nonetheless, their use in immobilization systems can be regarded as promising for use in bioethanol or distillates production.

Other Materials

Other methods of immobilization such as membrane systems, entrapment by various types of interaction (i.e., Van der Waals' forces, ionic bonds, hydrogen bridges) and multi-functional agents – several functionalities integrated into a single miniaturized device (i.e., glutaraldehyde-based system) – are scarcely treated. As earlier cited, Takaya et al. (2002) revealed a membrane-based bioreactor as a good candidate system for continuous dry wine production. Ligno-cellulosic materials from agricultural wastes can be valuable substrates for immobilization, after removal of the lignin fraction from the cellulose matrix by an alkaline treatment in view to create tubular cellulose–based (TC) nanoestructures.

YEAST PHYSIOLOGICAL CONSEQUENCES OF IMMOBILIZATION STRATEGIES

Cell growth, physiology, and metabolic alterations may be induced by immobilization although they are hard to predict (Melzoch et al., 1994; Norton and D'Amore, 1994; Walsh and Malone, 1995; Djordjevic et al., 2016).

Assays comparing immobilized and free cells have revealed effects on increase in stored polysaccharides, altered growth rates, lower yield of fermentation by-products, activation of yeast energetic metabolism, increased substrate uptake and

product yield, higher intracellular pH, increased resistance against toxic and inhibitory compounds, and increased invertase activity (Norton and D'Amore, 1994). Immobilization of yeast to various solid surfaces affects intrinsic cell growth rate, which either increased (Bandyopadhyay and Ghose, 1982) or decreased (Doran and Bailey, 1986). The pH in immobilized *S. cerevisiae* cells in alginate beads is lower than in free yeasts, 6.8 and 6.9, respectively, which was attributed to increased permeability of the cell membrane for protons, leading to a higher ATP utilization and activating glycolysis and glucose uptake (Galazzo and Bailey, 1990). This results in an increased enzyme activity and thus more substrate channeled to biomass and ethanol production. Norton and D'Amore (1994) reported an enhanced ethanol resistance, a partial removal of substrate inhibition by cell immobilization, and higher tolerance to toxic compounds. These authors suggested that an increased ethanol tolerance might be due to a modification in concentration of membrane fatty acid because of oxygen diffusion limitations or simply due to cell encapsulation by a protective layer of the immobilization material. On the other hand, the tolerance to toxic compounds can be indirectly related to osmotic stress that leads to intracellular production of compounds like polyols that regulate pressure, which also leads to diminished water activity and consequently increased tolerance to toxic chemicals (Norton and D'Amore, 1994). Finally, Lodato et al. (1999) showed higher thermal stability in immobilized yeasts.

In immobilized cell fermentations, increased ester and decreased fusel alcohols formation and the ratio of esters to alcohols have the highest influence on beverage technology (Bardi and Koutinas, 1994; Mallouchos et al., 2003). Some trials have been attempted to model the accumulation of dominant yeast metabolites produced by free and immobilized cells (Vassilev et al., 2013). Nagarajana et al. (2014) observed a permanent pattern of gene expression different from starving planktonic cells: highly expressing genes in cell wall reassembling and stress tolerance, glycolysis, but decreasing transcription of genes that regulate the cell cycle and in the tricarboxylic acid cycle. Consequently, changes in concentrations of metabolites are observed when using entrapped or adsorbed yeast cells. Special attention has to be given to compounds such as alcohols (ethanol, higher alcohols), carbonyl compounds (acetaldehyde, vicinal diketones), esters (acetate esters, medium-chain fatty acid esters), organic acids (medium-chain fatty acids), and sulfur compounds (hydrogen sulfide, sulfur dioxide, dimethyl sulfide) as they are those that most affect flavor during fermentation (Dufour et al., 2003; Nedović et al., 2015). In fermented beverages, the greatest aroma impact is due to increased esters, decrease of fusel alcohol concentrations, and ratio of esters/alcohols from fermenting yeast metabolism (Bardi and Koutinas, 1994; Mallouchos et al., 2003; Nedović et al., 2015). In white wine production, it was detected a significant difference in sensory properties among free and immobilized cells (Mallios et al., 2004; Tsakiris et al., 2004a,b; Genisheva et al., 2012). Kourkoutas et al. (2004b) noted a stronger flavor and aroma in semi-sweet wines when immobilizing yeasts. Kourkoutas et al. (2004a), Tsakiris et al. (2004a,b), and Gonzalez-Pombo et al. (2014) did not report an important influence on the pleasantness of wine. A slight difference was revealed in the scores for preference of the produced wines, where scores were higher when using immobilized cells compared to free cells (Tsakiris et al., 2004a,b). These authors also found that temperature is an important factor for wines elaborated with immobilized yeasts at lower temperatures, which were preferred by the consumers. Another aim of yeast immobilization in winemaking is the removal of the off-flavor aroma compounds, as well as the de-acidification of wines to enhance the organoleptic features of the final product (Genisheva et al., 2012, 2013, 2014; Vilela et al., 2013). Nedović et al. (2015) suggested more attention to be placed on evaluation of sensory quality of wine produced by free and immobilized cells with a trained panel or consumers along with instrumental analyses in order to assess the quality of final products and further support the development of acceptable products before being marketed.

NEW TRENDS OF YEAST IMMOBILIZATION IN WINEMAKING

During the last few years, novel yeast cell immobilization systems have been designed to adapt to winemaking purposes. New materials such as spherical gellan particles cross-linked with magnesium acetate were found suitable for AF of grape must (Iurciuc et al., 2016). In this way, Kumar et al. (2016) studied the use of nano-/micro-porous of cellulosic materials (TC) produced by delignification of mango (*Mangifera indica* L.), sal (*Shorea robusta* G.) sawdust and rice husk (*Oryza sativa* L.) in various food bioprocesses. They conclude that the porous structure of TC renders it suitable for use as carrier for yeast immobilization in AF and also as filter material in microorganism removal processes. The TCs used as *S. cerevisiae* cells immobilization carriers for AF of grape must and glucose media at 15°C provide satisfying fermentation rates, high ethanol content and productivity, and volatile by-products production. In addition, advanced applications in winemaking and co-immobilization of different organisms in different carriers, same carrier, or among each other (exploiting adherence properties of organisms) were recently described.

Canonico et al. (2016) co-immobilized non-*Saccharomyces* yeasts in Ca-alginate to perform sequential fermentations coupled with a final inoculation of free *S. cerevisiae* cells to reduce ethanol content in wine. The yeasts immobilized were Crabtree negative (sugar consumption by respiration and low ethanol yield) and naturally present on grapes and winemaking equipment. The strategy resulted in high reaction rates, avoidance of contamination where the sugar content was reduced to a 50% in 3 days and the ethanol up to 1.6% v/v, and in less prolonged time than in non-immobilized formats. An enhancement of the analytical profile of wine was observed for most of the yeasts immobilized. Although this produced promising results, Canonico et al. (2016) recommended further research because the system submitted uses high inoculation levels and expensive immobilization procedures, which increases the costs of the fermentation process. Then, Moreno et al. (2016) proposed to use the *S. cerevisiae* ability to auto-immobilize in biofilms (i.e., flor velum) to further consume ethanol from a red wine.

In this work, the authors observed a decrease in the ethanol content (also 1.6% v/v) and volatile acidity, favorable effect on the color and astringency, and differences in the content of 1-propanol, isobutanol, acetaldehyde, 1,1-diethoxiethane, and ethyl lactate after a short time (40 days) under velum aging conditions. From a sensory analysis, wines were well accepted by the younger consumers in a panel thus, concluding that flor yeasts auto-immobilization in form of biofilms can be used as fining agents supporting new perspectives for the elaboration of new wine types in an inexpensive manner. Another application of yeast immobilization (*Candida intermedia* yeast cells encapsulated in Ca-alginate and magnetic Ca-alginate beads) was discovered by Farbo et al. (2016) objectified to remove the mycotoxin ochratoxin A from rotten grape juice. Although they obtained significant reductions of over 80%, these authors observed a slow release of the mycotoxin by the yeast carriers.

By using the same carrier, *S. cerevisiae* and lactic acid bacteria *Oenococcus oeni* were confined to operate simultaneously AF and MLF with the purpose of enhancing safety and quality of wine (Servetas et al., 2013; Bleve et al., 2016). In this way, Servetas et al. (2013) co-immobilized *S. cerevisiae* and *O. oeni* in DCM carriers covered with starch gel composite and observed a high efficiency at low temperature fermentations (10°C) obtaining a wine characterized by an increased ester formation and lower higher alcohols. Bleve et al. (2016) co-immobilized the same microorganisms in Ca-alginate beads revealing an efficient performance of Negroamaro must fermentation with a decrease of the time needed to complete AF and MLF, low production of volatile acidity and similar organoleptic traits of the wine obtained than with those using sequential AF-MLF in free cell formats. The yeast and bacteria cells immobilized were reused up to three times with no activity loss. Also, *S. cerevisiae* and *O. oeni* entrapped cells into grape stems/skins were used in sequential AF and MLF obtaining wines with sweet and fruity flavors (Genisheva et al., 2014). Nevertheless, it must be considered that simultaneous AF and MLF could also have severe drawbacks sometimes leading to spoilage wines.

Novel concepts of organism co-immobilization without the need of an external support have arose. This kind of methodology exploits the ability of the organisms used to adhere to external bodies. This is the case of the co-immobilization of yeasts and filamentous fungus categorized as GRAS. It consists of the attachment of yeast cells to the mycelium of filamentous fungus (e.g., *Rhizopus* sp., *Aspergillus niger*, and *Penicillium* sp.) (Peinado et al., 2004, 2005, 2006; Nyman et al., 2013) that can be regarded as a natural immobilization system matrix and complies with several required features for the promotion of industrial application: abundant, cheap, storable for long-terms, non-destructive, food-grade, etc. Co-immobilizing *Penicillium chrysogenum* and yeast cells results in the formation of spherical bodies that are hollow, known as "yeast biocapsules" (**Figure 2**). The system minimizes changes to the yeast metabolism and/or yeast viability and enables diffusion of nutrients/products to and from the biocapsules due to the porous structure of the hypha framework (García-Martínez et al., 2011). The yeast biocapsule methodology exploits the natural adhesion properties of yeast (i.e., biofilm formation) and filamentous fungus cells so they attach to each other thus eliminating the need of external supports and decreasing the final price of the process. Future research on the function of the *FLO11* gene as well as other genes involved in biofilm formation, in flor yeast will help boost cell-immobilization methodologies by decreasing the release of yeast cells to the external medium (Nedović et al., 2015).

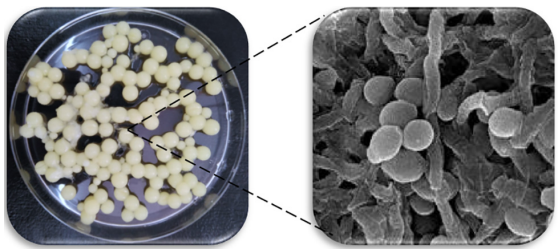

FIGURE 2 | Yeast biocapsules (3–5 mm in diameter) formed with *Saccharomyces cerevisiae* and *Penicillium chrysogenum* **(Left)** and section of the inner wall of a fresh biocapsule exhibiting intact hyphae (filamentous form) with yeast cells (oval shape) photographed in a scanning electron microscope SEM **(Right)**.

García-Martínez et al. (2011) demonstrated the death of the fungus when the yeast biocapsules were incubated in media supporting yeast fermentation by effect of direct contact between its hyphae and yeast cells, and to endure as a mere, but highly inert and stable support for yeast cells, which can ease their reuse. Because of these characteristics, yeast biocapsules have already been utilized in production of white wine, sparkling wine, and natural sweet wine as well as for bioethanol from starch and molasses (Peinado et al., 2005, 2006; García-Martínez et al., 2012, 2013, 2015; Puig-Pujol et al., 2013) in a lab-scale and have been considered a promising technique for industrial-scale fermentation purposes. Comparison of white grapes juice fermentations conducted by yeast biocapsules vs. free yeasts showed higher amounts of acetaldehyde produced by the biocapsules (84 vs. 63 mg/L, respectively), isobutanol (217 vs. 194 mg/l), L-proline (7.7 vs. 6.5 mM), and aspartic acid (0.42 vs. 0 mM) in final wine. All of these analyzed compounds ranged between the limits of concentration values described in the literature and no existence of off-flavors were reported (Peinado et al., 2005). López de Lerma et al. (2012) and García-Martínez et al. (2013) used osmotolerant *S. cerevisiae* strains to form biocapsules to elaborate sweet wine from raisin must to overcome the lag phase of yeasts under osmotic stress. Fermentations resulted in high concentrations of compounds related to osmoregulation like glycerol, acetaldehyde, acetoin among others, leading to an increased complexity of wine aroma (García-Martínez et al., 2013). Biocapsule immobilization was also compared to Ca-alginate beads for sparkling wine elaboration, producing the first wine with lower calcium ion content and improved enological characteristics (Puig-Pujol et al., 2013).

In wine sensory quality analyses, results vary from different studies (Mallios et al., 2004; Tsakiris et al., 2004a,b; Genisheva et al., 2012; Gonzalez-Pombo et al., 2014). Tsakiris et al. (2004a,b)

asked consumers to calculate the pleasantness of red wine samples elaborated with yeast cells immobilized and they noted scores slightly higher for immobilization although not statistically different. Knowledge about the aroma and flavor profile provided by wine yeasts combined with the utilization of mixed non-*Saccharomyces*/*Saccharomyces* starters in immobilized formats for sequential inoculations allow winemakers to use them in a scientifically controlled way to craft wine types that match consumer preferences in a diversified range of market sectors. The utilization of non-*Saccharomyces* yeast combined to *S. cerevisiae* (to avoid stuck fermentations) has been recommended to improve the quality and complexity of wine (Jolly et al., 2014; Capozzi et al., 2015). Hence, the utilization of controlled multi-starter fermentation using previously 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; Morata et al., 2012; Jolly et al., 2014). Yeast cell immobilization will ease sequential inoculations of these yeasts where the selected non-*Saccharomyces* and *Saccharomyces* yeasts are in high concentrations and active; and ferment for a given amount of time one after the other until *S. cerevisiae* is added to conclude the fermentation (Canonico et al., 2016). This practice will allow the non-*Saccharomyces* yeast longer time to express their particular metabolic footprint that would otherwise be inhibited by the stress of *Saccharomyces* competition.

In the last few years, Kandylis et al. (2010), Kourkoutas et al. (2010), and Tsaousi et al. (2010, 2011) have proven the potential of elongated periods of storage for thermally dried immobilized yeast cells in different carriers (delignified brewer's spent grains and DCM, GP, and freeze dried wheat) with neither loss of viability nor fermentation activity and making wines with similar organoleptic characteristics to those of fresh inocula, thereby accentuating the commercial potential for industrial usage. For these reasons, immobilization of microbial cells can improve cell metabolism even under stress conditions (e.g., high sugar content, low and high temperatures) and can be used for biological removal of detrimental compounds (i.e., de-acidification) or controlled liberation of flavor-active compounds, all of which improve the ability of the overall process and the quality of the end products. Indeed, the long-term storage of immobilized cells and their utilization at higher scales will boost the industrialization of immobilized technology in winemaking.

FINAL CONSIDERATIONS

Studies evidence the advantages of immobilized yeast cells in comparison with free yeast cells. Cell immobilization has been proven to be an interesting strategy to overcome some important inconveniences in fermented alcoholic beverages production. However, though many benefits are described and new technologies are still arising, there are not many applications for winemaking at an industrial level. Potential reasons could be as follows: (i) lack of feasibility at the cellar scale – some of the methods may be difficult to up-grade, (ii) insufficient effectivity of yeast cell adherence to the carriers of current immobilization technologies, (iii) high investments (economic and time) to integrate these technologies into traditional practices without a secure outcome, (iv) lack of advertisement on immobilized yeasts, and (v) limited knowledge in winemakers about the yeast immobilization techniques and their benefits.

This review shows how studies concerning immobilization supports and matrix properties, such as their solubility, chemical and mechanical stability, their degradation in different culture broths and physico-chemical conditions during their use in a bioreactor, should be addressed more in depth. In this respect, the spontaneous and inter-species biological co-immobilization system between a GRAS fungus and an industrial yeast strain open new perspectives as a new carrier for improvement of yeast immobilization systems. Furthermore, for the implementation to an industrial scale, a higher scientific knowledge is necessary regarding the influence of immobilization on the physiology of industrial yeast strains and about the metabolites excreted, especially those directly related with the sensorial attributes of the obtained beverages.

CONCLUSION

We think that immobilization systems used for yeast cells provide a revolutionary perspective for the next future in production of wine and other beverages. Their use to carry out addressed and controlled fermentation processes can contribute to innovate the production technology and the design and making of new and differentiated supreme quality products for consumers. The scarceness of industrial applications that exist in the present does not mean that research on yeast immobilization techniques should be abandoned. Aversely, investigation should be boosted in order to find the right immobilization technique for the right application in winemaking in order to exploit all the potential of these promising techniques.

AUTHOR CONTRIBUTIONS

JM-G performed the necessary literature searches, data compilation, and wrote the manuscript. TG-M, JCM, and JM coordinated the work and critically revised the manuscript before submission.

FUNDING

This work was supported by the "XXII Programa Propio de Fomento de la Investigación 2017" (MOD.4.1 P.P.2016 J.J. MORENO V.) from the University of Cordoba (Spain); funding from the Spain's Ministry of Education, Culture and Sport, Grants for training university teachers (FPU) (JM-G).

ACKNOWLEDGMENTS

The authors thank Minami Ogawa for her assistance with English language editing.

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