ADVANCES IN SUSTAINABLE VITICULTURE AND WINEMAKING MICROBIOLOGY

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ADVANCES IN SUSTAINABLE VITICULTURE AND WINEMAKING MICROBIOLOGY

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Advances in Sustainable Viticulture and Winemaking Microbiology is an international scientific research eBook on the context of sustainable viticulture and winemaking development from the microbiological point of view. The Editors welcome the lectors to read multidisciplinary articles that bridge viticulture and winemaking with microbial ecology, environmental and social sciences.

Manuscripts focus on novel findings underlining those relationships. The journal 'Frontiers in Microbiology' published original research articles that demonstrate a clear scientific breakthrough versus current knowledge. This eBook covers application fields such as sustainable viticulture, sustainable winemaking, the climatic global change, the preservation of natural resources and health, agriculture and biodiversity, ecological, economical and social impacts of beverages and food quality and security management and the geographical distribution of yeast and bacteria populations related to winemaking issues of agricultural changes.

'If wine was perfect, there would be no need for microorganisms for a sustainable viticulture and winemaking' - Gustavo Cordero-Bueso

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EDITORIAL
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Editorial: Microorganisms for a Sustainable Viticulture and Winemaking

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Editorial on the Research topic

Microorganisms for a Sustainable Viticulture and Winemaking

During the last decades, wine production in most countries is based on the use of commercial yeast and lactic acid bacteria strains leading to the colonization of the wineries and vineyards by these strains, and in the increasing use of chemical pesticides to control plant diseases or pests management. This signifies the consequent reduction of autochthonous microbial biodiversity. This signifies that wine styles could also become standardized, severely reducing the competitiveness of wines traditionally produced in the EU vs. the new wines elaborated in the emerging vine-growing areas. Moreover, the current climatic change, increasing of population, migration movements and economic changes need new strategies for viticulture and winemaking to ensure an economically or environmentally sustainable and healthy chain of production. Diversity and natural ecosystems could serve winemaking in many different ways, not all of which are well known. Thus, in this context increasing attention is being paid to species isolated from local vines or pristine environments and to their potential for stabilizing yields and reducing losses caused by plant diseases, pests and abiotic stresses and for safety and a better control of the fermentation processes using locally selected yeasts.

The research topic "Microorganisms for a sustainable viticulture and winemaking" belongs to the Food Microbiology section in the Frontiers in Microbiology journal. It covers a review and 14 original research papers. We present an overview of these papers starting with microbial populations associated with grape-berries and wines. Six of the contributions focused on the importance of the use of native microorganisms associated to grape-berries and uninoculated wines, as well as on the synergies and trade-offs that occur using selected native yeast strains as single or mixed starters. Vigentini et al. reported that in-bottle fermentation of sparkling wines is currently triggered by few commercialized *Saccharomyces cerevisiae* strains. This lack of diversity in *tirage* yeast cultures leads to a prevalent uniformity in sensory profiles of the final products. Authors exploited the natural multiplicity of yeast populations to introduce variability in sparkling wines throughout the re-fermentation step, considering it a convenient way for introducing differentiation to the final product without modifying the traditional technology. The work of Padilla et al. aimed at the reproduction of the native microbiota from the vineyard in the inoculum. Native selected *Saccharomyces* and non-*Saccharomyces* yeast species were inoculated sequentially into musts, and wines obtained were of similar quality and clearly differentiated by sensory analysis panelists. The fact that

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the proposed use of new starters using native yeast strains will almost invariably involve either simultaneous or sequential inoculation with *S. cerevisiae* has also driven the attention to the potential biological interactions between different starters during wine fermentation. Curiel et al. delved the response, under aerobic conditions, of *S. cerevisiae* to other two non-*Saccharomyces* species, *Hanseniaspora uvarum* and *Candida sake*, and focusing on the early stages of the interaction. Results point to some common features of the way *S. cerevisiae* modified its transcriptome in front of other yeast species, namely activation of glucose and nitrogen metabolism, being the later specific for aerobic conditions. Sadoudi et al. showed for the first time that the entire acetic acid and glycerol metabolic pathways can be modulated in *S. cerevisiae* by the presence of *Metschnikowia pulcherrima* at the beginning of fermentation. On the other hand, Lleixà et al. emphasized the importance of the concentration of nutrients on the evolution of mixed fermentations and points to the optimal conditions for a stable fermentation in which the inoculated yeasts survived until the end. According to García et al. choosing well the inoculation strategy between *S. cerevisiae* and non-*Saccharomyces* strains are critical to obtain a good quality wine. They analyzed, by real-time quantitative PCR (qPCR) combined with the use of specific primers, the dynamics of mixed and sequential cultures throughout the fermentation process at pilot scale using the Malvar white grape variety.

It is important to notice that the processes of yeast selection for using as wine fermentation starters have revealed a great phenotypic diversity both at the interspecific and intraspecific level, which is explained by a corresponding genetic variation among different yeast isolates. Guillamón and Barrio reviewed the mechanisms involved in generating genetic polymorphisms in yeasts, the molecular methods used to unveil genetic variation, and the utility of these polymorphisms to differentiate strains, populations, and species in order to infer the evolutionary history and the adaptive evolution of wine yeasts, and to identify their influence on their biotechnological and sensorial properties. Molecular tools have widely contributed to the interpretation of gene functionality within haploid isolates, but the genetics of metabolism in relevant polyploid yeast strains is still poorly understood. Vigentini et al. applied Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR-associated protein (Cas) system to two strains of *S. cerevisiae*, eliminating the *CAN1* arginine permease pathway to generate strains with reduced urea production. Moreover, other two original papers focused on models to predict the oenological potential of any given fermented beverage microbiome. In the first one, Bagheri et al. evaluated the complex wine microbiota by a model yeast consortium comprising eight species commonly encountered in South African grape musts and an ARISA based method to monitor their dynamics. The dynamics of these species were evaluated in synthetic must and Chenin blanc grape must fermentations in the presence or absence of *S. cerevisiae* using direct viable counts and ARISA. The data shown that *S. cerevisiae* specifically suppresses certain species while appearing to favor the persistence of other species. Thus, authors concluded that the wine ecosystem could be characterized by both mutually supportive and inhibitory species. Huang et al. proposed metatranscriptomics as a method to comprehensively explore the active microbial community members and key transcripts with significant functions in Chinese liquor starter production processes.

Native lactic acid bacteria (LAB) are also capable of growing during winemaking, thereby strongly affecting wine quality. Recently many winemakers are exploiting the potential of locally selected LAB strains able to be used as starters in detriment of commercial ones. The original research by Miranda-Castilleja et al. evidences the presence of local strains able to be used as starter cultures, and enable the assessment of the risks derived from the presence of spoilage LAB strains resistant to wine-like conditions. Other investigation carried out by Romero et al. revealed the presence of local strains distinguishable from commercial strains at the genetic/genomic level and having genomic traits that enforce their potential use as starter cultures in red wines.

With concern of safety and pest management, three original researches overview different prevention or correction strategies, using selected yeast, from the vineyard to the winery. The increasing level of hazardous residues in the environment and food chains has led the European Union to restrict the use of chemical fungicides. Thus, as stated before, exploiting new natural antagonistic microorganisms against fungal diseases could serve the agricultural production to reduce pre- and post-harvest losses, to boost safer practices for workers and to protect the consumers' health. Cordero-Bueso et al. evaluated the antagonistic potential of epiphytic yeasts against *Botrytis cinerea, Aspergillus carbonarius*, and *Penicillium expansum* pathogen species. In particular, yeast isolation was carried out from grape berries of *Vitis vinifera* ssp *sylvestris* populations, of the Eurasian area, and *V. vinifera* ssp *vinifera* cultivars from three different farming systems (organic, biodynamic, and conventional). They found six strains, all isolated from wild vines, with a notable antifungal action. On the other hand, it is well known that copper is widely used in agriculture as a traditional fungicide in organic farming to control downy mildew on grapes-berries, consequently it is possible to find this metal during all stages of the vinification process. Thus, Capece et al. found a wild yeast strain of *S. cerevisiae* able to complete the alcoholic fermentation and remove the copper from wine. This fact represents a biotechnological sustainable approach, as an alternative to the chemical-physical methods, ensuring at the same time a completed alcoholic fermentation and organoleptic quality of the wine. Furthermore, sulfur dioxide (SO_2) is used commonly to stabilize the final product, but limiting its use is advised to preserve human health and boost sustainability in winemaking. Valdetara et al. investigated the influence of SO_2 in relation with pH and ethanol factors on the expression of several genes and volatile phenol production in *Dekkera bruxellensis* under different model wines throughout a response surface methodology. The obtained results could be useful to improve the SO_2 management at the grape harvesting and during winemaking to minimize the *D. bruxellensis* spoilage.

The varied contributions to this Research Topic are evidence of the study undertaken by researchers that embracing a

sustainable agriculture would bring to the field of food microbiology, while warning that, at least for now, some selected microorganisms could replace agro-chemicals and standardized fermented beverages. Several of the issues surrounding new bioscience techniques, novel information about the selection of yeast starters, and alternatives to the use of some chemical compounds were also raised. We hope that research topic adequately informs readers about the benefits that nature offers to the field of food microbiology and about the many challenges that have yet to be overcome in this field.

AUTHOR CONTRIBUTIONS

GC-B wrote and drafted the Editorial. PI-C and GS drafted and revised the final version of the Editorial.

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ORIGINAL RESEARCH
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Use of Native Yeast Strains for In-Bottle Fermentation to Face the Uniformity in Sparkling Wine Production

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The in-bottle fermentation of sparkling wines is currently triggered by few commercialized *Saccharomyces cerevisiae* strains. This lack of diversity in *tirage* yeast cultures leads to a prevalent uniformity in sensory profiles of the end products. The aim of this study has been to exploit the natural multiplicity of yeast populations in order to introduce variability in sparkling wines throughout the re-fermentation step. A collection of 133 *S. cerevisiae* strains were screened on the basis of technological criteria (fermenting power and vigor, SO_2 tolerance, alcohol tolerance, flocculence) and qualitative features (acetic acid, glycerol and H_2S productions). These activities allowed the selection of yeasts capable of dominating the in-bottle fermentation in actual cellar conditions: in particular, the performances of FX and FY strains (isolated in Franciacorta area), and OX and OY strains (isolated in Oltrepò Pavese area), were compared to those of habitually used starter cultures (IOC18-2007, EC1118, Lalvin DV10), by involving nine wineries belonging to the two Consortia of Appellation of Origin. The microbiological analyses of samples have revealed that the indigenous strains showed an increased latency period and a higher cultivability along the aging time than the commercial starter cultures do. Results of chemical analyses and sensory evaluation of the samples after 18 months *sur lies* have shown that significant differences ($p < 0.05$) were present among the strains for alcoholic strength, carbon dioxide overpressure and pleasantness, whereas they were not observed for residual sugars content, titratable acidity or volatile acidity. Indigenous *S. cerevisiae* exhibited comparable values respect to the commercial starter cultures. The ANOVA has also proven that the base wine formulation is a key factor, by significantly affecting ($p < 0.01$) some oenological parameters of wine, like alcoholic strength, volatile acidity, carbon dioxide overpressure, titratable acidity and dry extract. The use of native yeast strains for the re-fermentation step can be considered a convenient way for introducing differentiation to the final product without modifying the traditional technology. In a perspective of "precision enology," where the wine is designed on specific vine cultivars and microorganisms, this work underlines that exploring yeast biodiversity is a strategic activity to improve the production.

Keywords: Franciacorta, in-bottle fermentation, Oltrepò Pavese, *Saccharomyces cerevisiae*, sparkling wine, yeast strain selection

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INTRODUCTION

The widespread use of selected cultures, commonly found on the market as Active Dry Yeast, is probably the most important innovation that allowed a more effective management of the fermentative process in winemaking since the last century (Pretorius, 2000; Fleet, 2008; Suárez-Lepe and Morata, 2012). Nevertheless, this oenological practice has determined a decrease of diversity in microbial populations involved in fermentation with a consequent reduction of their impact on the sensory characteristics of the final product (Csoma et al., 2010; Di Maio et al., 2012). Actually, it has been widely recognized that each yeast species can contribute to the formation of aromatic compounds through peculiar metabolic pathways and differences in flavor production can be observed at the strain level (Romano et al., 2003; Molina et al., 2009). Despite the high number of starter cultures sold on the market, the available yeast strains are less than what we can think; indeed, manufacturers of different brands often designate the same strain with different codes or names (Fernández-Espinar et al., 2001; Vigentini et al., 2009). The question becomes trickier for the sparkling wines made by the so-called traditional method (*méthode Champenoise*) that require a second in-bottle fermentation of a base wine followed by a prolonged aging over lees. In this case, the commercialized yeast strains are a small number and mostly ascribing to one species, *Saccharomyces cerevisiae* (Torresi et al., 2011; Vigentini et al., 2015; Perpetuini et al., 2016). This condition has led to a widespread homology in organoleptic features of sparkling wines and to a loss of diversity, without exploiting the potential of microorganisms to obtain innovative products by low-aromatic vine cultivars. To overcome these issues, some winemakers used to manage a small amount of must by spontaneous fermentation to enrich the flavor profiles of base wines with the contribution of native yeasts (Vigentini et al., 2014), though the influence of the environmental microorganisms is hardly recognizable. In recent years, many researches have been focused on the selection of indigenous strains to be used as a starter in particular style of wine or in specific regions, with the aim of providing sensory characteristics attributable to the territory of belonging (Capece et al., 2010; Settanni et al., 2012; Suzzi et al., 2012; Tristezza et al., 2012; Rodríguez-Palero et al., 2013; Furdikova et al., 2014; Ilieva et al., 2017). This goal is not easy to carry on for sparkling wine production by traditional method because of the following reasons: first, the starting material is often a mixture of wines and additives (*liqueur de tirage*), formulated by an oenologist according to the cellar style (Pozo-Bayón et al., 2009; Torresi et al., 2011). Besides, the final addition of *liqueur de dosage* can strongly affect the sensory traits (Kemp et al., 2014). Second, several winemakers are convinced that the yeast role in the *prise de mousse* step is only useful for generating the over pressure into the bottle, without significantly influencing the aromatic features. Third, the strain selection for the second fermentation requires long times of testing to verify the effect on characteristics of the sparkling wines and the interactions among environmental and technological factors are difficult to be elucidated (Borrull et al., 2015, 2016).

The in-bottle aging is a complex phenomenon that involves the pivotal roles of the temperature, the base wine formulation and the yeast strain; definitely, an effect on the synthesis and release of aromatic compounds, the cell autolysis, the foaming quality and the bubbling properties of the final product have been demonstrated (Alexandre and Guilloux-Benatier, 2006; Pozo-Bayón et al., 2009; Torresi et al., 2011; Kemp et al., 2014; Perpetuini et al., 2016). In addition, the cellular aptitude to flock is a key point for the selection of strains to be used in traditional method, as it is useful to facilitate the separation process of yeast lees into the bottle by natural settling. The study of genes coding for the flocculent phenotype and their expression in *S. cerevisiae* have revealed the strain specific nature of this property (Tofalo et al., 2014, 2016), even if a high variability in behavior patterns has been observed depending on the environmental conditions and aging time.

The aims of this study were to select indigenous yeast strains throughout consecutive screening steps based on technological and qualitative criteria for sparkling wine-making and to compare the fermentative performances of these strains with those already used by the wine industry in real cellar situations. In particular, we performed the experimental trials at nine wineries of Franciacorta and Oltrepò Pavese areas in Lombardy region, which is the largest Italian district where sparkling wines are produced by traditional method (Vigentini et al., 2014; Foschino et al., 2015), through the involvement of the respective consortia of Appellation of Origin.

MATERIALS AND METHODS

Strain Collection

One hundred and thirty three *S. cerevisiae* strains identified and genotyped in a previous work (Vigentini et al., 2015), were chosen based on their distinctive inter-delta profiles obtained by capillary electrophoresis. Fresh cultures of each strain grown in YPD broth (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose, pH 6.5) at 26°C, for 24 h in orbital agitation (120 rpm), were centrifuged at 3,500 *g* for 15 min and then the cells were resuspended in the same broth added with 25% (v/v) glycerol. Cell suspensions were stored at −80°C or on YPD agar (18 g/L) at 4°C for short-term storage.

Selection for Oenological Traits

Technological characteristics like fermenting power, fermenting vigor and resistance to sulfur dioxide, were preliminarily investigated in order to select strains with oenological potential for sparkling white wine production according to the OIV-OENO Resolution, 370-2012 (2012).

The fermenting power, expressed as % (v/v) ethanol produced, was daily evaluated by monitoring the weight loss for 3 weeks at 18°C in YPD broth added with 260 g/L glucose in static conditions. A 250 mL flask, sealed with a Müller trap and containing 100 mL of the growth broth, was inoculated with 1% (v/v) of a fresh culture, realized as previously described, in order to obtain approximately 1×10^6 CFU/mL starting concentration. The fermenting vigor, expressing the speed at which yeast starts the fermentation, was determined as grams of CO_2 lost in 48 h

from the beginning of the trial. The resistance to sulfur dioxide was examined by spotting 5 μL of fresh culture, onto YPD plates acidified at pH 3.5 with tartaric acid and added with 15 g/L agar. Variable amounts of sterile solution of potassium metabisulfate were previously supplemented to the medium in order to obtain doses of total SO_2 ranging from 100 to 300 mg/L. Resistance degree to sulfur dioxide was reported as the maximum dose at which the yeast exhibited an evident growth after incubation at 26°C for 72 h. A control test without adding solution of potassium metabisulfate was carried out.

A second step of investigation, limited to the strains that passed the first screening phase, was carried out on the characteristics that influence the wine quality like acetic acid, glycerol and hydrogen sulfide productions. The acetic acid and glycerol amounts were assayed in the supernatants at the end of the fermentations of the first set of analysis. Two mL aliquots of cell cultures were centrifuged at 3500 *g* for 15 min and specific enzymatic kits based on spectrophotometric UV method were used (Jenway, UV-visible spectrophotometer, model 7315, Bibby Scientific Limited, Stone, UK), according to the supplier's recommendations (Megazyme International, Bray, Ireland). The synthesis of hydrogen sulfide was estimated by spotting 5 μL of a fresh culture, on BIGGY agar plates (Oxoid limited, Basingstoke, UK). After incubation at 26°C for 72 h the color of the colonies may range from white-cream until brown-black in function of increasing amounts of hydrogen sulfide produced.

A third step of selection, limited to those strains that passed the second screening phase, was performed by assessing the ability of cells to grow in presence of ethanol and by characterizing the flocculent phenotype. The alcohol tolerance test was performed in 100 mL bottles with 75 mL YEPD broth acidified at pH 3.5 with tartaric acid and containing 10% ethanol (v/v), by inoculating a 1% (v/v) of a fresh culture in order to realize an approximately 1×10^6 CFU/mL starting concentration. After inoculation, samples were incubated at 15°C in static conditions and cell growth was monitored every 5 days by Optical Density measurements at 600 nm in U.V-Visible spectrophotometer (Jenway). Flocculation test was carried out according to the protocol of Suzzi and Romano (1991) with some modifications: after the evidence of cell growth ($OD_{600\ nm} > 1.0$) in samples used for the alcohol tolerance test, 3 mL of microbial suspension were taken from there, centrifuged at 2,000 *g* per 5 min and the pellet was resuspended in 3 mL of flocculation buffer (50 mmol/L Na acetate/acetic acid, 5 mmol/L $CaSO_4$, pH 4.5). The $OD_{600\ nm}$ values were immediately measured and after 15 min, by leaving the cuvette at room temperature in a static position. The degree of flocculence for each strain was calculated as follows: $F = OD_{600\ nm\ after\ 15\ min} / OD_{600\ nm\ at\ starting\ time}$ per 100 with scores ranging 0–10 (very flocculent, corresponding to point 4 of Suzzi and Romano's scale), 10–30 (moderately flocculent, point 3), 30–70 (weakly flocculent, point 2), 70–90 (poorly flocculent, point 1), 90–100 (non-flocculent, point 0).

Set Up of the *Tirage* Experiments

Based on the results previously obtained, four strains (FX and FY isolated in Franciacorta, OX and OY isolated in Oltrepò Pavese areas) were selected to be used as starter cultures for the re-fermentation trials of base wines in nine different wineries (**Table 1**). Each tested strain was pre-inoculated in 20 mL of YEPD broth at 26°C for 24 h in shaking state at 120 rpm; then 2 mL of this culture were transferred to 500 mL polycarbonate Erlenmeyer flasks with DuoCAP® (TriForest, Irvine, USA), containing 200 mL of YEPD broth, and incubated at 26°C for 48 h in orbital agitation (120 rpm). After OD measurement at 600 nm, a volume corresponding to a concentration of 5×10^9 cells per mL was centrifuged (Hettich Zentrifugen, Rotina 380 R, Germany) at 3,500 *g* for 15 min; the pellets were then resuspended in 25 mL of YEPD broth and stored at 4°C. The same protocol was carried out for the strains of the commercial starter culture habitually utilized in the relative cellar (**Table 1**), in order to compare the performances under the same conditions. Each cellar used its own base wine, prevalently made with Chardonnay cultivar for Franciacorta wineries and Pinot Noir for Oltrepò Pavese ones (**Table 1**). In each winery three trials were performed in parallel, two by inoculating the indigenous strains of the corresponding territory and one with the usual starter culture strain (**Table 1**). For each trial, 50 L of clarified base wine, with different ethanol content (**Table 1**), was added

TABLE 1 | Information about wine-making of experimental sparkling wine samples.

Winery	Vine-growing area	Prevalent grape cultivar in base wine	Ethanol content (% v/v) in base wine	Indigenous selected strains		Starter culture strain habitually inoculated
I	Franciacorta	Chardonnay	10.5	FX	FY	IOC18-2007†
II	Franciacorta	Chardonnay	11.5	FX	FY	Not disclosed
III	Franciacorta	Chardonnay	11.5	FX	FY	IOC18-2007
IV	Franciacorta	Chardonnay	11.0	FX	FY	EC1118‡
V	Franciacorta	Chardonnay	11.0	FX	FY	DV10‡
VI	Oltrepò Pavese	Pinot noir	11.0	OX	OY	IOC18-2007
VII	Oltrepò Pavese	Pinot noir	11.0	OX	OY	IOC18-2007
VIII	Oltrepò Pavese	Pinot noir	11.0	OX	OY	EC1118
IX	Oltrepò Pavese	Croà	10.5	OX	OY	DV10

† *Institut Oenologique de Champagne, Épernay, France.*
‡ *Lalvin®, Lallemand Oenology, Petaluma, USA.*

with approximately 24 g/L of sucrose and sterilized by filtering. A *pied de cuvee* for each tested strain was prepared by the following steps: 25 mL of the previously concentrated cell suspension were diluted in 250 mL of sterile distilled water, pre-warmed at 30°C; after 30 min, 250 mL of base wine, pre-warmed at 24°C, were added and maintained at the same temperature in a thermostatic room; after 4 h, 500 mL of base wine, pre-warmed at 24°C, and 1 g of yeast autolysate containing ammonium salts, amino acids, thiamine and pantothenic acid (Proteofast, BioEnologia 2.0 S.r.l., Oderzo, Treviso, Italy) were added and thoroughly mixed; after 4 h, 500 mL of base wine, pre-warmed at 24°C, and 2 g of yeast autolysate were added and thoroughly mixed. After a night at 24°C, 1 L of base wine at 20°C and 4 g of yeast autolysate were added and thoroughly mixed. Lastly, the whole *pied de cuvee* (2.525 L) was poured into the 50 L base wine mass, added with 30 mL of adjuvant 83 Liquide (Station Oenotechnique de Champagne, Magenta, France) and mixed thoroughly to form the *liqueur de tirage*. For each trial, approximately 70 Champagne bottles (750 mL type) were filled and equipped with plastic caps (*bidules*), sealed with crown caps and maintained at cellar temperature for 18 months in each cellar.

Monitoring the *Prise De Mousse* Experiments by Microbiological Analysis

The trend of the second fermentation was monitored for each trial by sampling two bottles at the starting time, after 2 weeks and then every month until the fourth one. Cell concentration was determined for each sample by plate count technique (OIV-OENO Resolution, 206/2010, 2010). After appropriate dilution in Peptoned Water (Merck, Germany) 100 μL of sample were spread onto WL agar plates (Merck) and incubated at 25°C for 3 days. Then, after counting, up to four colonies grown in plates at the highest dilutions were randomly isolated by twice streaking, in order to identify the dominant strains through a molecular typing technique. DNA extraction was carried out according to the protocol of Vigentini et al. (2014) and the amplification of inter-delta regions (δ-PCR) was performed to discriminate the isolates (Legras and Karst, 2003). After electrophoretical separation as reported by Vigentini et al. (2014), the obtained inter-delta profiles were analyzed using Quantity One Software (Bio-Rad, CA, USA).

Cell viability was estimated by microscopic technique after applying a methylene blue staining (OIV-OENO Resolution, 206/2010, 2010). Appropriate dilutions of the samples were observed in a Burker counting chamber at a magnification of 400 X (Microscope Standard 25, Zeiss, Germany), within 15 min contact with the stain. Cell viability was expressed as the percentage ratio between the number of the not stained cells (live) and the number of the total observed cells.

Chemical Analysis

At the end of the aging *sur lies* (18 months), two bottles of wine samples for each trials were analyzed for: alcoholic strength (% v/v), glucose and fructose content (g/L), titratable acidity, expressed as tartaric acid (g/L), volatile acidity, expressed as acetic acid (g/L) and total sulfur dioxide (mg/L) according to the standard protocol proposed by OIV (2014). Carbon dioxide overpressure (bar) was measured in one sample per cellar by aphrometric technique (OIV, 2014).

Sensory Evaluation

The sensory evaluation was performed in different sessions on bottled samples at 18 months of aging *sur lies* by a panel of at least 10 skilled judges working at the wineries involved in the project or collaborating with the wine consortia. Yeast precipitates (lees) were previously removed from the tested samples by riddling and disgorging operations; *liqueur d'expedition* was not added. The wine quality was estimated by defining aroma descriptors that were chosen by the taster panels in a previous session according to the rules of respective Appellation of Origin Committees, Consorzio Franciacorta (http://www.franciacorta.net/en/) and Consorzio Tutela Vini Oltrepò Pavese (http://www.vinoltrepo.org/it/eng/). Samples were presented in a blind randomized sequence. Then judges were asked to score the samples on a scale of a pleasantness distributed on a decimal scoring, where point 0 meant extremely unpleasant and point 10 extremely pleasant, by considering the odorous characteristics and the taste, separately.

Statistical Analysis

The effect of some factors, such as the yeast strain inoculated for the developing of the second fermentation, the wine base formulation and cellar conditions, the prevalent grape cultivar worked for the base wine preparation, on some chemical parameters and sensorial evaluations were investigated by one-way ANOVA (Camussi et al., 1986) according to the general linear model. Results of microbiological counts were transformed in the respective decimal logarithms to match a normal distribution of values. Data were processed with Statgraphic® Plus 5.1 for Windows (StatPoint, Inc., Herndon, Virginia, USA). When the effect was significant ($p < 0.05$), differences between means were separated by LSD test of multiple comparisons.

RESULTS

Strain Selection

Figure 1 shows the results obtained through the fermenting power (A) and the fermenting vigor (B) assays. Two-thirds of the 133 investigated strains proved to be able to produce more than 12% (v/v) alcohol in the tested conditions, with an arithmetic mean of 12.4% (v/v). In particular, 68 strains exceeded the median value of 12.6% (v/v). Generally, the *S. cerevisiae* isolates did not exhibit a high fermenting vigor since the average value was 1.53 g of CO_2 per 100 mL within 48 h, even if 27% of them generated more than 2 g; 65 strains were those that overcame the median value of 1.15 g. As regards the tolerance test to sulfur dioxide, 97, 78, and 29% of strains could grow at 100, 200, and 300 mg/L of total SO_2,respectively. Consequently, 46 strains that simultaneously displayed to exceed the median value of the fermenting power, the median value of the fermenting vigor and the high value of resistance to sulfur dioxide, were selected for the next phase of screening.

Quantification assays of the acetic acid and glycerol productions are shown in **Figure 2**. The mean value of the acetic acid production was 0.41 g/L. In the tested conditions, only

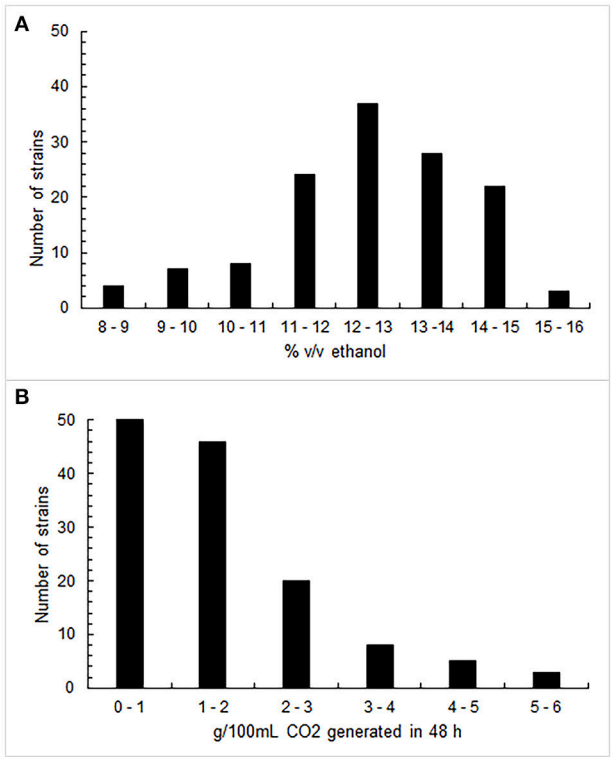

FIGURE 1 | Distribution of fermenting power values **(A)**, expressed as % (v/v) ethanol, and of fermenting vigor values **(B)**, expressed as CO_2 g/100 mL generated in 48 h, by 133 *S. cerevisiae* strains.

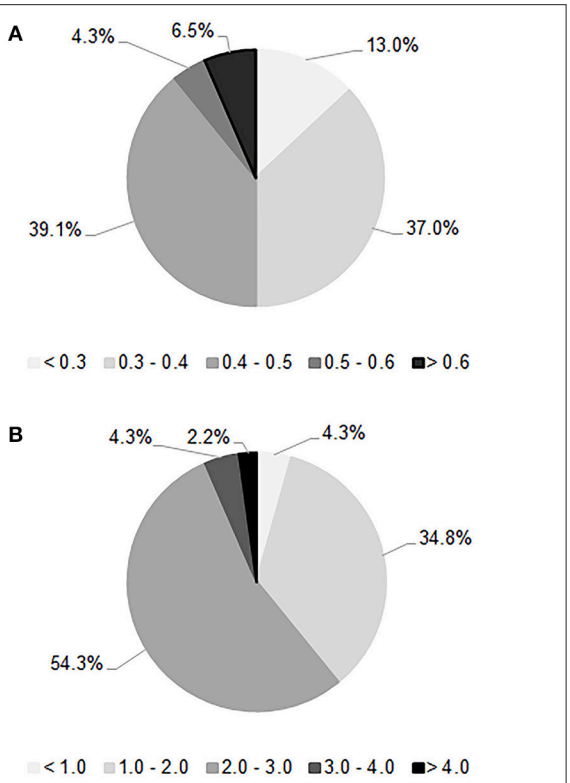

FIGURE 2 | Distribution of the acetic acid **(A)** and glycerol **(B)** amounts, expressed as g/L, produced by 46 *S. cerevisiae* selected strains after the first phase of screening.

six strains developed a low level of volatile acidity (<0.3 g/L), which is crucial for sparkling wine quality. On the other hand, the average amount of glycerol production was 2.18 g/L, a low value compared to the data reported in literature (Scanes et al., 1998; Suárez-Lepe and Morata, 2012); only three strains proved to be able to generate more than 3 g/L of glycerol. The hydrogen sulfide test revealed that 78% of strains were high synthesizers of this compound since they generated brown colonies, while 20% were low producers with formation of beige-cream colored colonies; only one strain did not produce hydrogen sulfide. The choice was oriented toward the lowest producers of acetic acid and H_2S and highest producers of glycerol. Based on these outcomes, 16 strains were taken for the next step of selection consisting of alcohol tolerance and flocculation tests. All of them reached an $OD_{600\,nm} > 1.0$ within 10 days of incubation at 15°C in the acidified medium added with ethanol at 10% (v/v), by demonstrating reliability to start the second fermentation. As regards the flocculation test only one strain showed a degree of flocculence of point 2, while the others proved to be poorly flocculent (point 1) in 13% of cases or non-flocculent phenotype (point 0) in 81%. Thus, two strains isolated in both investigated territories, named FX and FY from Franciacorta area and OX and OY from Oltrepò Pavese area that presented the best scores in the all considered parameters were designated for the in-bottle fermentation trials.

Monitoring of the In-Bottle Fermentation Trials

The oenological performances of the four selected strains were tested in *prise de mousse* experiments after *tirage* operation carried out in 750 mL bottles. Five Franciacorta wineries, for the FX and FY strains, and four Oltrepò Pavese wineries, for the OX and OY strains, were involved in the experimental plan according to the decision of the winemakers Consortia (**Table 1**). The starter culture IOC18-2007, EC1118 or Lalvin DV10, which was habitually used by the single cellar, was prepared in the same conditions as the indigenous strain and it was chosen as control test (**Table 1**). The average temperature of the cellars was 14.5°C ± 2°C. Samples were analyzed by determining cell counts, cell vitality and genetic identification of the dominant strains. The cell concentration in the samples inoculated with FX and FY strains showed similar trends (**Figure 3A**), by unveiling a slower increase in plate counts at the beginning of the trials respect to the control tests inoculated with the commercial starter cultures. Furthermore, the enumeration of cultivable cells of both Franciacorta indigenous strains remained higher than 5 Log CFU/mL at 2 months and approximately at 4 Log CFU/mL after 3 months of aging, exhibiting significant different log counts ($p < 0.05$) respect to the references strains. After 4 months IOC18-2007, EC1118 and Lalvin DV10 strains were no longer detectable by plate count technique (**Figure 3A**).

Microscopic observations revealed that lower ratios of viable cells were present in the bottles inoculated with FX and FY strains in comparison to those prepared with the conventional yeasts, up to 2 weeks of incubation. Conversely, about 20% of cell population of indigenous Franciacorta strains remained metabolically active until 2 months, which was not the case for the common starter cultures (**Figure 3B**). After 120 days, 100% of cells appeared not viable for any strains. The analysis of the DNA amplification profiles of the inter-delta regions confirmed the dominance of the inoculated strains for each trial and all along the aging period, until it was possible to isolate colonies (data not shown).

The cell concentration in the bottles elaborated in Oltrepò Pavese cellars displayed a homogeneous behavior since no significant difference came out from the samples inoculated with the different yeasts. However, OX and OY strains always preserved a cultivability higher than the commercial starter cultures of approximately one order of magnitude from 1 to 4 month (**Figure 3C**). The results of staining test evinced comparable percent values of cell viability for both Oltrepò Pavese indigenous strains and the conventional ones throughout the monitored period. For all samples, after 120 days it was no longer possible to find colonies, whereas after 90 days viable cells could not be observed anymore (**Figure 3D**). The genotypic patterns obtained from δ-PCR analysis allowed to establish that the inoculated strains persisted as dominant yeast population in each trial during the aging time (data not shown).

Quality Evaluation of Sparkling Wine Samples

The mean values and relative standard deviation of some oenological parameters obtained from the chemical and sensory analyses of the experimental samples for different strains and different wineries are reported in **Tables 2, 3**, respectively. Data were subjected to one-way ANOVA in order to evaluate the effect of the "strain" inoculated for the second in-bottle fermentation, the "winery" factor, intended as the set of additives and cellar environment related to wine-making operation, or the "grape" variety, mainly used to produce the base wine. As regards the "strain" factor (**Table 2**), the average datum of alcoholic strength in sparkling wines inoculated with OX strain was significantly lower ($p < 0.05$) than those inoculated with FX, OY, EC1118 and DV10 strains. Similarly, the pressure of carbon dioxide reached inside the bottles inoculated with FX, FY and DV10 strains was significantly higher ($p < 0.05$) than that inoculated with OX and OY. Conversely, no significant differences were observed among mean values in residual sugars content (g/L), or titratable acidity (g/L), or volatile acidity (g/L). Also the average data of total SO_2 (mg/L) and dry extract (g/L) did not reveal significant differences among the samples inoculated

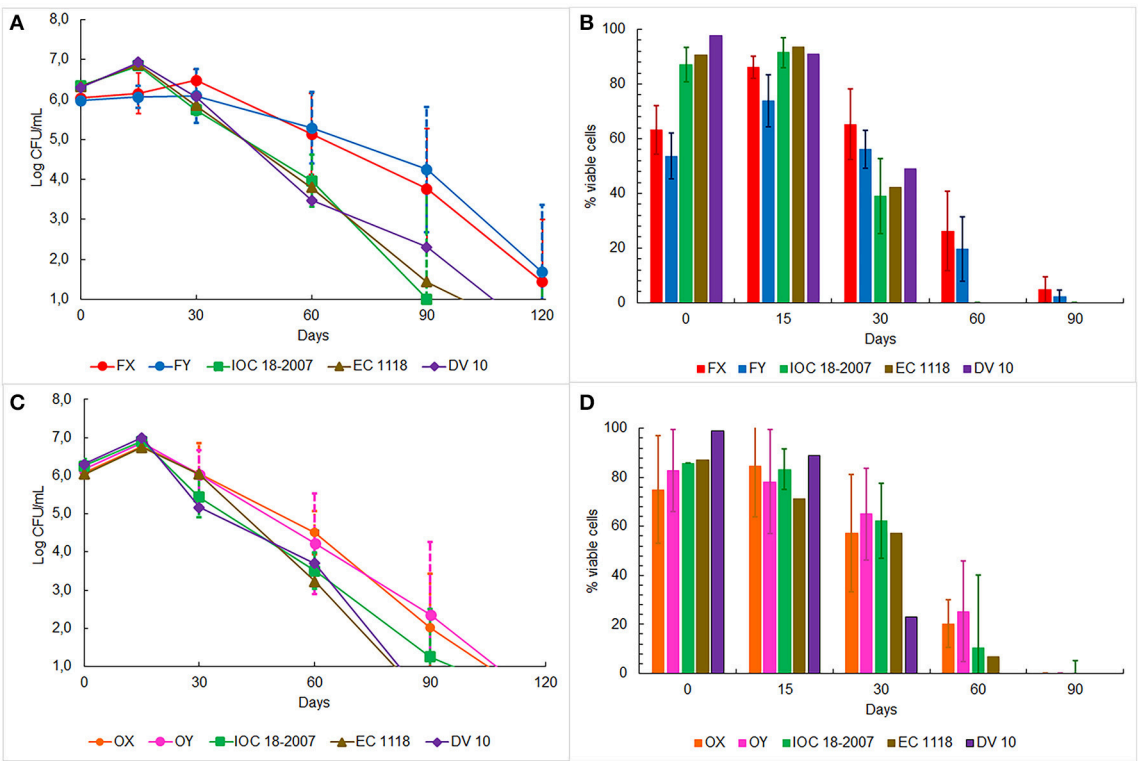

FIGURE 3 | Monitoring of yeast plate counts in base wine samples inoculated with: **(A)** FX and FY strains from Franciacorta area, **(C)** OX and OY strains from Oltrepò Pavese area and other commercial strains. Yeast cell viability in the same base wine samples inoculated with: **(B)** FX and FY strains from Franciacorta area **(D)** OX and OY strains from Oltrepò Pavese area and commercial strains.

TABLE 2 | Mean (± standard deviation) of oenological parameters obtained from sparkling wine samples, inoculated with different strains, after 18 months of aging on the lees in nine cellars of Franciacorta and Oltrepò Pavese areas.

Strain	FX	FY	OX	OY	IOC 18-2007	EC 1118	DV10
Winery	I, II, III, IV, V	I, II, III, IV, V	VI, VII, VIII, IX	VI, VII, VIII, IX	I, III, VI, VII	IV, VIII	V, IX
Alcoholic strength (% v/v)	12.5^{b} (± 0.5)	$12.3^{a,b}$ (± 0.6)	11.9^{a} (± 0.4)	12.5^{b} (± 0.3)	$12.3^{a,b}$ (± 0.6)	12.5^{b} (± 0.1)	12.7^{b} (± 0.2)
Glucose and fructose content (g/L)	0.9 (± 0.7)	1.4 (± 1.0)	2.2 (± 1.9)	1.9 (± 1.1)	0.5 (± 0.2)	1.5 (± 0.3)	0.7 (± 0.5)
Titratable acidity (g/L)	7.2 (± 0.6)	6.9 (± 0.6)	6.7 (± 0.8)	6.7 (± 1.1)	7.2 (± 0.5)	6.1 (± 0.4)	6.9 (± 0.7)
Volatile acidity (g/L)	0.41 (± 0.15)	0.45 (± 0.20)	0.56 (± 0.12)	0.43 (± 0.05)	0.42 (± 0.09)	0.58 (± 0.18)	0.41 (± 0.07)
Total SO_2 (mg/L)	44 (± 19)	44 (± 20)	40 (± 17)	39 (± 10)	32 (± 5)	46 (± 15)	63 (± 15)
Dry extract (g/L)	18.6 (± 1.1)	18.7 (± 1.1)	19.3 (± 1.7)	18.8 (± 1.1)	18.6 (± 1.4)	18.6 (± 0.4)	19.6 (± 0.6)
CO_2 overpressure (bar)	7.2^{d} (± 0.5)	$6.8^{c,d}$ (± 0.8)	5.6^{a} (± 0.5)	$5.9^{a,b}$ (± 0.5)	$6.4^{a,b,c}$ (± 1.1)	$6.3^{a,b,c}$ (± 0.9)	$6.8^{b,c,d}$ (± 1.0)
Olfactive pleasantness	6.0^{a} (±1.3)	5.8^{a} (±1.2)	5.0^{b} (±1.1)	5.7^{a} (±1.6)	$5.4^{a,b}$ (±1.8)	$5.6^{a,b}$ (±1.2)	5.9^{a} (±0.9)
Gustative pleasantness	5.9^{a} (±1.7)	5.8^{a} (±1.7)	$5.4^{a,b}$ (±1.4)	4.9^{b} (±1.6)	$5.4^{a,b}$ (±1.7)	4.6^{b} (±1.7)	6.0^{a} (±1.2)

Values on the same row with different superscripts letters are significantly different (P ≤ 0.05).

TABLE 3 | Mean (± standard deviation) of oenological parameters obtained from sparkling wine samples, made in different wineries of Franciacorta and Oltrepò Pavese areas, after 18 months of aging on the lees of different strains.

Winery	I	II	III	IV	V	VI	VII	VIII	IX
Strains	FX, FY, IOC 18-2007	FX, FY, strain not disclosed	FX, FY, IOC 18-2007	FX, FY, EC1118	FX, FY, DV10	OX, OY, IOC 18-2007	OX, OY, IOC 18-2007	OX, OY, EC1118	OX, OY, DV10
Alcoholic strength (% v/v)	11.7^{a} (± 0.3)	13.0^{e} (± 0.2)	$12.8^{d,e}$ (± 0.2)	$12.3^{b,c}$ (± 0.3)	$12.4^{b,c}$ (± 0.4)	$12.5^{c,d}$ (± 0.4)	$12.2^{b,c}$ (± 0.2)	$12.4^{b,c}$ (± 0.3)	$12.0^{a,b}$ (± 0.6)
Glucose and fructose content (g/L)	$1.5^{a,b}$ (± 1.0)	0.4^{a} (± 0.3)	$0.8^{a,b}$ (± 0.5)	$1.8^{b,c}$ (± 0.5)	$0.7^{a,b}$ (± 0.5)	0.6^{a} (± 0.6)	$0.7^{a,b}$ (± 0.7)	2.7^{c} (± 1.3)	2.8^{c} (± 1.8)
Titratable acidity (g/L)	6.7^{b} (± 0.4)	7.0^{c} (± 0.3)	7.5^{d} (± 0.2)	6.5^{b} (± 0.2)	7.8^{e} (± 0.3)	7.9^{e} (± 0.6)	7.2^{c} (± 0.4)	5.9^{a} (± 0.4)	6.6^{b} (± 0.4)
volatile acidity (g/L)	0.48^{c} (± 0.10)	$0.36^{a,b}$ (± 0.07)	0.29^{a} (± 0.07)	0.69^{d} (± 0.09)	$0.37^{a,b}$ (± 0.07)	$0.42^{b,c}$ (± 0.04)	0.51^{c} (± 0.09)	0.50^{c} (± 0.08)	0.50^{c} (± 0.16)
Total SO_2 (mg/L)	$30^{a,b}$ (± 8)	40^{c} (± 7)	25^{a} (± 6)	56^{d} (± 6)	72^{e} (± 8)	$33^{a,b,c}$ (± 6)	$37^{b,c}$ (± 4)	$36^{b,c}$ (± 5)	51^{d} (± 6)
Dry extract (g/L)	$18.6^{b,c}$ (± 0.6)	$17.8^{a,b}$ (± 0.5)	19.8^{d} (± 0.6)	$18.2^{b,c}$ (± 0.5)	19.7^{d} (± 0.6)	19.8^{d} (± 1.1)	17.3^{a} (± 0.6)	18.8^{c} (± 0.5)	19.8^{d} (± 0.8)
CO_2 overpressure (bar)	7.0^{d} (± 0.4)	7.7^{e} (± 0.3)	$7.4^{d,e}$ (± 0.4)	6.3^{c} (± 0.8)	$7.2^{d,e}$ (± 0.5)	6.3^{c} (± 0.4)	$5.8^{b,c}$ (± 0.4)	$5.6^{a,b}$ (± 0.4)	5.2^{a} (± 0.4)
Olfactive pleasantness	$5.9^{b,c}$ (±1.0)	$5.9^{b,c}$ (±1.3)	6.2^{c} (±1.2)	$5.9^{b,c}$ (±1.2)	$5.9^{b,c}$ (±1.3)	6.1^{c} (±1.4)	4.4^{a} (±1.5)	5.2^{b} (±0.9)	$5.4^{b,c}$ (±0.9)
Gustative pleasantness	$5.9^{c,d}$ (±1.6)	4.8^{a} (±1.5)	$5.8^{b,c,d}$ (±1.5)	$5.9^{c,d}$ (±2.2)	6.4^{d} (±1.6)	$5.5^{a,b,c}$ (±1.8)	$5.1^{a,b}$ (±1.5)	4.8^{a} (±0.9)	$5.2^{a,b}$ (±1.2)

Values on the same row with different superscripts letters are significantly different (P ≤ 0.05).

with different strains. The results of sensory test for the smell pleasantness gave significantly ($p < 0.05$) higher scores to the samples re-fermented with strains FX, FY, OY, and DV10. The values obtained from the tasting evaluation confirmed a higher agreeableness ($p < 0.05$) for the sparkling wines inoculated with strains FX, FY and DV10. The "winery" factor (**Table 3**) proved to be heavily engaged by determining significant differences in alcoholic strength ($p < 0.01$), residual sugars content ($p < 0.05$), volatile acidity ($p < 0.01$) and CO_2 overpressure ($p < 0.01$). Also the average data of titratable acidity ($p < 0.01$), total SO_2 ($p < 0.05$) and dry extract ($p < 0.01$) revealed important differences among samples prepared in different wineries, showing that

the formulation of the base wine, the cellar practices and the environmental conditions deeply affected the outcomes. The results of the sensory evaluation confirmed the substantial impact of how the wine-making was carried out in the single cellar; indeed significant differences were found among the scores that were attributed to the samples of each winery, by displaying $p < 0.01$ for the perception of volatile compounds and $p < 0.05$ in the case of taste sensations. Finally, the factor "grape cultivar" used to prepare the base wine seemed to significantly influence the following parameters: residual sugars amount ($p < 0.01$), where the samples prevalently made with *Chardonnay* and *Pinot Noir* showed mean values of 1.03 and 1.36 g/L respectively, vs. a mean value of 2.87 g/L for those prepared with *Croà*; level of total SO_2 ($p < 0.05$) with averages data of 44, 35 and 51 mg/L for *Chardonnay*, *Pinot Noir* and *Croà* wines, respectively; carbon dioxide overpressure ($p < 0.01$) since the mean value in *Chardonnay* based samples (7.1 bar) was higher than in those of *Pinot noir* (5.7 bar) and *Croà* (5.8 bar). Both the scores obtained in sensory tests for olfactive and gustative pleasantness from sparkling wines prevalently produced with *Chardonnay* variety resulted higher ($p < 0.05$) than those made with *Pinot Noir* and *Croà*.

DISCUSSION

The previous results of an investigation (Vigentini et al., 2015) on the indigenous microbiota in wine-making environment of Franciacorta and Oltrepò Pavese areas, have revealed a high level of genomic diversity within the species *S. cerevisiae*, through polymorphism analysis of the interdelta regions by capillary electrophoresis. Likewise, in this work, the determination of some phenotypic characteristics on the same *S. cerevisiae* strains have confirmed the presence of a large range of values in metabolite production, such as fermenting power, fermenting vigor, acetic acid, glycerol, and hydrogen sulfide, or in resistance to sulfur dioxide. The observation of this intraspecific biodiversity provides a wealth for the potential exploitation to obtain strains tailored to the needs of the wine producer (Pretorius, 2000; Fleet, 2008). Anyway, the adoption of selection criteria results in a hard activity when the strains to be investigated are hundreds, since the priorities planning and the choice of the tasks to achieve the goals become conclusive. In the present work, a polyphasic approach was carried out by considering each strain and the overcoming of the threshold of the median value for some oenological parameter per each phase of the study. Primarily, the selection has been addressed to *S. cerevisiae* as it is considered the most capable yeast species to realize a secondary fermentation starting from high alcohol concentration and in the presence of sulfur dioxide. The second selection occurred for the strains that showed values higher than the median ones for other quality parameters important for sparkling wine-making, such as the low production of acetic acid, high production of glycerol, and low formation of hydrogen sulfide. Again, those strains that have exceeded the median values were chosen for the evaluation of the resistance to ethanol and the flocculent phenotype. Finally, in order to meet a request of the Appellation of Origin Committees of the winemakers, the belonging to the territory was the last criterion used to decide which strains should be tested in *prise de mousse* trials under actual cellar conditions.

The experimental plan stated that each winery had to perform the in-bottle fermentation experiments inoculating its own base wine with the two selected indigenous strains, isolated in the relevant vine-growing area, *plus* the starter strain normally used in its own cellar, according to a protocol previously planned and shared with the oenologists. This allowed us to compare the data obtained from different strains in the same operative conditions, as well as to evaluate the outcome from the same strain in different wineries by assessing its performance in different environments under real operative conditions.

As regards the cell counts and the strain identification, the results reveal that all selected strains are capable of developing and dominating the in-bottle fermentation. However, it should be noted that the Franciacorta indigenous strains show an increased latency period and a higher cultivability than the others along the aging time do. The natural autolysis of yeast, which can be estimated by the drop in percent cell viability, occurred after 2/3 months from the inoculation time, as expected at this temperature (Alexandre and Guilloux-Benatier, 2006).

The ANOVA of results from the analyzed samples at the end of the aging time (18th month) evinces that significant differences among the strains are present for some oenological parameters like the final alcohol content, the achieved carbon dioxide pressure and the sensorial traits. Interestingly, the indigenous strains get a valuation comparable to the one of the conventional starter cultures, or superior as in the case of FX strain from Franciacorta area. This confirms that the strain is a key element affecting the quality of the product, also in sparkling wine by traditional method, as already reported by few authors (Martínez-Rodríguez et al., 2002; Martí-Raga et al., 2016). Nevertheless, the comparison of data observed in samples managed in different cellars with the same strain proves that the "winery," described as the set of the base wine formulation and the environmental conditions, is the most conditioning factor since significant differences are found in all investigated oenological parameters. These data sustain how much the oenologist's choices are fundamental in selecting the ingredients, assembling the *cuvée* and managing the cellar practices for the quality of the final product. For some parameters, also the prevalent grape cultivar used to make the base wine appears to significantly influence the characteristics of the sample wines.

Although some oenological aspects were not considered in this work, this investigation demonstrates the possibility of recovering indigenous *S. cerevisiae* strains in the environment, that exhibit technological and quality traits suitable for the traditional method, especially the pursuing of the in-bottle fermentation at low temperature starting from a high alcohol content.

Increasing the choice of available strains meets the needs of the sparkling wines industries directed toward an expanding global market searching a differentiation of sensory quality and a recognition of a link with the territory of production. Indeed, the change of the yeast for the second fermentation can be easily

introduced to improve or to obtain a typicality of the product without modifying the traditional technology (Pozo-Bayón et al., 2009; Kemp et al., 2014). This goes in the direction of an enology of precision where the wine is designed by combining the specific vine cultivar with a peculiar technology and exploiting the potential metabolic activities of specific microorganisms; over all that it is true for non-aromatic varieties (Vigentini et al., 2016), as in many sparkling wine productions.

Finally, the natural occurrence of native alcohol-tolerant yeasts in the environment may leads the oenologist toward the design of innovative procedure for sparkling wine-making, in order to maximize the potential of microbial diversity present in the current vintage or belonging to the territory. As suggestion, it could be possible to make spontaneous fermentation in a volume of selected must from healthy grapes and, at the end of fermentation, to collect the indigenous microbial populations by centrifugation. Then, this part containing the natural mixture of high ethanol resistant strains could be re-inoculated as starter culture into the base wine for the *tirage* operation.

AUTHOR CONTRIBUTIONS

IV contributed to the design and organization of the work, to the management of lab work, to the data collection and analysis, to draft and review the manuscript; SB and FV contributed to the microbiological, chemical, molecular and sensory analyses of the samples, to the assistance to cellar work at the wineries and to draft the manuscript; MF and CAP, each for its own Consortium, contributed to the design of the work, to the management of cellar work at the wineries and to the organization of sensory sessions; CP contributed to the management of lab work, to the data collection and analysis, to draft the manuscript; RF contributed to the design and organization of the work, to the data collection and analysis, to draft and review the manuscript, and ensured that all questions related to the accuracy or integrity of any part of the work were appropriately investigated and resolved.

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Sequential Inoculation of Native Non-*Saccharomyces* and *Saccharomyces cerevisiae* Strains for Wine Making

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The use of non-*Saccharomyces* yeast for wine making is becoming a common trend in many innovative wineries. The application is normally aimed at increasing aromas, glycerol, reducing acidity, and other improvements. This manuscript focuses on the reproduction of the native microbiota from the vineyard in the inoculum. Thus, native selected yeasts (*Hanseniaspora uvarum, Metschnikowia pulcherrima, Torulaspora delbrueckii, Starmerella bacillaris* species and three different strains of *Saccharomyces cerevisiae*) were inoculated sequentially, or only *S. cerevisiae* (three native strains together or one commercial) was used. Inoculations were performed both in laboratory conditions with synthetic must (400 mL) as well as in industrial conditions (2000 kg of grapes) in red winemaking in two different varieties, Grenache and Carignan. The results showed that all the inoculated *S. cerevisiae* strains were found at the end of the vinifications, and when non-*Saccharomyces* yeasts were inoculated, they were found in appreciable populations at mid-fermentation. The final wines produced could be clearly differentiated by sensory analysis and were of similar quality, in terms of sensory analysis panelists' appreciation.

Keywords: indigenous yeast, *Torulaspora*, *Metschnikowia, Hanseniaspora, Starmerella*, Priorat, wine

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INTRODUCTION

Due to the increasingly competitive global market, there is a trend for local wine producers to attempt to link their products with geographical identity (Harvey et al., 2014), which has been identified as the *terroir,* including soil, climate, grape varieties and microbial population (Bokulich et al., 2013). Native microorganisms, particularly yeasts, have been highlighted as key factors for preserving typicality, characteristic flavors and the high quality of wines (Tofalo et al., 2014), which could be considered the microbial fingerprint. However, this microbial fingerprint is not probably static and can change along the time and climatic conditions of the harvest as can be seen by comparing in the Priorat region results form our group (Torija et al., 2001; Padilla et al., 2016). Also new results obtained in a 3 years study (Vigentini et al., 2015) are opening the debate if the microbial population permanently remains in vineyards.

The Priorat Qualified Appellation of Origin (DOQ in Catalan language) is a traditional area for wine production located in the south of Catalonia, Spain, where Carignan and Grenache are the typical and characteristic red grape varieties. Although limited data are available concerning

the use of locally selected yeast for must inoculation in Catalonia, several studies developed in different wine-producing areas have noted the use of native yeasts as an innovative approach to obtain wines reflecting *terroir* (Vilanova and Massneuf-Pomarède, 2005; Carrascosa et al., 2012; Scacco et al., 2012).

The use of locally selected yeast is normally based on a study on natural biodiversity. Yeast biodiversity during the spontaneous fermentation of grape juice includes the presence of different species. It has been widely reported that non-*Saccharomyces* species dominate the first phase of alcoholic fermentation, and some of these yeasts can also be present at advanced stages, even while the species *Saccharomyces cerevisiae* dominates the process (Fleet, 1993). This extensive yeast biodiversity is the reason supporting the design and implementation of yeast starter cultures that are not pure or single-species. The defense of the wine typicality should actually include a combination of non-*Saccharomyces* and *S. cerevisiae* strains with the aim to obtain wines exhibiting complexity but avoiding the risks related to natural fermentations (Comitini et al., 2011; Tristezza et al., 2011; Suzzi et al., 2012; Gobbi et al., 2013; Medina et al., 2013).

Thus, the proper design of an autochthonous starter culture is essential to reproduce the local sensory properties, including the incorporation of a mixture of different non-*Saccharomyces* species and different strains of *S. cerevisiae* to mimic spontaneous alcoholic fermentations. Among non-*Saccharomyces* species, *Hanseniaspora uvarum*, *Starmerella bacillaris* (previously known as *Candida zemplinina*), *Torulaspora delbrueckii* and *Metschnikowia pulcherrima* have been isolated in different wines (Lopandic et al., 2008; Kraková et al., 2012; Albertin et al., 2014) and have been described as characteristic of the Priorat (Torija et al., 2001; Wang et al., 2015; Padilla et al., 2016; Portillo and Mas, 2016). However, the combination of several non-*Saccharomyces* and strains of *S. cerevisiae* can be challenged by the winemaking conditions (i.e., SO_2 dosage, temperature, etc. . .) as well as the initial yeast population in grapes (Constantí et al., 1998; Vigentini et al., 2014). Thus, special care in the winery has to be taken for this kind of procedures.

This work aims to test the industrial use of locally selected yeast strains reproducing the vineyard for wine production in the Priorat DOQ. For this purpose, a specific multistarter culture consisting of different strains of *S. cerevisiae* and non-*Saccharomyces* species mimicking Priorat natural musts has been developed. This study was done using synthetic must in order to have all the conditions of incubation and sterility under control as well as natural Grenache and Carignan grape juices at industrial scale. The mix of different species was used to inoculate the four non-*Saccharomyces* species and sequentially (24 h later) the mix of three different *S. cerevisiae* strains. Additionally, control fermentations containing only the three native *S. cerevisiae* strains or a *S. cerevisiae* commercial strain have been performed to evaluate the contribution of non-*Saccharomyces* and native inoculum to fermentation kinetics, yeast dynamics, and different oenological parameters as well as the production of major volatile compounds. Additionally, a sensory evaluation based on triangle tests was performed.

MATERIALS AND METHODS

Strains

Four non-*Saccharomyces* yeast strains: *H. uvarum* CECT 13130, *S. bacillaris* CECT 13129, *T. delbrueckii* CECT 13135 and *M. pulcherrima* CECT 13131; and three *S. cerevisiae* strains: CECT 13132, CECT 13133 and CECT 13134, were used in this work. All strains were previously isolated from DOQ Priorat spontaneous fermentations (Padilla et al., 2016) and deposited in the Spanish Type Culture Collection (CECT). The non-*Saccharomyces* species were selected by the absence of off-odor production (especially acetic acid), prevalence in musts during fermentations and ester production. Instead, resistance to high sugar concentration was the main criteria for selection of *S. cerevisiae* strains, which is one of the main characteristics of Priorat musts, but also competitiveness in front of other *Saccharomyces* strains and the production of esters and acetates in single fermentations (Torija et al., 2001). Additionally, commercial *S. cerevisiae* wine strains GR in Grenache (provided by AB Mauri, Sydney, NSW, Australia), CA in Carignan or QA23 in Synthetic must (both from Lallemand Inc., Montreal, QC, Canada) were included in this study as a control. Yeasts were maintained in glycerol stocks at −80°C.

Biomass Production

Native yeasts were grown in plates with 25 cm of diameter containing YPD medium (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose, 17 g/L agar) at 28°C before use. Plates were washed with saline solution (NaCl 0.9% w/v) for yeast collecting, and cells were then quantified and used as inocula for laboratory and industrial vinifications. Commercial *S. cerevisiae* strains were purchased as active dry yeast and rehydrated following the manufacturer's instructions.

Fermentation and Sampling

The laboratory scale fermentations were conduct using synthetic must (as reported in Andorrà et al., 2012) with nitrogen content of 300 mg/L and 200 g/L of total sugar and pH adjusted to 3.3. Fermentations were performed in triplicate in continuous shaking at 120 rpm at 25°C in 500 mL glass bottles filled each one with 400 mL of synthetic must and covered with cotton caps. The inoculation process is described in **Table 1**, monitoring and sampling was done as in the industrial scale.

On the other hand, six industrial fermentations were conducted in stainless steel tanks filled with 2000 kg of crushed grapes, rendering 1050 L of Grenache (GR) or Carignan (CA) wine in a cellar from DOQ Priorat. Due to the specific characteristics of the vineyards in Priorat, this volume is very common in the area and is the volume routinely used in this cellar. Before inoculation, musts were chemically analyzed. The musts had a density around 1100 g/L, pH between 3.19 and 3.29 with total acidity of 4.6 and 5.2 g/L, which are typical values from the area. Due to the low levels of yeast assimilable nitrogen (66 and 80 mg/L), juices were gradually supplemented throughout the alcoholic fermentation with a total of 50 mg inorganic nitrogen/L (as Diammonium Phosphate) and 15 mg

organic nitrogen/L (as yeast lysates). For each must variety, three vinifications containing different yeast strain combinations were performed and monitored (**Table 1**). The A fermentations (A-SM = in Synthetic must, A-GR = in Grenache must and A-CA = in Carignan must) contained a combination of the seven native strains, which were sequentially inoculated. At time 0, non-*Saccharomyces* strains were added into the must, mimicking the natural yeast composition found in previous studies, and the mixture of *S. cerevisiae* was incorporated 24 h later. In contrast, the B fermentations (B-SM, B-GR and B-CA) contained only the mixture of the three *S. cerevisiae* autochthonous strains. Experiments with commercial *S. cerevisiae* strains (C-SM, C-GR and C-CA) were conducted as a control for each type of must.

From each bottle and tank, daily samples were taken to monitor sugar concentration by measuring must density using an electronic densitometer (Mettler-Toledo S.A.E., Barcelona, Spain). Additionally, samples of the grape juice before inoculation (day 0), 1 day after inoculation with non-*Saccharomyces* in the case of mixed fermentations (24 h; day 1), 1 day after inoculation with *S. cerevisiae* (24 h Sc; day 1 or 2), at a mid-fermentation point (M; day 4–5) and at the end of the fermentation (F; day 8–9) were also aseptically withdrawn for yeast counting and molecular identification. Moreover during industrial fermentations, cells from 1 mL at each sampling point were collected after centrifugation (Spectrafuge, Labnet, United States) at 9200 g for 5 min for quantitative PCR (qPCR) analysis.

The synthetic wines were analyzed after the alcoholic fermentation. The final industrial wines were stabilized for 30 days at 4°C, and then 30 ppm of sulfur dioxide was added as potassium metabisulfite, and the final product was bottled. These conditions were maintained for 2 months until the sensory evaluation took place.

Yeast Content and Isolation

Yeast counts were conducted in duplicate on solid YPD and agar-Lysine (LYS) plates (Oxoid, United Kingdom, prepared according to manufacturer's instructions) after serial decimal dilution with distilled sterile water of the samples. Plates were incubated at 28°C for 3 days. For yeast isolation and identification, from plates containing 30–300 colonies approximately, 25 colonies from each medium and each sampling point were picked randomly.

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

Yeast isolates were identified by PCR-RFLP analysis of 5.8S-ITS rDNA according to Esteve-Zarzoso et al. (1999), using primers ITS1 and ITS4 (White et al., 1990). PCR products were digested, without further purification, with the restriction enzymes *Cfo*I, *Hae*III, *Dde*I, and *Hin*fI. The PCR products and their restriction fragments were separated by gel electrophoresis on 1.5 and 3% (w/v) agarose gels, respectively. The sizes of the DNA fragments were estimated by comparison against a DNA ladder (100 bp Roche Diagnostics GmBh, Mannheim, Germany). The obtained restriction profiles were compared with the profiles reported in Esteve-Zarzoso et al. (1999) and in the Yeast-id database[1].

Sequencing of the D1/D2 domains of 26S rRNA gene was conducted to confirm yeast identification using primers NL1 and NL4 (Kurtzman and Robnett, 1998). The PCR product was purified and sequenced by Macrogen Inc. (Seoul, South Korea) using an ABI3730XL automated capillary DNA sequencer. The sequences were compared with the ones in GenBank using the BLASTN tool (NCBI) and deposited in GenBank database with the accession numbers described in Padilla et al. (2016).

Yeast Typing

The isolates from the dominant yeast species were genetically characterized. *S. cerevisiae* strains were typified by the analysis of inter-delta regions, as described by Legras and Karst (2003) using the primers delta12 and delta21. DNA was extracted from yeast cultures grown in YPD broth for 24 h at 28°C (Querol et al., 1992). Interdelta PCR products were separated by electrophoresis on 2% (w/v) agarose gels. The sizes of the DNA fragments were estimated by comparison against a DNA ladder (100 bp Roche Diagnostics GmBh, Mannheim, Germany).

[1]http://www.yeast-id.com

TABLE 1 | Yeast composition of starter cultures employed in different fermentation modalities (cells/mL).

	Grenache			Carignan and Synthetic must		
Yeast strains	**A**	**B**	**C**	**A**	**B**	**C**
H. uvarum CECT 13130	1.2×10^5			1.2×10^6		
S. bacillaris CECT 13129	6×10^4			6×10^5		
T. delbruecki CECT 13135	10^4			10^5		
M. pulcherrima CECT 13131	10^4			10^5		
S. cerevisiae CECT 13132	7×10^4	7×10^4		7×10^5	7×10^5	
S. cerevisiae CECT 13133	7×10^4	7×10^4		7×10^5	7×10^5	
S. cerevisiae CECT 13134	7×10^4	7×10^4		7×10^5	7×10^5	
S. cerevisiae GR			2×10^6			
S. cerevisiae CA						2×10^6
S. cerevisiae QA 23						2×10^6

Quantitative PCR

Yeast DNA was extracted from 1 ml pelleted cells using the DNeasy PLANT kit (Qiagen, United States). Quantitative PCR (qPCR) was performed in a 7300 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, United States). Power SybrGreen master mix was used according to the manufacturer's instructions (Applied Biosystems, Foster City, CA, United States). An AB 0–600 96-well optical plate (Thermo Scientific, Waltham, MA, United States) was used for the reaction. The primers used for each species were those described by Hierro et al. (2007) (*Saccharomyces* and *Hanseniaspora*), Andorrà et al. (2010) (*S. bacillaris*), Zott et al. (2010) (*T. delbrueckii*), and Díaz et al. (2013) (*M. pulcherrima*). The cycle threshold (CT) was automatically determined. All samples were analyzed in duplicate, and cell concentrations were quantified by CT measurement using the calibration curves of a relevant concentration series of yeast cells for each species (see calibration curves for each species in Supplementary Table S1).

Chemical Analysis of Musts and Wines

Density, pH, total acidity and ethanol were determined according to the methods in the Compendium of International Methods of Analysis of Musts and Wines (OIV, 2015). Yeast assimilable nitrogen was measured according to the formol method (Gump et al., 2000). Sugars (glucose and fructose), acetic acid and glycerol were quantified using the Miura one enzymatic autoanalyzer (BioGamma I.S.E. S.r.L., Rome, Italy) with the corresponding enzymatic kits (BioSystems S.A., Barcelona, Spain).

Determination of Volatile Compound Production

The six final wines obtained using industrial conditions were analyzed for major volatile compounds by gas chromatographic–flame ionization detection (GC-FID) by an external analytical service (L.A.A.E., Zaragoza, Spain) according to Ortega et al. (2001). In summary, 3 mL of each wine were diluted with 7 mL of water, salted with 4.5 g of ammonium sulfate and extracted with 0.2 mL of dichloromethane. The extract was injected in the split mode into a Varian CP-3800 GC system (Palo Alto, CA, United States), separated on a DB-WAX polyethylene glycol column (30 m × 0.32 mm and 0.5 μm film thickness) from J&W Scientific (Folsom, CA, United States), and detected by FID.

Sensory Analysis of the Industrial Wines Obtained

The panel for wine sensory evaluation consisted of two groups of tasters. Group A consisted of eleven judges (six females and five males) recruited from the Faculty of Oenology of the University Rovira i Virgili. Group B consisted of six oenologists from cellars belonging to the Priorat DOQ (four females and two males). Panelists were placed in individual sensory booths. Fifty milliliters of each wine was served at room temperature, and the order of presentation was randomized. For each grape variety, two different discriminating triangle tests were presented, one containing samples from fermentations A and B and the other from B and C.

Statistical Analysis

Significant differences in sensory analysis were defined using the critical number of correct answers for the triangle test (Roessler et al., 1948).

RESULTS

Yeasts in Natural Musts

A specific characteristic of these juices was the very healthy status of the grapes, which reached concentrations of 2×10^3 (GR) to 4×10^4 (CA) CFU/mL when plated. Yeast populations in the grapes of the area are generally higher, approximately 10^5 cells/g grapes or mL of must. A total of 153 colonies were identified from these musts. This low yeast population, especially in GR juices, led to the isolation of many different yeast species, the most abundant being *Rhodotorula mucilaginosa* (30%), *Debaryomyces hansenii* (21%), and *M. pulcherrima* (19%). Additionally, other species such as *Wickerhamomyces anomalus* and *Zygoascus hellenicus* were isolated in minor numbers (less than 10%). Instead, in the case of CA musts, the more common *H. uvarum* (74%) and *S. bacillaris* (25%) were present, reaching 99% of the isolates, and only one additional isolate of *M. pulcherrima* was found.

Fermentation Kinetics and Yeast Population

Total yeast counts (YPD), non-*Saccharomyces* yeast counts (LYS) and must density throughout all fermentations are shown in **Figure 1**. Values at time 0 correspond to must samples before inoculation in the case of industrial fermentations, while for synthetic must fermentations correspond to the inoculated population. In all cases, the typical growth kinetic was observed, exhibiting high total yeast viability until the end of the fermentations, with values of approximately 10^8 CFU/mL. In contrast, there was no growth of non-*Saccharomyces* species at this point, with counts at the middle fermentation point ranging between 10^5 and 10^7 CFU/mL. When non-*Saccharomyces* yeasts were inoculated (**Figure 1A**), the population recovered in LYS plates reached concentrations of 10^6 (A-CA) to 10^8 (A-SM) CFU/mL. These non-*Saccharomyces* populations decreased when *Saccharomyces* was inoculated to synthetic must fermentations; however, in natural musts this high population size was maintained until the middle of fermentation, declining afterward. Additionally, three strains of *S. cerevisiae* were sequentially inoculated after 24 h. When the three *S. cerevisiae* native strains were inoculated (**Figure 1A**), according to the type of must used, a similar pattern was observed. In all fermentations the non-*Saccharomyces* population was able to increase during the 1st days to decrease afterward. However, in synthetic must, the decreased appears shortly after *S. cerevisiae* inoculation, whereas in natural musts these decreases were later.

Mixed fermentation in synthetic must revealed the maximum yeast diversity on YPD plates at mid fermentation. During the

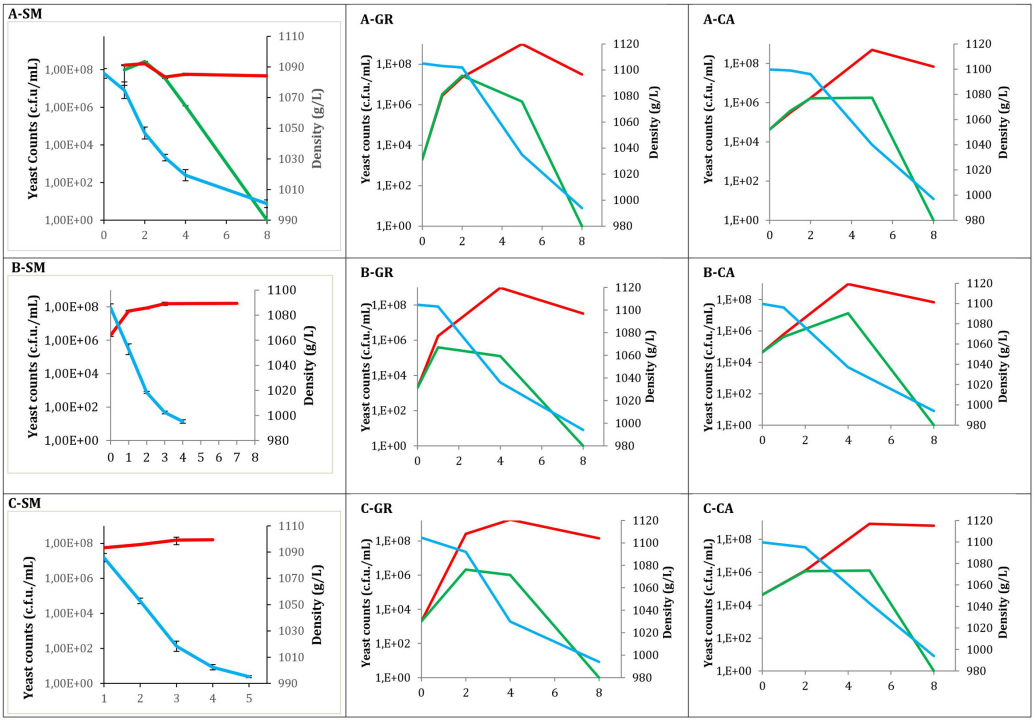

FIGURE 1 | Evolution of different fermentations modalities. Total yeast (—) counts obtained from YPD; Non-*Saccharomyces* yeast (—) counts on LYS; and Density (—). **(A)** Mixed fermentation (four species of non-*Saccharomyces* and the three strains of *Saccharomyces*), **(B)** Fermentation performed using the three native strains of *Saccharomyces*, **(C)** Fermentations conducted by industrial yeast starter belonging to *Saccharomyces*. Fermentations were performed using different musts. SM, Synthetic must; GR, Grenache must; CA, Carignan must.

initial sampling points, a high presence of *H. uvarum* was detected; however, these non-*Saccharomyces* yeast species were not identified at the last sampling point, in which all of the colonies were identified as *S. cerevisiae*.

When the three selected *S. cerevisiae* strains were used (**Figure 1B**) the non-*Saccharomyces* populations had a similar pattern, with Grenache must samples reaching slightly lower populations. When the commercial yeast strains were used (**Figure 1C**) the pattern was also very similar to the inoculation of the three *S. cerevisiae* strains.

The fermentation kinetics observed by density monitoring showed that the fermentations finished within 8 days when a mixture of *Saccharomyces* and non-*Saccharomyces* are present, independently of the origin (natural or synthetic) of the must. However, for fermentation using *Saccharomyces* inoculation this time is reduced to 4 days (synthetic must). This fact can be explained for the presence of lag phase when non-*Saccharomyces* yeasts are present. In the case of GR mixed fermentation, the sugar consumption was slightly slower compared with pure *S. cerevisiae*, although the process finished at the same time. For the three CA experiments, a similar fermentative pattern was observed.

Yeast Population Dynamics

As expected, the colonies recovered on YPD medium (**Figure 2**), at later time sampling points were identified as *S. cerevisiae* (100%). Only *S. cerevisiae* was recovered from the colonies isolated in synthetic must fermentations inoculated with this species (data not shown). However, in natural must fermentations, a clear difference was observed between mixed (**Figure 2A**) and pure *S. cerevisiae* vinifications (**Figure 2B**). In the natural must fermentations inoculated with a mixture of non-*Saccharomyces*, a higher biodiversity during the first stages of the process was observed compared with those inoculated with *S. cerevisiae*. In the case of A-GR, all inoculated non-*Saccharomyces* species, *H. uvarum* (50%), *S. bacillaris* (17%), *T. delbrueckii* (8%), and *M. pulcherrima* (25%), were recovered after 1 day of inoculation (24 h); however, in A-CA, all inoculated yeasts except *T. delbrueckii* were found, with values of 52% for *H. uvarum*, 40% for *S. bacillaris* and 8% for *M. pulcherrima*. Additionally, *H. guilliermondii* was also recovered at the middle point in A-GR. This high biodiversity present in mixed inocula fermentations decreased as the fermentation proceeded. The percentages of non-*Saccharomyces* species found were between 65 and 80% 24 h after inoculation with *S. cerevisiae* and 12 and 20% at the mid-fermentation point.

Once *S. cerevisiae* was incorporated into the must, it was possible to isolate it after 24 h (24 h Sc). In A-GR as well as in A-CA, *S. cerevisiae* gradually dominated the process. In the fermentation with the native strains of *S. cerevisiae* B-GR, only the yeast *S. cerevisiae* was isolated at the three sampling points. In contrast, in B-CA, the imposition occurred gradually,

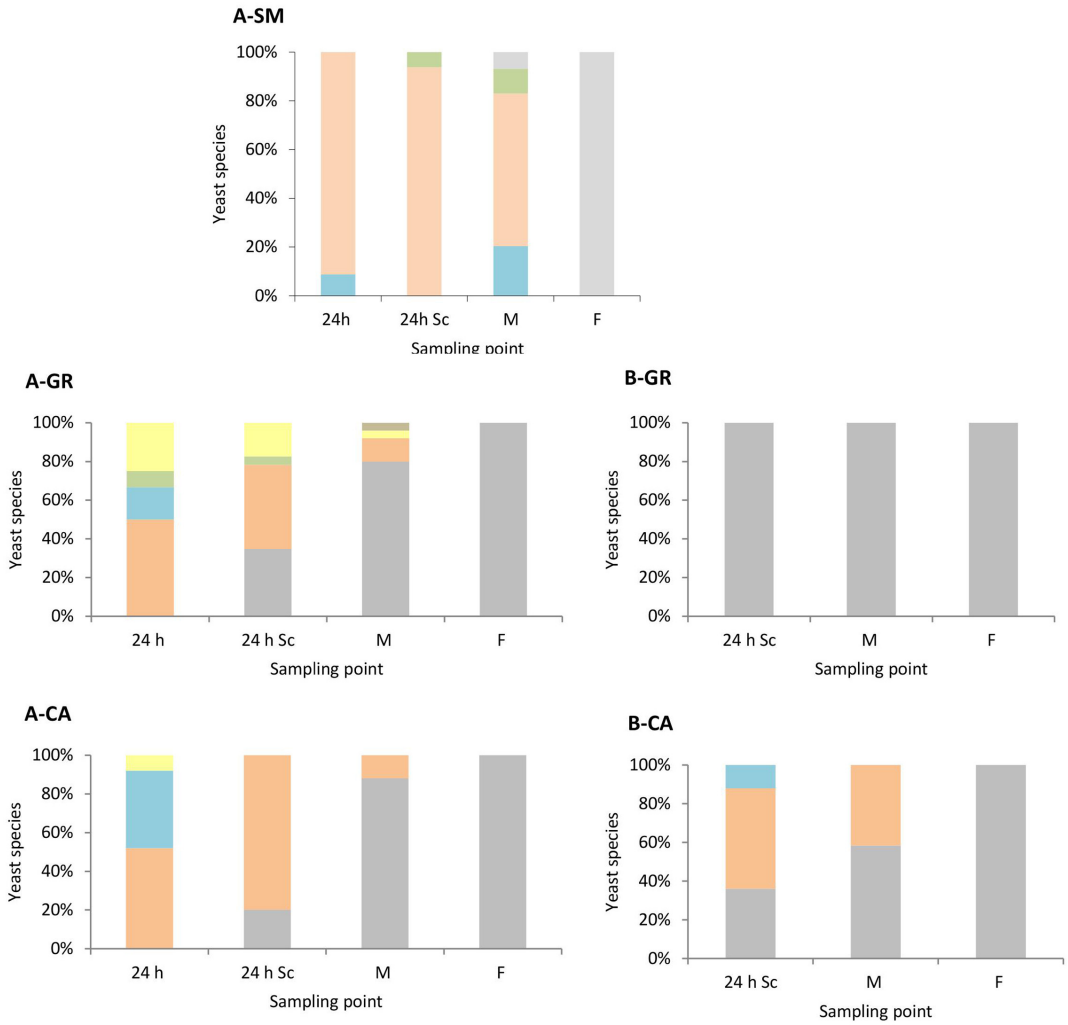

FIGURE 2 | Yeast species population dynamics established by RFLP-ITS-PCR of YPD-cultured isolates. **(A)** Mixed fermentation (four species of non-*Saccharomyces* and the three strains of *Saccharomyces*), **(B)** Fermentation performed using the three native strains of *Saccharomyces*. SM, Synthetic must; GR, Grenache must; CA, Carignan must. The species identified were *Hanseniaspora guilliermondii*, *Metschnikowia pulcherrima*, *Torulaspora delbrueckii*, *Starmerella bacillaris*, *Hanseniaspora uvarum*, *Saccharomyces cerevisiae*. Columns are indicated as: 24 h (24 h after non-*Saccharomyces* inoculation), 24 h Sc (24 h after *S. cerevisiae* inoculation), M (middle fermentation), F (end of fermentation).

as non-*Saccharomyces* species were also recovered up to the mid-fermentation point (42%).

These results were confirmed when the yeast population dynamics in LYS media were analyzed, where non-*Saccharomyces* species were isolated until the mid-fermentation point but undetectable at later stages (**Figure 3**). In synthetic must fermentations only *H. uvarum* and *S. bacillaris* were detected, showing an increase of the *S. bacillaris* presence as the fermentation proceeded (**Figure 3A**). In natural must fermentations inoculated with non-*Saccharomyces* yeast (**Figure 3A**) showed some species diversity at the beginning of the fermentation compared with pure *S. cerevisiae* fermentations (**Figure 3B**), although the most abundant yeast in all cases was *H. uvarum*, with proportions of approximately 86% and 95% in the GR and CA varieties, respectively. Among non-*Saccharomyces* species, this yeast dominated throughout fermentation, with just one non-inoculated species, *H. guilliermondii*, detected in small percentages at mid-fermentation in the case of GR wines.

The yeast dynamics for natural must fermentations were also analyzed by culture-independent techniques, specifically by qPCR (**Figure 4**). The data obtained from qPCR analysis overall agree with the plating results, with some particularities. First, the quantification of yeast in both musts in GR showed the presence of *Hanseniaspora species* at higher levels (1×10^4 cells/mL) than the colonies recovered on plates (2×10^3 CFU/mL). Furthermore, *S. cerevisiae* initial counts detected by qPCR were at concentrations that had to be detected in plates, although no *S. cerevisiae* isolates were identified using the RFLPs of 5.8S ITS rDNA. However, when *S. bacillaris* was recovered on plates, its

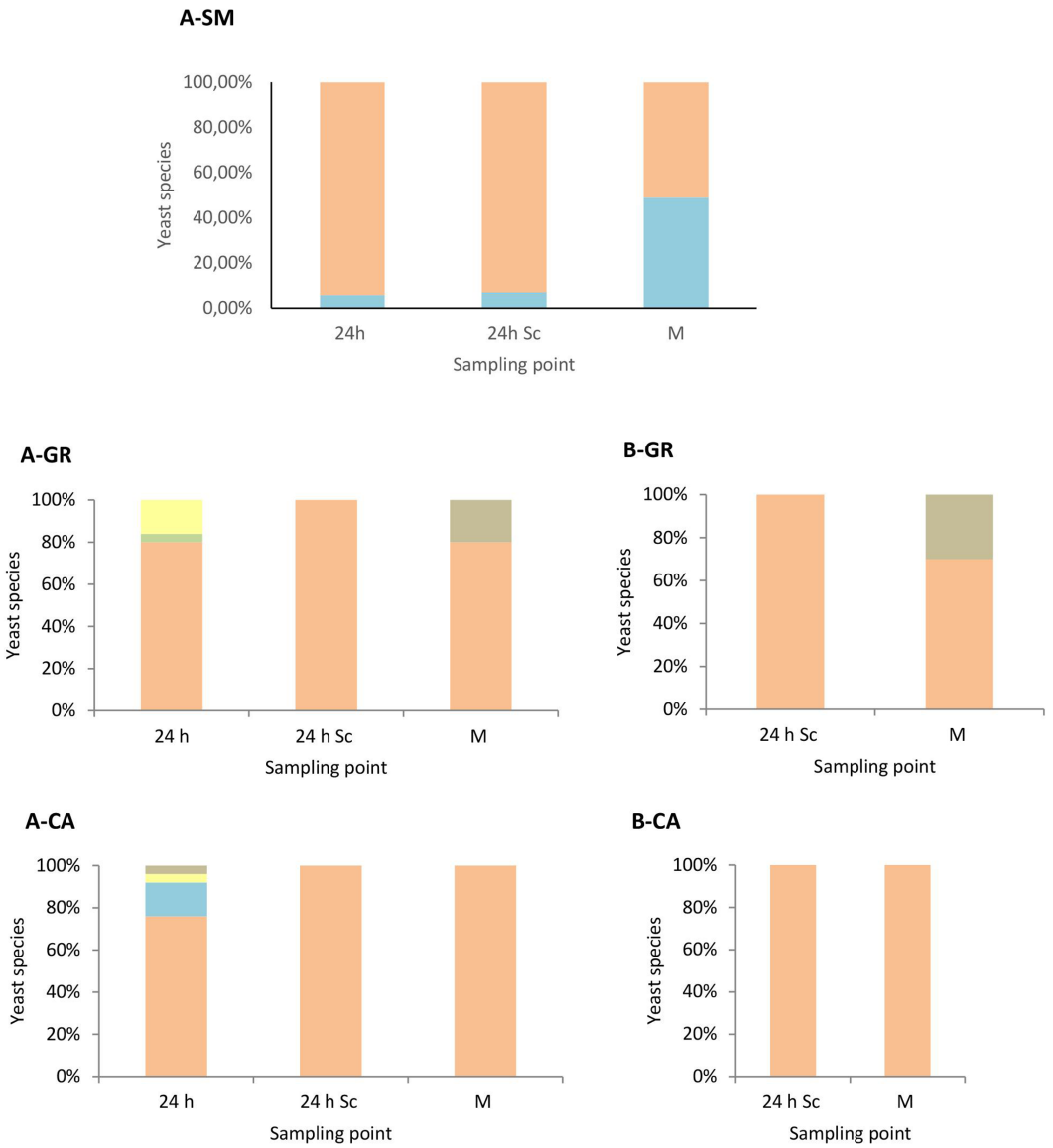

FIGURE 3 | Yeast species population dynamics established by RFLP-ITS-PCR of LYS-cultured isolates. **(A)** Mixed fermentation (four species of non-*Saccharomyces* and the three strains of *Saccharomyces*), **(B)** Fermentation performed using the three native strains of *Saccharomyces*. SM, Synthetic must; GR, Grenache must; CA, Carignan must. The species identified were *Hanseniaspora guilliermondii*, *Metschnikowia pulcherrima*, *Torulaspora delbrueckii*, *Starmerella bacillaris*, *Hanseniaspora uvarum*, *Saccharomyces cerevisiae*. Columns are indicated as: 24 h (24 h after non-*Saccharomyces* inoculation), 24 h Sc (24 h after *S. cerevisiae* inoculation), M (middle fermentation), F (end of fermentation).

quantification by qPCR was very low. In contrast, in the CA musts, the massive presence of *Hanseniaspora* cells agreed with the observations on plates, as well as the numbers of *S. bacillaris*, although in this case the qPCR counts were slightly lower than expected.

The increase of cell concentration due to inoculation was observable in all cases when the addition of the starter culture was performed. When the non-*Saccharomyces* species were inoculated, the observed increase agreed with the inoculated populations, except for *S. bacillaris* in GR, likely due to its low presence in the grape juice (**Figure 4A**). In the musts inoculated with autochthonous *S. cerevisiae* (**Figure 4B**), the increase was also seen in the quantification with qPCR. Finally, the levels of non-*Saccharomyces* during all these fermentations were very similar to the levels detected on LYS plates, with a clear dominance of *H. uvarum*. The presence of non-*Saccharomyces* throughout B-CA fermentation is remarkable, likely due to the presence of higher populations of these yeasts in the CA must.

Yeast Typing

To test the dominance of the major inoculated species, *S. cerevisiae* strains isolated during the fermentations were

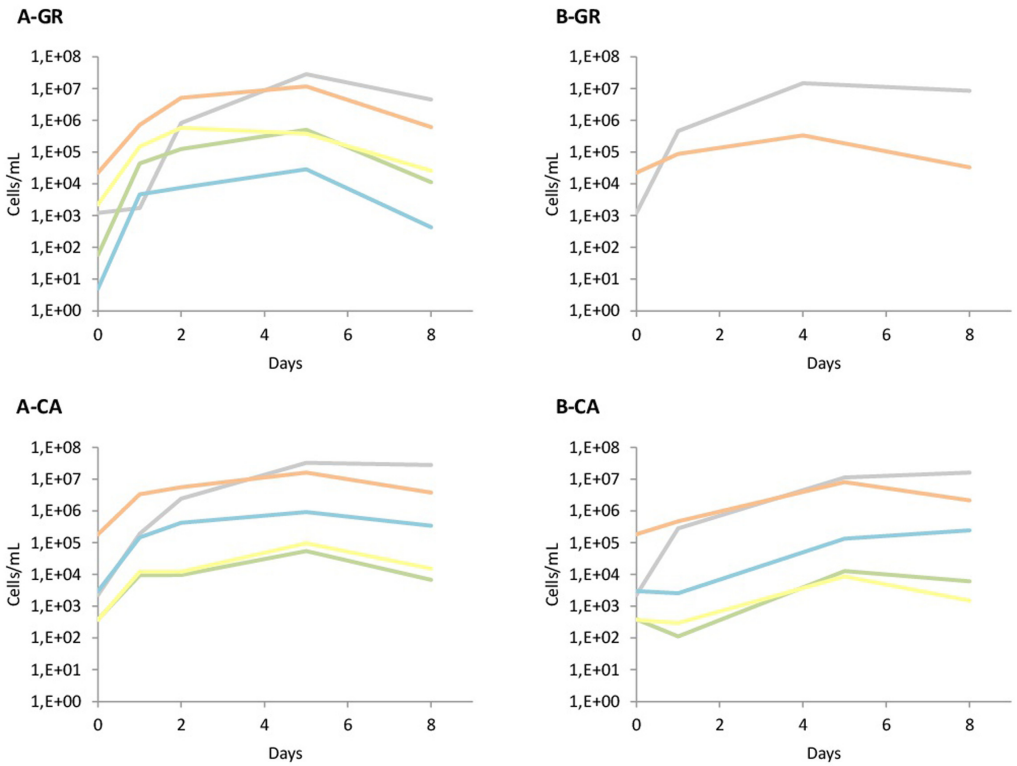

FIGURE 4 | Population dynamics of different yeast species established by qPCR. The species tested were Saccharomyces spp. (—); *Hanseniaspora* spp. (—); *S. bacillaris* (—); *T. delbrueckii* (—); *M. pulcherrima* (—) . **(A)** Mixed fermentation (four species of non-*Saccharomyces* and the three strains of *Saccharomyces*), **(B)** Fermentation performed using the three native strains of *Saccharomyces*. GR, Grenache must, CA, Carignan must.

typified at strain level. In the case of *S. cerevisiae*, the analysis was performed using the colonies at the end of fermentations. In fermentations A and B, the interdelta fingerprint of *S. cerevisiae* colonies isolated at the end of the process corresponded with the three native strains inoculated, CECT 13132, CECT 13133 and CECT 13134. Although the three profiles were present at the end of the fermentations, the main profile recovered was that of the strain CECT 12132, followed by CECT 12134 and CECT 12133 (**Table 2**). In the fermentations with the commercial strain, only the inoculated strain was recovered (data not shown).

TABLE 2 | Percentages of the inoculated *S. cerevisiae* strains recovered at the end of different fermentations.

	CECT 13132	CECT 13133	CECT 13134
A-SM	54	18	27
B-SM	56	25	18
A-GR	48	8	44
B-GR	63	8	29
A-CA	68	14	18
B-CA	64	12	24

A, Mixed fermentation (four species of non-Saccharomyces and the three strains of Saccharomyces); B, Fermentation performed using the three native strains of Saccharomyces. Fermentations were performed using different musts: SM, Synthetic must; GR, Grenache; CA, Carignan.

Chemical Analysis of Wines

The main oenological parameters of the wines obtained are shown in **Table 3**. All wines contained less than 2 g/L of residual sugars. Additionally, all wines presented an alcohol level expected according to the sugar content of the musts. Small variations were observed in the synthetic and CA wines, with alcohol content from 12.43 to 12.83% for synthetic must or from 13.9 to 14.3% for Carignan wines. Within each wine, the natural must fermentations performed with commercial *S. cerevisiae* strains contained higher levels of glycerol and acetic acid than fermentations performed with autochthonous strains, however, when synthetic must was used, the highest values were exhibited by the mixed fermentation (**Table 3**).

Volatile Compound Production in Industrial Fermentations

The volatile profiles of the six final wines were evaluated (Supplementary Table S2). A total of twenty-nine volatile compounds were quantified and classified into esters (10), alcohols (8), acids (7), carbonyl compounds (3) and lactones (1). Among esters, the most abundant in all fermentations was ethyl acetate, followed by ethyl lactate. However, ethyl and isoamyl acetates, ethyl hexanoate and ethyl butyrate were detected above the odor threshold only in GR wines. In the case of alcohols, isoamyl alcohol, isobutanol and ß-phenylethanol were the main

TABLE 3 | Analytical parameters of final wines.

	Glucose+Fructose (g/L)	Glycerol (g/L)	Acetic acid (g/L)	Alcohol (% v/v)	pH
A-SM	3.79 ± 1.50	11.06 ± 0.21	0.84 ± 0.01	12.83 ± 0.11	3.23 ± 0.01
B-SM	0.11 ± 0.15	8.58 ± 0.10	0.44 ± 0.03	12.43 ± 0.11	3.21 ± 0.01
C-SM	0.17 ± 0.10	9.80 ± 1.12	0.64 ± 0.03	12.50 ± 0.34	3.19 ± 0.02
A-GR	1.09	6.01	0.27	14.9	3.20
B-GR	0.25	5.41	0.31	14.9	3.20
C-GR	0.17	7.10	0.42	14.9	3.13
A-CA	0.18	7.92	0.45	14.3	3.20
B-CA	0.45	8.13	0.44	13.9	3.18
C-CA	0.15	8.97	0.56	13.9	3.16

*Abbreviations as in **Table 2**. Standard deviations were indicated only when triplicates has been used (Synthetic Must fermentations).*

alcohols detected in all wines. All of them and methionol were present above the odor threshold, except for isobutanol in GR-C wine. Acetic acid was by far the most abundant acid in both wine varieties. All except isobutyric acid and decanoic acid were present above the odor threshold. Additionally, the major carbonyl compounds acetaldehyde and butyrolactone were present in all fermentations in a similar range, but the latter was detected below the odor threshold.

Sensory Analysis of the Industrial Wines

The wines obtained at industrial scale for the three different treatments underwent sensory evaluation by triangle tests. **Table 4** presents the results obtained for the two different varieties. Statistically significant differences among the wines were found in three of the four tests performed, as more than 10 of 17 panelists were able to differentiate wines produced with different inocula. In the case of GR wines, native *S. cerevisiae* fermentations were different from the fermentation produced with the commercial strain of *S. cerevisiae*. When the CA variety was tasted, the wines presented in both sensory tests were perceived as different.

DISCUSSION

In this work, the effects of native multi-starter yeast inocula on industrial and laboratory alcoholic fermentations have been studied. Concerning fermentation kinetics and total yeast population, similar results were obtained for the fermentations, and thus similar behavior was found between commercial and native yeast inocula. Additionally, the data obtained followed the typical growth pattern, with values of total yeasts at the end of the alcoholic fermentation close to 10^8 CFU/mL. This value is consistent with results obtained from pure *S. cerevisiae* fermentations as well as from combined *S. cerevisiae* and non-*Saccharomyces* vinifications (Beltran et al., 2002; Gobbi et al., 2013; Belda et al., 2015). However, minor differences have been observed when natural and synthetic must were compared. Natural must was the best medium to grow the *Saccharomyces* yeast, because the recovery on YPD plates was more than 60% at mid fermentation, while in synthetic must the presence of *Saccharomyces* was reduced to 5%. However, *S. cerevisiae* was the only isolated at the end of all fermentations. Although synthetic must tries to mimic natural musts, these are more complex and most likely will be richer in nutrients, which could be a determining factor.

The low yeast population size, such as the observed in GR musts, is normally related to low recovery of the main non-*Saccharomyces* species (*H. uvarum* and *S. bacillaris*), which allows minor species to be easily detected (Beltran et al., 2002). Alternatively, CA must exhibits the typical Priorat microbial fingerprint consisting mainly of *H. uvarum* and *S. bacillaris* (Padilla et al., 2016). It is important to highlight that all non-*Saccharomyces* species isolated at this point have been previously reported on grapes or wine fermentations from the Priorat region (Torija et al., 2001) and are fairly universal, as reviewed by Jolly et al. (2014).

When comparing the populations obtained using culture-dependent and culture-independent techniques in fresh must samples, total yeast plate counts were approximately 1-log lower than qPCR data when yeast populations were low. The qPCR of the *Saccharomyces* spp. population in both varieties found approximately 10^3 cells/mL, but no isolates from these species were recovered from the fresh juice. Similar qPCR results had been reported during the characterization of Merlot musts, but in that case, the culturing of *S. cerevisiae* was directly excluded due to the choice of a non-*Saccharomyces* growth media (Zott et al., 2010). However, the qPCR determination of *H. uvarum* population overestimated it at this initial point, as previously reported, and therefore our data support the suggestion that qPCR is a more sensitive method concerning detection of this species (Zott et al., 2010). In contrast, *S. bacillaris* was slightly underestimated. The reasons for these differences could be different: on one side the differential growth of different species

TABLE 4 | Triangle test evaluation of final industrial wines.

Triangle test	Correct answers (Total)
A-GR against B-GR	7 (17)
B-GR against C-GR	12** (17)
A-CA against B-CA	12** (17)
B-CA against C-CA	10* (17)

*Abrreviations as in **Table 2**. **Significant difference p-value < 0.01. *Significant difference p-value < 0.05.*

on plates, and on the other side due to limited specificity of the qPCR probes and the method efficiency.

Yeast counts and population dynamics after the incorporation of native yeast were also monitored. The initial growth of the non-*Saccharomyces* yeasts was only observed clearly using synthetic must, while the use of natural must seems to be more restrictive to the growth of this type of yeasts. However, this fact is not so clear in all fermentations, because the increase of non-*Saccharomyces* populations by plating has been detected in the GR fermentations inoculated with commercial starter and CA fermentations inoculated with native starter, while this increase is no so evident in other fermentations. The overall detection and quantification of yeast during different fermentation strategies by both culture-dependent and independent methods were very similar, as also reported by Zott et al. (2010), likely due to the high yeast population levels and small number of dominant species. However, small differences need to be further described. One of the main differences is that the non-*Saccharomyces* yeasts were detected up to mid fermentation by plating but until the end of the fermentation by qPCR analysis, as previously reported (Hierro et al., 2006). In these fermentations, *Hanseniaspora* spp. values ranged from 3×10^4 to 3×10^6 cells/mL at the end of the different industrial fermentations, while *S. bacillaris* counts were approximately 3×10^5 cells/mL in the final CA wines, which was in agreement with previous studies (Hierro et al., 2007; Andorrà et al., 2010; Zott et al., 2010). Additionally, *T. delbrueckii* was detected and quantified by qPCR in fermentations A-GR, A-CA and B-CA, but it was only isolated from experiment A-GR at 24 and 48 h after non-*Saccharomyces* inoculation. At these points, qPCR detected *T. delbrueckii* populations at 4×10^4 and 1×10^5 cells/mL, values above the cell concentrations found in CA fermentations. Disagreements in the detection of this species in plates and qPCR were also reported by Zott et al. (2010).

In addition to yeast identification, isolates from the main species were typified to assess the dominance of the starter culture. In experiments where a mixture of three native *S. cerevisiae* strains was inoculated, 100% of *S. cerevisiae* isolates exhibited the electrophoretic pattern of the inoculated strains. This result indicates that the three native *S. cerevisiae* strains included in the yeast consortium coexisted throughout the alcoholic fermentation and dominated the process, being in all types of fermentations (synthetic, Grenache and Carignan) a clear predominance of CECT13132, independently of the composition of the must or the presence of other native yeasts. Similarly, in a study conducted in Albariño white wines where three native strains were singly inoculated, all strains were recovered, and the percentage of imposition was between 90 and 100% in the different stages of fermentation (Carrascosa et al., 2012). In contrast, other authors (Esteve-Zarzoso et al., 2000; Sun et al., 2015) have reported that not all commercial yeast starters can dominate the fermentations in comparison with natural yeast present or isolated from their area, showing that the native microbiota prevailed over the commercial starter culture used, mainly isolated from other oenological region. This result supports the idea that autochthonous yeasts are well adapted to particular fermentation conditions, and thus their incorporation in a mixed inoculum is highly recommendable.

Wines produced with commercial strains rendered higher levels of glycerol and acetic acid, but in all cases, the final content was acceptable. These commercial strains are among the most used in the region and are able to perform the alcoholic fermentation of high sugar content to dryness. In the case of GR, all fermentations produced the same final alcohol content. However, for CA wines, there were some differences in the alcohol production. This result could be due to the heterogeneity of the starting must, which could include slight differences in the sugar content.

The volatile profiles of the different wines were also studied. The total acid content was higher in fermentations conducted using commercial *S. cerevisiae* strains, in agreement with results obtained in the general chemical characterization. However, the concentrations of the other analyzed volatile compounds were very similar among different treatments. The contributions of *H. uvarum*, *C. zemplinina*, *T. delbrueckii,* and *M. pulcherrima* to wine aroma have been studied (Comitini et al., 2011; Andorrà et al., 2012; González-Royo et al., 2014; Loira et al., 2014; Renault et al., 2015). Most studies concluded that the incorporation of these species exhibited a positive impact on aroma development. Nevertheless, most articles focused on evaluating the effects of single strains or mixed starters composed of one *S. cerevisiae* strain and one non-*Saccharomyces* species. Therefore, the interactions among different non-*Saccharomyces* wine yeast species need to be further elucidated. The results obtained in this paper highlight that complex interactions among yeast strains are likely to occur during the industrial fermentation of grape juice, and thus it is difficult to identify clear trends among different inoculation strategies. Still, the sensory evaluation concluded that most of the wines produced could be identified as different from the organoleptic point of view. However, high ethanol content and the full body characterize the Priorat wines, which is the consequence of its high complexity. Thus, although tasters could differentiate all the produced wines, there was not a significant preference: in all the cases the preferences were close to 50% of the tasters that identified the differences.

AUTHOR CONTRIBUTIONS

BP, LZ, AF, RP, and BE-Z performed the experiments; BP, BE-Z, GB, and AM designed the experiments, analyzed and interpreted the results; wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.01293/full#supplementary-material

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Different Non-*Saccharomyces* Yeast Species Stimulate Nutrient Consumption in *S. cerevisiae* Mixed Cultures

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The growing interest of the winemaking industry on the use of non-*Saccharomyces* starters has prompted several studies about the physiological features of this diverse group of microorganisms. The fact that the proposed use of these new starters will almost invariably involve either simultaneous or sequential inoculation with *Saccharomyces cerevisiae* has also driven the attention to the potential biological interactions between different starters during wine fermentation. Our current understanding is that alternative yeast starters will affect wine features by both direct and indirect mechanisms (through metabolic or other types of interactions with *S. cerevisiae*). There are still few studies addressing the question of yeast–yeast interactions in winemaking by a transcriptomic approach. In a previous report, we revealed early responses of *S. cerevisiae* and *Torulaspora delbrueckii* to the presence of each other under anaerobic conditions, mainly the overexpression of genes related with sugar consumption and cell proliferation. We have now studied the response, under aerobic conditions, of *S. cerevisiae* to other two non-*Saccharomyces* species, *Hanseniaspora uvarum* and *Candida sake*, keeping *T. delbrueckii* as a reference; and always focusing on the early stages of the interaction. Results point to some common features of the way *S. cerevisiae* modifies its transcriptome in front of other yeast species, namely activation of glucose and nitrogen metabolism, being the later specific for aerobic conditions.

Keywords: interspecific interaction, biotic stress, non-*Saccharomyces*, mixed starter, wine fermentation

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INTRODUCTION

Employment of non-*Saccharomyces* yeast starters constitutes a growing trend in the winemaking industry. They are proposed as a means to improve aromatic complexity, so recovering some of the features of spontaneous fermentation, while minimizing the risk of microbial spoilage associated to it (Ciani and Comitini, 2011). The potential benefits have been linked to specific yeast species, with commercial strains belonging to *Torulaspora delbrueckii*, *Pichia kluyveri* or *Lachancea thermotolerans*, among other species. In addition to its contribution to improved aromatic profile, non-*Saccharomyces* strains have been proposed to improve glycerol or mannoprotein content, volatile acidity, color stability, or alcohol level reduction (Ciani and Comitini, 2011; Morales et al., 2015; Ciani et al., 2016).

In terms of microbial interactions, there is a substantial difference between conventional inoculated wine production, in which *Saccharomyces cerevisiae* dominates from almost the beginning of fermentation; and the use of non-*Saccharomyces* starters (either in co-inoculation or sequential inoculation), which results in two different species represented by comparable cell numbers for a relatively long period. Consequently, the contribution of the inoculation of non-*Saccharomyces* strains to winemaking can be either direct or indirect, through biological interactions with *S. cerevisiae*. Some recently described examples include a synergic interaction between *S. cerevisiae* and *T. delbrueckii* resulting in increased levels of 3-sulfanylhexan-1-ol (Renault et al., 2015, 2016) or in a decrease of volatile acidity and higher isoamyl acetate production (Taillandier et al., 2014); synergic interactions between *Debaryomyces vanrijiae* or *Candida sake* and *S. cerevisiae* resulting in enhanced aroma profile (Maturano et al., 2015).

Co-inoculation involving *S. cerevisiae* and other wine yeast species, nearly always results in the disappearance or loss of viability of non-*Saccharomyces* cells (Albergaria et al., 2010; Taillandier et al., 2014; Wang et al., 2015, 2016). Although this dominance can be mainly explained by the indirect impact of sugar consumption rates, nutrient depletion, and ethanol production; some direct mechanisms for yeast species antagonism have also been described. For example, killer factors have been known in *S. cerevisiae* for many years. These secreted peptides, encoded by extrachromosomal elements, affect a limited number of yeast species (van Vuuren and Jacobs, 1992; Pérez et al., 2001). Similar toxins have been described for some other yeast species (Velázquez et al., 2015). In addition, a peptide fragment of the *S. cerevisiae* glycolytic enzyme GAPDH was recently shown to inhibit growth of several wine bacterial and yeast species (Albergaria et al., 2010; Branco et al., 2014).

A few studies have addressed microbial interactions in winemaking by transcriptomic approaches. *S. cerevisiae* was shown to reduce its global transcription activity in co-inoculation with *Hanseniaspora guilliermondii* (Barbosa et al., 2015). In addition, these authors showed that the response of *S. cerevisiae* involved the up-regulation of genes related with biosynthesis of vitamins, and down-regulation of genes involved in the uptake and biosynthesis of amino acids. Rossouw et al. (2012) also identified altered gene expression in *S. cerevisiae* in response to the metabolic activity of *Oenococcus oeni*. The same group identified co-flocculation as a possible mechanism of specific yeast–yeast interspecific interactions (Rossouw et al., 2015). More recently, Pérez-Torrado et al. (2017) analyzed the interaction between different co-inoculated strains of *S. cerevisiae*. The results provided insight on the dominance phenomenon between strains of the same species, highlighting the importance of cell-to-cell contact and differential sulphite production in this process.

The few available genome-wide studies of the interaction between *S. cerevisiae* and non-*Saccharomyces* yeasts (microarray based) analyzed transcription after at least 1 or 3 days of contact between *S. cerevisiae* and the other microorganism. In a recent work, we addressed earlier stages of fermentations co-inoculated with *S. cerevisiae* and *T. delbrueckii*. We found a remarkable transcriptional reprograming for both yeast strains in the presence of each other, as soon as 2 h after being put into contact (Tronchoni et al., 2017). In this work, we have focused on the early transcriptional responses of *S. cerevisiae* to strains belonging to three different yeast species, *T. delbrueckii*, *Hanseniaspora uvarum*, and *C. sake*. The first species is currently the most widely employed alternative yeast starter for winemaking (e.g., Belda et al., 2015). It was kept as a reference to account for the differences in fermentation conditions between our previous work and the current one (Tronchoni et al., 2017). Species of the genus *Hanseniaspora* are ubiquitous in the winemaking environment, and some of them have been proposed as wine yeast starters (Ciani et al., 2016). *C. sake* has been studied as a promising species for alcohol level reduction in wine by promoting respiratory metabolism (Rodrigues et al., 2016).

MATERIALS AND METHODS

Strains and Media

Four yeast strains have been used in this work, *S. cerevisiae* FX10 (Laffort, SA), a widely used industrial wine yeast strain, *T. delbrueckii* CECT 11199 (CBS 1146), *C. sake* CECT 11909 (CBS 159), and *H. uvarum* CECT 10389 (MCYC 1857). Synthetic must contained (per liter): glucose: 100 g; fructose: 100 g; malic acid: 6 g; citric acid 6 g; YNB w/o aa; w/o $(NH_4)_2SO_4$ 1.7 g; nitrogen sources (Asp 29 mg; Glu 80 mg; Ser 52 mg; Gln 333 mg; Hys 31 mg; Gly 12 mg; Thr 50 mg; Arg 296.28 mg; Ala 97 mg; Tyr 13 mg; Cys 18.2 mg; Val 29 mg; Met 21 mg; Trp 116 mg; Phe 25 mg; Ile 22 mg; Leu 32 mg; Lys 13.72 mg; Pro 400 mg; NH_4Cl 306 mg); anaerobic factors (ergosterol 15 mg; oleic acid 5 mg; tween 80 0.5 mL); inositol 18 mg; pH adjusted at 3.5 with NaOH.

Cultivation Conditions

Pre-cultures were grown in YPD (1% yeast extract, 2% peptone, and 2% glucose) medium for 48 h at 25°C and 150 rpm. Before co-inoculation, pre-cultures were centrifuged at 2200 × *g*, for 15 min at room temperature, and washed twice with distilled water. Mixed cultures constituted by *S. cerevisiae* and one of the assayed strains were inoculated to a total initial optical density (OD_{600nm}) of 0.2 (0.1 for each strain) in 200 mL of synthetic must medium contained in 250 mL flasks with wide aluminum foil caps allowing aeration. Single culture of *S. cerevisiae* strain was inoculated to an OD_{600nm} of 0.2 to match conditions in mixed cultures. Flasks were incubated at 25°C under agitation (250 rpm) during 3 h. Experiments were performed in triplicate. The viability of the different populations in the mixed cultures was confirmed after 24 h of co-cultivation. Cells from the mixed cultures were plated at 25°C at different dilutions to ensure individual colony growth and then re-plated at 37°C were *S. cerevisiae* cells can be differentiated from the other yeast species that do not growth at this temperature. This confirmed that after 24 h both yeast species were present in the media.

RNAseq, Data Analyses, and Statistics

After 3 h of cultivation, total flasks volumes were centrifuged and collected cells washed twice with distilled water before samples were submerged in liquid nitrogen and stored at

−80°C for total RNA isolation. Total RNA from the biological triplicates was extracted using RNeasy® mini kit (QIAGEN) and subjected to DNAase treatment using the Ambion DNA-free™ kit according to the manufacturers' instructions. Concentration, purity, and integrity of RNA samples were determined by spectrophotometric analysis considering the absorbance ratio at 260/280 nm and at 230/260 nm. Library preparation and sequencing of RNA was performed at the Genomics Core Facility in the Center for Biomedical Research of La Rioja (CIBIR). After poly-A filtering, libraries were generated for the different conditions, triplicates of *S. cerevisiae* single cultures and triplicates of *S. cerevisiae* co-cultivated with *T. delbrueckii*, *C. sake,* and *H. uvarum*. From these libraries, 100-bp pair-end sequence reads were produced with Illumina HiSeq 2000. All raw RNA-Seq data have been deposited in NCBI under Sequence Read Archive SRR5422019 (BioProject PRJNA381847) accession number.

Alignment of reads to the S288c R64 *S. cerevisiae* yeast reference genome assembly was carried out using TopHat2 v.2.0.13 (Kim et al., 2013). Only uniquely mapped single copy, ≤1 polymorphism per 25 bp reads with quality≥ 20 were kept for further analysis. The htseq-count tool (v.0.5.4p5) from HTSeq (Anders et al., 2015) was used to estimate unambiguous read count per genome assembly annotated transcript. Normalization following the trimmed mean of M-values (TMM) method (Robinson and Oshlack, 2010), as well as a time-points DEGs searches (adjusted Benjamini–Hochberg $P \leq 0.05$ and ≥twofold change) were performed in edgeR v.2.2.6 (Robinson et al., 2010). Finally, fragments per kb of exon per million fragments mapped (FPKM) was calculated using Cuffdiff v.2.2.1 (Trapnell et al., 2013) and low-expressed transcripts were filtered out when FPKM was <1 in both samples. In order to confirm that there was no cross mapping from the co-cultivation, a quimeric genome from *S. cerevisiae* and the non-*Saccharomyces* species was created when the genome was available (*T. delbrueckii* and *H. uvarum*). Almost the same genes (98%) appeared DE comparing both strategies, quimeric and regular mapping.

Different sets of genes were considered for analysis purposes. We refer to significantly up- or down-regulated genes for those that have a log fold change (LogFC) ≥ 0.5 or ≤ -0.5 and an adjusted p-value ≤ 0.05. When we refer to highly up- or down-regulated genes (a more restrictive category), showing a LogFC ≥ 1 or ≤ -1 and an adjusted p-value ≤ 0.05. The response of *S. cerevisiae* to each different strain has been analyzed using the more restrictive dataset, including gene ontology analysis. For comparative analysis among the different datasets the broader database has been used (Venn Diagram). Gene expression values showing higher adjusted p-values were never taken into consideration for data analysis or discussion, independently of the associated LogFC.

Principal component analysis (PCA) was done using AltAnalyze software (2.1.0) (Emig et al., 2010). AltAnalyze was feeded with the normalized RNAseq data transcripts per million (TPM). The remaining statistical analyses were done using STATA-SE. Venn diagram was done by using Venny 2.1 on-line tool software (Oliveros, 2007–2015). GO term analysis was performed using YeastMine (Balakrishnan et al., 2012). The *p-values* were corrected for multiple testing by the Bonferroni test for functional associations and GO analyses. The statistical level of significance was set at p-value ≤ 0.05. Then, GO terms were grouped in biomodules by GO/Module (Yang et al., 2011) to prioritize Gene Ontology.

RESULTS

Experimental Set-up

In this work, we have analyzed the effect of three different yeast species over the *S. cerevisiae* transcriptome when grown together at early stages of an aerobic synthetic must fermentation. *T. delbrueckii*, *C. sake,* and *H. uvarum* were chosen to be co-inoculated with *S. cerevisiae* at equal cell density. After 3 h, cells were sampled and the transcriptome of the *S. cerevisiae* cells from mixed and single cultures was compared by RNAseq analysis. These three yeast species are often isolated from grape must at early stages of fermentation, and are hence natural competitors of *S. cerevisiae* (Fleet, 2003; Jolly et al., 2014). In a previous work the transcriptome of *S. cerevisiae* and *T. delbrueckii* was analyzed after 2 and 12 h of anaerobic co-cultivation. Comparison of single and mixed cultures showed that genes from "Glucose Fermentation Pathway" were overexpressed in both species due to the presence of the other yeast in the media. Overexpression in *S. cerevisiae* is noticed in the first sampling point and in *T. delbrueckii* in the second one. Even though *T. delbrueckii* shows good fermentative fitness in pure culture, it is quickly overtaken by *S. cerevisiae*, perhaps because the earlier reply of *S. cerevisiae*. This made us wonder if the observed quick response of *S. cerevisiae* to the presence of *T. delbrueckii* was specific for this yeast species or similar responses could be obtained with different yeasts. For this reason, other yeast species present at early stages of grape must fermentation but phylogenetically more distant than *T. delbrueckii* (Masneuf-Pomarede et al., 2016) were chosen.

Genes under NCR Are Induced in the Presence of *T. delbrueckii*

T. delbrueckii was used to keep a reference to our published work (Tronchoni et al., 2017) but based on our previous results, the selection of an early time point was set to 3 instead of 2 h. The number of overexpressed genes was similar, with only 44 genes being highly up-regulated (Supplementary File S1). Several genes from the "Glucose Fermentation Pathway" appear significantly up-regulated as well as several genes encoding for glucose transporter proteins, as previously described (Supplementary File S1). Under standard conditions, genes related to fermentation of glucose and its transport into the cell are tightly regulated by the extracellular concentration of glucose through carbon catabolite repression (CCR) (Gancedo, 1998). For instance, high-affinity glucose transporters are only expressed when the concentration of this sugar is low (Ozcan, 2002; Kayikci and Nielsen, 2015). Interestingly, seven out of the eight genes involved in glucose uptake and metabolism are under the control of this carbon source that repress its expression when sugar concentration is high. Based on the glucose concentration

of the media, around 200 g/L, these genes should be down-regulated. This result points to a partial relieve of CCR in *S. cerevisiae* by the presence of *T. delbrueckii*.

In addition, Gene Ontology categories enriched for *S. cerevisiae* genes up-regulated in the presence of *T. delbrueckii* were mostly related with nitrogen metabolism, specifically allantoin catabolism (**Table 1**). Actually, most genes induced by co-cultivation with *T. delbrueckii* were involved in utilization of alternative nitrogen sources; and are under nitrogen catabolite repression (NCR) control. The relevance of the activation of these genes is such that five out of the main up-regulated and even half of the highly up-regulated genes were under the control of this transcription factor *GLN3* (Supplementary File S1), required for the expression of genes involved in the use of non-preferred nitrogen sources (Magasanik and Kaiser, 2002). Among them, genes showing the highest over-expression values belong to the *DAL* family. Indeed, the entire pathway for allantoin catabolism was overexpressed.

Like the CCR and NCR dependent genes mentioned above, expression of other genes expected to show low activity in rich medium, especially after only 3 h of incubation (i.e., before actual consumption of carbon or nitrogen sources might be observed), was also highly induced in *S. cerevisiae* by co-cultivation with *T. delbrueckii*. Among them, we found other NCR dependent genes like those coding for proline permease, *PUT4*; proline oxidase, *PUT1*; general amino acid permease, *GAP1*; GABA permease, *UGA4*; or a putative allantoate permease; as well as some high affinity permeases and metal transporters like those for inorganic phosphate, *PHO84*; sulfate, *SUL1*; copper, *CTR3*; or cysteine, *YCT1* (Supplementary File S1).

Genes Involved in Cell Replication Are Up-regulated by Co-cultivation with *C. sake*

No significant gene ontology enrichment was found for the only 20 genes showing a high overexpression in *S. cerevisiae* when co-cultivated with *C. sake*. About half of them were shared with the list of highly overexpressed genes coming from *T. delbrueckii* co-cultivation, including genes already discussed above, related with the allantoin pathway, nitrogen uptake or non-preferred nitrogen sources, and genes involved in glucose uptake and metabolism (Supplementary File S1). Some of these genes in common showed higher expression compared to the *T. delbrueckii* experiment, like *CHA1*, involved in the use of nitrogen sources (serine or threonine). Other highly overexpressed genes shared between *C. sake* and *T. delbrueckii* co-cultivation are all involved in replication (specially RNA helicases, but also rRNA and ribosome biogenesis, or Start checkpoint), or related with cell wall (*TIP1*), and membrane lipid composition (*OLE1*, *ERG5*, *ERG3*, *ERG11*, *ERG1*, and *ERG25*) (Supplementary File S1). The up-regulation of these genes suggests another possible strategy of *S. cerevisiae* to improve competitiveness in grape must, besides or complementary to the activation of genes required for sugar and nitrogen consumption. This would consist of an increase in relative membrane surface (through increased cell numbers), which will help accelerate nutrient uptake in detriment of other yeasts.

In contrast to the *T. delbrueckii* experiment, in *C. sake* competition, there are more genes highly down-regulated than up-regulated (34 genes). These genes are summarized in the GO term categories "carboxylic acid transmembrane transport" and "sulfur compound metabolic process" (Supplementary File S1). Some of the genes under these two categories are *MET1*, *MET2*, *MET8*, *MET32*, *ISU2*, or *SUL2*. They encode methionine and sulfur permeases, and are involved in methionine synthesis, or the synthesis of iron-sulfur proteins. Thus, the transcriptional response of *S. cerevisiae* to co-cultivation with *T. delbrueckii* or *C. sake* is similar, considering overexpressed genes. However, there are clear differences among the down-regulated genes. Actually, from the 34 genes highly down-regulated in the *C. sake* experiment, only four appear in the *T. delbrueckii* down-regulated dataset, while other two genes appear as up-regulated (Supplementary File S1).

Co-cultivation with *H. uvarum* Triggers the Expression of Genes under "Response to Stress" Category

Co-cultivation with *H. uvarum* resulted in a low gene expression profile as seen with *T. delbrueckii* and *C. sake*, with 29 genes showing high overexpression, 6 genes in common with *T. delbrueckii,* and only 3 with *C. sake* (Supplementary File S1). Among the 29 genes significantly highly up-regulated in these cultures 12 out of them belong to the GO categories "response to stimulus" and/or "response to stress." Thus, many of the most

TABLE 1 | Gene Ontology enrichment for *Saccharomyces cerevisiae* in co-cultivation with different non-*Saccharomyces* yeasts.

Co-cultivated yeast species	Regulation	GO IDs	Significance	GO terms
Torulaspora delbrueckii	Up	GO:0006144	0.001	Purine nucleobase metabolic process
		GO:0000256	0.000	Allantoin catabolic process
Candida sake	Down	GO:0006790	0.002	Sulfur compound metabolic process
		GO:0003333	0.009	Amino acid transmembrane transport
		GO:0046942	0.026	Carboxylic acid transport
		GO:0098656	0.029	Anion transmembrane transport
Hanseniaspora uvarum	Down	GO:0009086	0.001	Methionine biosynthetic process
		GO:0019379	0.000	Sulfate assimilation, phosphoadenylyl sulfate reduction
		GO:0070814	0.004	Hydrogen sulfide biosynthetic process

overexpressed genes are involved in resistance to several stresses, for instance, pleiotropic drug resistance (2 genes), heat (4 genes), DNA replication stress or DNA damage (6 genes) or osmotic stress (Hog1 dependent, 2 genes). It is also worth mentioning three genes coding for cell wall mannoproteins (*TIR1*, *TIP1*, and *DAN1*), among the 10 most overexpressed genes. These mannoproteins belong to the Srp1/Tip1 family and have been described to respond to different stresses like cold stress and to be stimulated in the adaptation to hypoxia (Sertil et al., 1997; ter Linde et al., 1999; Abramova N. et al., 2001; Abramova N.E. et al., 2001; Tai et al., 2005). The results obtained for *H. uvarum* co-cultivation suggest that some cell wall proteins might be also important for the adaptation of *S. cerevisiae* to biotic stress.

As seen with *C. sake* mixed cultures, transcriptional response to co-cultivation with *H. uvarum* results in the repression of a great number of genes in *S. cerevisiae*, 31 in this case highly down-regulated (Supplementary File S1). This response is similar to that observed for *C. sake*, with several genes showing a reverse behavior, as compared to the *T. delbrueckii* experiment (7 genes). Some of these genes were important in the discussion of the effect of *T. delbrueckii* over *S. cerevisiae* in mixed cultures made above (*HSP12* and *PDC5*) and in our previous work (Tronchoni et al., 2017), like *HSP12* (described by us and others in *S. cerevisiae* – *S. cerevisiae* interactions).

Different Yeast Species Promote a Different Response in *S. cerevisiae* Although Some Similarities Can Be Observed

Total LogFC datasets without threshold restrictions were used to perform a PCA to compare the responses of *S. cerevisiae* to the different yeasts in co-cultivation (**Figure 1**). Results cluster each independent replicate together for each yeast, although there is a higher dispersion of *T. delbrueckii* and *H. uvarum* compared with *C. sake*. Principal Component 1 (PC1) explaining 62.1% of the variance separates *T. delbrueckii* and *C. sake* from *H. uvarum*, and PC2 explaining 15.2% of the variance, *T. delbrueckii* and *H. uvarum* from *C. sake*. Thus, each yeast ends up in a different section of the PCA. Highlighting a particular response of *S. cerevisiae* when co-cultivated with different yeast species.

Although PCA results define a different response depending on the yeast mixed culture, the co-cultivation experiments shared global transcriptomic characteristics as well as genes behaving in a similar manner. One of the main characteristics shared by all the experiments carried out so far is that it is a moderate response, gene expression fold changes are low as well as the number of significant genes. Venn diagram showing significant up-regulated genes (LogFC $\geq$ 0.5; *p*-adjusted $\leq$ 0.05) for the three species tested shows the degree of similarity described (**Figure 2**). Although there are genes in common among them, the percentage varies from the 35% of the genes being in common between *T. delbrueckii* and *H. uvarum*, both with similar number of up-regulated genes to the much lower number of genes shared with *C. sake* or among the three of them. On the other hand, Venn diagram for down-regulated genes (LogFC ≤ -0.5; *p*-adjusted $\leq$ 0.05) shows a much more heterogeneous response to co-cultivation depending on the competing yeast species.

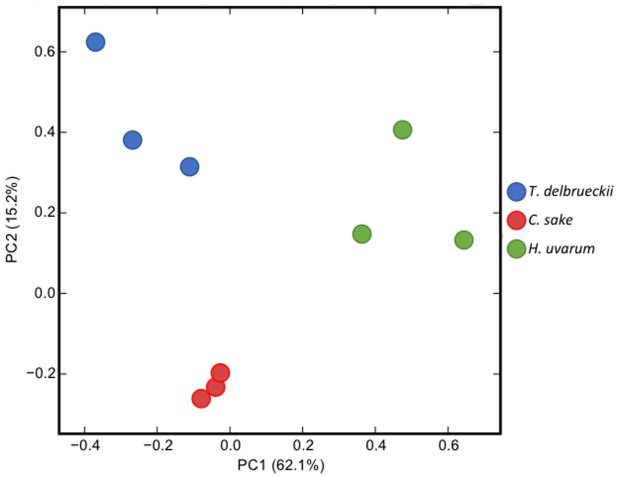

FIGURE 1 | Principal component analysis (PCA) of the normalized RNAseq data transcripts per million (TPM) of *Saccharomyces cerevisiae* in response to co-cultivation with different yeasts species.

A general picture can be drawn from the short list of genes equally up- or down-regulated in *S. cerevisiae* in response to co-cultivation among the three experiments (**Figure 2**). From the 12 genes commonly up-regulated, 3 are involved in glucose uptake and glycolysis, and according to literature should be repressed by high levels of glucose (after 3 h of co-cultivation in synthetic must, glucose concentration is close to 200 g/L). *HXT12*, a high-affinity glucose transporter and both cytoplasmic and mitochondrial aldehyde dehydrogenases; other three involved in membrane lipid metabolism, *OLE1* (monounsaturated fatty acid synthesis), *FAA4* (long-chain fatty acyl-CoA synthetase), and *ERG5* (ergosterol biosynthesis pathway); also, the major cell wall mannoprotein *TIP1*; two more related with the nitrogen sources available, *CHA1* (serine or threonine) and *PUT1* (proline). The three remaining genes codified for a protein required for antifungal drug resistance (*COS111*), a membrane protein involved in zinc ion homeostasis (*IZH1*) and *INA1* a putative protein of unknown function which paralog is *FAT3*, a protein required for fatty acid uptake. Therefore, as has been described previously for each individual yeast co-cultivation, this set of genes can be summarized in glucose uptake, membrane and cell wall biogenesis, and nitrogen utilization. Thus, despite the clean separation of the three yeast species co-cultivated by the PCA there are some trends common to all experiments. This can be seen in the expression of the *DAL* family of metabolic genes. Plotting LogFC for the different co-cultivation vs. single *S. cerevisiae* cultures of chromosome IX reveals the induction of this gene cluster (**Figure 3**), showing higher overexpression values for *T. delbrueckii*, lower values for *C. sake* despite a clear trend is observed, and just one gene significative for *H. uvarum*.

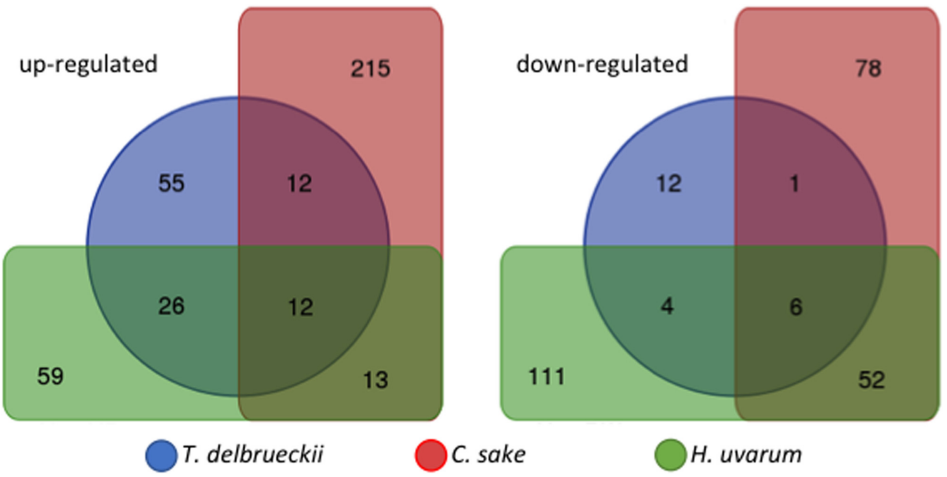

FIGURE 2 | Venn diagram showing up-regulated and down-regulated significant genes (LogFC ≥ 0.5 or ≤−0.5; *p*-adjusted ≤ 0.05) of *S. cerevisiae* in response to co-cultivation with different yeasts species.

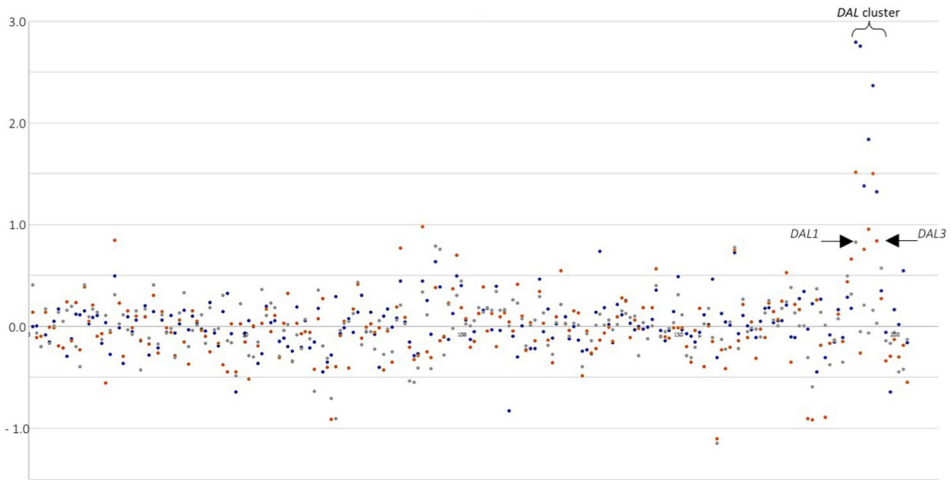

FIGURE 3 | Differential expression (LogFC) for genes in chromosome IX of *S. cerevisiae* in response to co-cultivation with different yeasts species. *DAL* cluster is highlighted. Arrows point first and last gene in the cluster. Blue dots: *T. delbrueckii* mixed culture. Orange dots: *C. sake* mixed cultures. Gray dots: *H. uvarum* mixed culture.

DISCUSSION

In a previous article, we analyzed the transcriptional response to co-cultivation of *S. cerevisiae* and *T. delbrueckii*. The study focused in the initial stages of wine fermentation, before *S. cerevisiae* completely dominated the mixed cultures. Both species showed a clear response to the presence of each other, even though the portion of the genome showing altered transcriptional levels was relatively small. Changes in the transcription pattern suggested a stimulation of metabolic activity and growth. Specifically, gene expression of the glucose fermentation pathway was induced. This was observed for both yeast species. However, the timing was different, with *T. delbrueckii* showing a delayed response (12 h) as compared to *S. cerevisiae*.

The early response of *S. cerevisiae* after 2 h of co-cultivation decided us to focus at this first time point in this new work. The selection of an early time point ensures that the gene expression changes are responding to the direct presence of the other yeast species instead of other more indirect signals like faster nutrient depletion from the media. In order to allow higher transcriptomic changes compared to previous results, cells were collected at 3 h of co-cultivation. Here we addressed the species-specificity of this early response to biotic stress, by co-cultivating *S. cerevisiae* with phylogenetically more distant yeast species. Three different species, common in the wine fermentation environment, were selected, *T. delbrueckii*, in order to have a reference to previous experiments, C. *sake*, and *H. uvarum* (Masneuf-Pomarede et al., 2016). *H. uvarum* was chosen because is one of the most abundant yeast species found

on grapes and in grape must (Albertin et al., 2015), therefore usually present when *S. cerevisiae* is inoculated, but also because it has been proposed as a non-*Saccharomyces* starter (Tristezza et al., 2016). *C. sake* has also been proposed to be co-inoculated with *S. cerevisiae* in order to improve and differentiate the wine fermentation process (Maturano et al., 2015; Rodrigues et al., 2016). This experiment was carried out under aerobic regime to better understand the behavior of yeast under this condition, that has been proposed as an alternative to reduce alcohol in wines by using non-*Saccharomyces* species in co-cultivation. In this occasion, the stimulation of metabolic activity previously seen for *S. cerevisiae* in co-cultivation with *T. delbrueckii* was confirmed in this work, not only for glucose metabolism but also for nitrogen metabolism. Several of the induced genes are described as being under NCR control. Apparently under conditions of co-cultivation with *T. delbrueckii*, *S. cerevisiae* partially relieves the nitrogen and glucose catabolite repression, up-regulating a series of genes that, in pure culture, are usually expressed in later stages of growth in grape must, when the concentration of easily assimilated carbon and nitrogen sources has decreased. An explanation for this could be that *S. cerevisiae* is responding by increasing the flux of nutrients (glucose and nitrogen) to reduce their availability for *T. delbrueckii*. A common response observed in both species in the different time points (2 and 12 h) in our previous work showed *HSP12* as a possible marker for co-cultivation. Current results confirm its induction after 3 h, but do not support the view of *HSP12* induction as a general response to co-cultivation in *S. cerevisiae*, since its expression was down-regulated in front of the other yeast species. However, overexpression of *HSP12* might depend on the competition strength of the strain in co-cultivation, or on the nature of the relationships established between the two strains in the mixed culture (cooperative or antagonistic). Therefore, up-regulation of *HSP12* will only take place under conditions of co-cultivation that may pose a challenge to the growth of *S. cerevisiae* cells.

The effect of the other two phylogenetically more distant yeasts, *C. sake* and *H. uvarum*, over *S. cerevisiae* gene expression was also examined after 3 h of co-cultivation under aerobic conditions. A set of genes related to glucose and nitrogen metabolism as observed in *T. delbrueckii*, appeared also overexpressed in the mixed culture, as compared to *S. cerevisiae* single cultures, although it does not involve as many genes as in the case of *T. delbrueckii*, and the overexpression levels are also lower. On the other hand, among the genes up-regulated there were several not observed for *T. delbrueckii*. The specific set of genes responding to co-cultivation with *C. sake* play different functions, including cell replication (genes involved in ribosome biogenesis, RNA helicases or Start checkpoint) or genes related to membrane maintenance. This could be pointing to a second strategy, compatible and complementary to the metabolic stimulation. The increase in population size by accelerating cell division. This would help increase the uptake of nutrients, decreasing their availability for competitor yeasts. Co-cultivation with *H. uvarum*, also induced genes that point to cell duplication as a target to improve competitiveness by *S. cerevisiae*.

Overexpression of the DAL family of genes has revealed in this work as a diagnostic feature of the relief of nitrogen catabolite repression in response to co-cultivation under aerobic conditions. Allantoin metabolic and catabolic processes appear as significantly enriched GO terms in response to *T. delbrueckii* (**Table 1**). And, although not statistically significant, the same trend was observed in the response of *S. cerevisiae* to the other two yeast species, as illustrated in **Figure 3**. The weaker impact on DAL expression levels in *C. sake* or *H. uvarum* co-cultures, as compared to *T. delbrueckii* might be related to the closer phylogenetic proximity of the later with *S. cerevisiae*, or the nature of the established interactions (positive or negative). This pathway has been described before to be relevant in yeast–yeast interactions. In comparisons of *S. cerevisiae* single cultures vs. mixed cultures with a non-*Saccharomyces* species (*H. guilliermondii*), the allantoin pathway was significant in single cultures after 24 h of cultivation (Barbosa et al., 2015). In our previous work that matches better their experimental conditions under anaerobiosis, after 2 h of co-cultivation, single cultures of *S. cerevisiae* had some genes from the allantoin pathway significantly up-regulated, but not enough genes to have the GO-term significantly expressed. On the contrary, in this work, it is the co-cultivation after 3 h what triggers this pathway in *S. cerevisiae*. Since the DAL gene cluster is up-regulated this time as well as many genes related to nitrogen metabolism and the uptake of non-preferred nitrogen sources, these differences should be due to the aeration regime selected under co-cultivation conditions in each experiment. It is concluded that the culture conditions have a strong impact on the way *S. cerevisiae* responds to the presence of competing yeast species. Indeed, overexpression of NCR dependent genes, including DAL genes, was not appreciated in experiments performed under anaerobic conditions (Tronchoni et al., 2017). Probably this is related to the stimulation of biomass production due to oxygen availability and partial respiratory metabolism. Further stimulation due to co-cultivation would hence lead to an increased demand of nitrogen, and the consequent overexpression of genes required for the assimilation of alternative nitrogen sources.

S. cerevisiae and *T. delbrueckii* responses to each other were similar in our previous work. They also showed similarities with other yeast–yeast interactions between *S. cerevisiae* strains (Rivero et al., 2015). This work confirms our previous results with *T. delbrueckii*, but also that, despite some similarities, the differences are enough to distinguish the effect of each yeast species. Interestingly there are also examples, like the DAL family of genes, were the same genes are involved in the responses of all species, but with clear differences in the intensity of the response. Therefore, although co-culture with different yeasts produces a similar response, this is not the exact same, and at least with the yeasts analyzed in this work each one induces a particular profile of gene expression in *S. cerevisiae*.

This work confirmed metabolic stimulation in *S. cerevisiae* as a consequence of co-cultivation with different wine yeast species, in synthetic must. This response was stronger for *T. delbrueckii*, which is a close phylogenetic relative of *S. cerevisiae*, than for not so closely related species. This response involves, by one side, overexpression of genes in the gluco-fermentative pathway; and by the other side, a partial relief of NCR. The later seems to depend on oxygen availability. In addition, the response to

C. sake and *H. uvarum* suggests a complementary strategy, enhancing cell duplication rates. Our results contribute to better understanding the behavior of starter yeasts in co-culture (*S. cerevisiae* with non-*Saccharomyces* strains), a promising winemaking practice whose application is steadily increasing in the cellars.

AVAILABILITY OF DATA AND MATERIAL

The data set supporting the results of this article is available in the NCBI repository under Sequence Read Archive SRR5422019 (BioProject PRJNA381847) accession number (http://www.ncbi.nlm.nih.gov/Traces/sra/sra.cgi?view=announcement). The data set supporting the results of this article is included in the article (and its Additional files).

AUTHOR CONTRIBUTIONS

JT, RG, PM, and JC conceived and designed the study. JC performed the experiments. JT and JC, analyzed the data. JT, RG, and PM interpreted the results and wrote the manuscript. All authors discussed and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2017.02121/full#supplementary-material

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frontiers in Microbiology

ORIGINAL RESEARCH
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Metschnikowia pulcherrima Influences the Expression of Genes Involved in PDH Bypass and Glyceropyruvic Fermentation in *Saccharomyces cerevisiae*

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Previous studies reported that the use of *Metschnikowia pulcherrima* in sequential culture fermentation with *Saccharomyces cerevisiae* mainly induced a reduction of volatile acidity in wine. The impact of the presence of this yeast on the metabolic pathway involved in pyruvate dehydrogenase (PDH) bypass and glycerol production in *S. cerevisiae* has never been investigated. In this work, we compared acetic acid and glycerol production kinetics between pure *S. cerevisiae* culture and its sequential culture with *M. pulcherrima* during alcoholic fermentation. In parallel, the expression levels of the principal genes involved in PDH bypass and glyceropyruvic fermentation in *S. cerevisiae* were investigated. A sequential culture of *M. pulcherrima*/*S. cerevisiae* at an inoculation ratio of 10:1 produced 40% less acetic acid than pure *S. cerevisiae* culture and led to the enhancement of glycerol content (12% higher). High expression levels of pyruvate decarboxylase *PDC1* and *PDC5*, acetaldehyde dehydrogenase *ALD6*, alcohol dehydrogenase *ADH1* and glycerol-3-phosphate dehydrogenase *PDC1* genes during the first 3 days of fermentation in sequential culture conditions are highlighted. Despite the complexity of correlating gene expression levels to acetic acid formation kinetics, we demonstrate that the acetic acid production pathway is altered by sequential culture conditions. Moreover, we show for the first time that the entire acetic acid and glycerol metabolic pathway can be modulated in *S. cerevisiae* by the presence of *M. pulcherrima* at the beginning of fermentation.

Keywords: sequential culture *Metschnikowia pulcherrima*/*Saccharomyces cerevisiae*, acetic acid, glycerol, alcoholic fermentation, quantitative RT-PCR

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INTRODUCTION

Complex interactions between organisms occur when fermentations are conducted with different yeasts (Fleet, 2003; Alexandre et al., 2004; Liu et al., 2015; Albergaria and Arneborg, 2016; Ciani et al., 2016). Considerable differences have been shown in the metabolism of *Saccharomyces cerevisiae* in single and in co-culture with non-*Saccharomyces* yeasts. Moreira et al. (2005) reported

an increase in the quantity of desirable compounds, such as higher alcohols and esters, when *S. cerevisiae* was co-fermented with *Hanseniaspora uvarum*. A previous study (Sadoudi et al., 2012) based on the analysis of 48 volatile compounds belonging to different chemical families, highlighted the existence of different types of interactions independent of biomass production between non-*Saccharomyces* yeasts co-cultured with *S. cerevisiae*. More precisely, a positive interaction (synergistic effect) between *Metschnikowia pulcherrima* and *S. cerevisiae* resulted in a higher level of aromatic compounds than the sum of the aromatic compounds present in each monoculture. In addition, in a sequential *M. pulcherrima*/*S. cerevisiae* culture, acetic acid production was significantly lower compared to that obtained with a *S. cerevisiae* monoculture. Different studies reported low acetic acid production for certain non-*Saccharomyces* yeasts (*M. pulcherrima*, *Torulaspora delbrueckii*, *Starmerella bacillaris*) and their capacity in culture with *S. cerevisiae* to produce lower acetic acid concentrations in comparison to *S. cerevisiae* monoculture (Bely et al., 2008; Comitini et al., 2011; Milanovic et al., 2012; Rantsiou et al., 2012). These studies suggest that the acetic acid metabolic pathway can be affected by interactions occurring between yeasts, leading to a decrease in the amount of acetic acid. However, little is known as yet of the impact of sequential non-*Saccharomyces*/*S. cerevisiae* culture on the genes involved in the acetic acid metabolic pathway of *S. cerevisiae*.

Acetic acid is the principal volatile acid of wine. It has a negative impact on yeast fermentative performance and affects the quality of some wines when present above a given concentration (Rasmussen et al., 1995). The OIV (2010) states that the maximum acceptable limit for volatile acidity for most wines is 1.2 g l^{-1} of acetic acid. Unfortunately, higher levels are sometimes produced, depending on the strain (Erasmus et al., 2004; Orlić et al., 2010), on grape or must composition (Delfini and Costa, 1993) and on the winemaking process (Barbosa et al., 2009). Therefore, strains with reduced acetate production would have a high enological value. Studies on the production of volatile acidity by *S. cerevisiae* in winemaking conditions showed that this acid is mainly formed at the beginning of alcoholic fermentation (Alexandre et al., 2004; Bely et al., 2008). Acetic acid is formed rapidly during the fermentation of the first 50–100 g l^{-1} of sugar, but part of it is metabolized by *S. cerevisiae* (Ribéreau-Gayon et al., 2006). This yeast can also assimilate acetic acid added at the beginning of alcoholic fermentation (Vasserot et al., 2010).

Acetic acid is a by-product of alcoholic fermentation produced *via* the pyruvate dehydrogenase (PDH) bypass (**Figure 1**). It is produced at the onset of anaerobic growth conditions, as a reducing equivalents regeneration mechanism (NADH and NADPH) essential for maintaining the redox balance (Remize et al., 2000). Enzymes involved in the PDH bypass include pyruvate decarboxylase (Pdc), acetaldehyde dehydrogenase (Ald), and acetyl-CoA synthetase (Acs) (**Figure 1**). The PDH complex leads to the formation of acetyl-CoA in the mitochondria through the oxidative decarboxylation of pyruvate. However, *S. cerevisiae* is unable to transport acetyl-CoA out of the mitochondria. Moreover, cytosolic $NADP^+$-dependent Ald is active during alcoholic fermentation, while PDH activity is limited under anaerobic conditions (Remize et al., 2000). Therefore, the PDH bypass is necessary for providing acetyl-CoA in the cytosolic compartment which is used, *inter alia*, in lipid synthesis (for a review, see Pronk et al., 1996).

Pdc catalyzes the decarboxylation of pyruvate to acetaldehyde and carbon dioxide. In *S. cerevisiae*, Pdc is encoded by three structural genes, *PDC1*, *PDC5*, and *PDC6* which encode Pdc1, Pdc5, and Pdc6 isoforms, respectively (Hohmann, 1991; Pronk et al., 1996). Pdc1 and Pdc5 are 88% identical (Hohmann and Cederberg, 1990). Pdc1 is the predominant isoenzyme form, performing 80–90% of the activity in cells. The Pdc6p is an active Pdc (Hohmann, 1991; Zeng et al., 1993; Baburina et al., 1994) but is not apparently involved in glucose fermentation and its role remains unclear (Hohmann, 1991). The regulatory genes *PDC2*, *PDC3*, and *PCD4* encode probably positive transcriptional regulators required for high-level expression of structural *PDC1* and *PDC5* genes (Milanovic et al., 2012).

Ald is responsible for the conversion of acetaldehyde to acetate. The *S. cerevisiae* Ald family counts five isoenzymes localized in the mitochondria or the cytosol. Ald6 and Ald4 have been shown to be the main cytosolic and mitochondrial Ald, respectively. Cytosolic Ald is encoded by *ALD2*, *ALD3*, and *ALD6* (occasionally named *ALD1*) genes and the mitochondrial enzymes are encoded by *ALD4* (occasionally named *ALD7*) and *ALD5* genes (Navarro-Aviño et al., 1999). Ald6 uses the $NADP^+$ co-enzyme, activated by Mg^{2+}, and is not glucose-repressed (Dickinson, 1996; Meaden et al., 1997). Ald4 uses both the NAD^+ and $NADP^+$ co-enzymes activated by K^+ and thiols, and it is highly glucose-repressed (Jacobson and Bernofsky, 1974). Numerous studies stated that cytosolic Ald is responsible for the formation of acetate from glucose and that mitochondrial enzymes are involved during growth on ethanol or glycerol as carbon sources (Saigal et al., 1991; Wang et al., 1998). Remize et al. (2000) showed that a strain deleted in the *ALD6* gene led to a considerable decrease in acetate yield. The absence of Ald6p was compensated by mitochondrial isoforms, involving the transcriptional activation of the *ALD4* gene (Saint-Prix et al., 2004). More recently, it was demonstrated that the fermentation stress response gene *AAF1* regulates acid acetic production under standard laboratory conditions. This gene encodes a probable transcription factor, containing a C2-H2 zinc finger domain at the N-terminus. Indeed, *AAF1* regulates the expression of *ALD4* and *ALD6* (Walkey et al., 2012). The deletion of this gene significantly reduced acetic acid levels without increasing the acetaldehyde concentration in wine (Luo et al., 2013).

Acs catalyzes the formation of acetyl-CoA from acetate. *S. cerevisiae* contains two structural genes *ACS1* and *ACS2*, each encoding an active Acs (Van den Berg et al., 1996). It has been shown that Acs is an essential enzyme in *S. cerevisiae*. A disruption of both *ACS1* and *ACS2* genes is lethal (Van den Berg and Steensma, 1995).

An imbalance of reduction equivalents at the beginning of *S. cerevisiae* growth in must, due to the initial lack of alcohol dehydrogenase, triggers another mechanism: glycerol production (Gancedo and Serrano, 1989) (**Figure 1**). Dihydroxyacetone phosphate, the substrate for the glycerol formation pathway, can be provided either by the glycolytic degradation of sugar or by

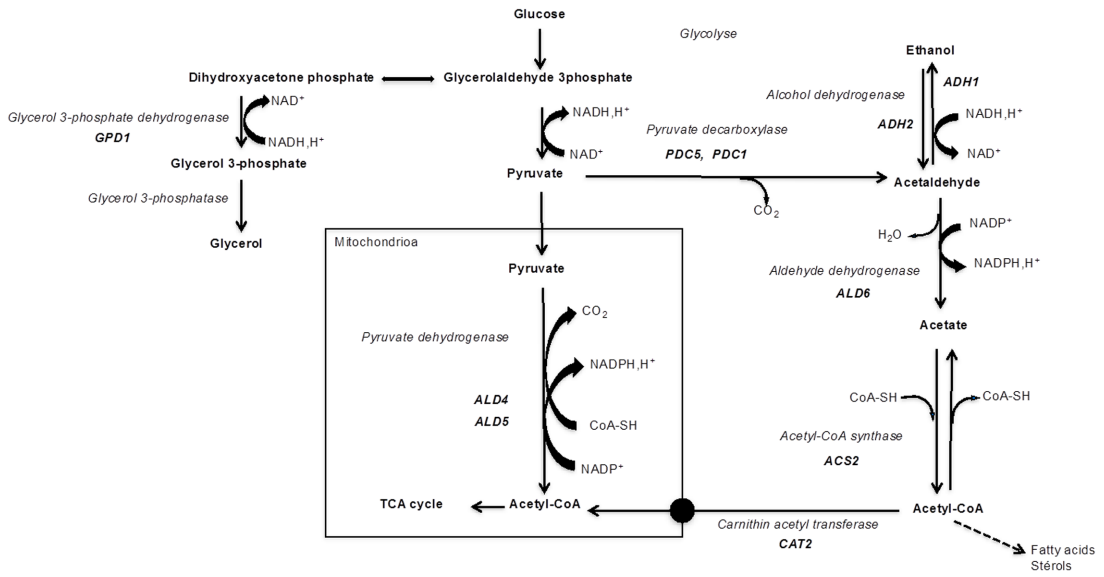

FIGURE 1 | Enzymes and main genes involved in PDH bypass and in glycerol production (glyceropyruvic fermentation).

gluconeogenic flux when non-fermentable carbon sources are used (Nevoigt and Stahl, 1997). Dihydroxyacetone phosphate is converted to glycerol-3-phosphate, which is an intermediate for glycerol formation. Two homologous genes *GPD1* and *GPD2* encode the isoenzymes glycerol-3-phosphate dehydrogenase (Gpd). *GPD1* expression is induced by osmotic stress. The repressor/activator Rap1p was demonstrated to be an important determinant of induced transcriptional activities of the *GPD1* promoter (Eriksson et al., 2000). Expression of *GPD2* is not affected by changes in external osmolarity, but it is stimulated by anoxic conditions (Ansell et al., 1997). A recent study by Pérez-Torrado et al. (2016) showed the induction of *GPD1* after the first hour of growth in wine fermentation conditions for different *Saccharomyces* species. For the *GPD2* gene, the time and the level of induction seem to be species- or strain-dependent. Moreover, some strains do not seem to activate this gene which presents very low mRNA levels.

In the present study, we performed sequential fermentations, combining *M. pulcherrima* and *S. cerevisiae* strains, in order to evaluate the effect of the presence of *M. pulcherrima* on the production of acetic acid and glycerol during alcoholic fermentation. Moreover, the impact of this sequential culture on the expression of genes in *S. cerevisiae* encoding enzymes involved in acetic acid and glycerol pathways during alcoholic fermentation was investigated.

MATERIALS AND METHODS

Yeast Strains

The commercial strain *S. cerevisiae* PB2023 (SPINDAL-AEB group) was used as control strain. The non-*Saccharomyces M. pulcherrima* MCR-24 strain (accession number: JX234570) used in this study was previously isolated from Pinot Noir grape must. This strain was selected for its alcoholic fermentation performance (completion of alcoholic fermentation producing around 11% v/v ethanol) and its low acetic acid production (Sadoudi et al., 2012).

Media

Sauvignon Blanc grape must (112 g l^{-1} glucose, 109 g l^{-1} fructose, 3.1 g l^{-1} L-malic acid, 378 mg l^{-1} total nitrogen, pH 3.35) supplemented with sulfur dioxide (30 mg l^{-1}) was used in the fermentation tests. The must was pasteurized at 100°C for 10 min and the effectiveness of this treatment was verified by plating on YPD solid medium (20 g l^{-1} glucose, 5 g l^{-1} yeast extract, 10 g l^{-1} peptone, 0.2 g l^{-1} chloramphenicol, agar 20 g l^{-1}). YPD liquid medium was used for yeast pre-cultures before inoculation in musts.

YPD solid medium was used for viable cell counting (non-*Saccharomyces* or *S. cerevisiae* yeasts) during mono-culture fermentations and total viable cell counting (both non-*Saccharomyces* and *S. cerevisiae* yeasts) during sequential fermentations.

Lysine agar (LA) medium [66 g l^{-1} Lysine medium (Oxoid), 10 ml 50% potassium lactate, 0.11 ml 90% lactic acid, and 0.2 g l^{-1} chloramphenicol] was used for viable cell counting of non-*Saccharomyces* yeast during sequential fermentation. LA medium is a selective medium which limits the growth of *S. cerevisiae* (Lin, 1975). The number of *S. cerevisiae* cells was given as the difference between the total plate count using YPD agar and the plate count using LA.

Fermentation Conditions and Sampling

Fermentations were carried out for *S. cerevisiae* PB2023 in pure culture and *M. pulcherrima* MCR-24/*S. cerevisiae* PB2023 in mixed cultures.

Pure Cultures

Pure cultures were carried out in 500 ml Erlenmeyer flasks containing 350 ml of Sauvignon Blanc grape must and closed with dense cotton plugs. Yeasts were pre-cultured in YPD medium at 30°C for 48 h and then inoculated in musts at a concentration of 10^6 cells ml^{-1}. Fermentations were carried out in triplicate at 20°C, without shaking. Fermentation progress and yeast growth were monitored throughout the fermentation process by measuring sugar concentration and by viable cells counts.

Sequential Cultures

Sequential fermentations were carried out in 500 ml Erlenmeyer flasks containing 350 ml of the same must as described above. Before must inoculation, *S. cerevisiae* PB2023 and *M. pulcherrima* MCR-24 were pre-cultured in YPD medium for 48h. *M. pulcherrima* MCR 24 and *S. cerevisiae* PB2023 were then sequentially inoculated at a ratio of 10:1. *M. pulcherrima* MCR 24 was inoculated at 10^7 cells ml^{-1} and after 48 h, *S. cerevisiae* PB2023 was introduced at 10^6 cells ml^{-1}. Each experiment was performed in triplicate at 20°C under static conditions. Fermentation progress and yeast growth were monitored throughout the fermentation process by measuring sugar concentration and by viable cell counts, as described previously.

Sampling

Samples of the fermenting must were taken at different stages of fermentation (−2, −1, 0, 1, 2, 3, 4, 5, 6, and 8 days of fermentation) from each fermentation trial. Day "−2" corresponds to the day of inoculation with *M. pulcherrima* MCR 24 strain and day "0" corresponds to the day when *S. cerevisiae* PB2023 was added. One part of each sample was used to determine the cell number. The other part of the sample was centrifuged at 1000 rpm for 5 min at 4°C. Supernatants were stored at −20°C and analyzed later to determine residual sugar, ethanol, glycerol, and acetic acid concentrations. The cell pellet was collected for RNA extraction. The RNA extractions were performed from the day "1" of fermentation until the end of the process.

Enological Parameter Analysis

Glucose, fructose, ethanol, glycerol, and acetic acid were determined using enzymatic kits following the manufacturer's instructions (Bio-SenTec, France). Total acidity was determined by the potentiometric method. The wine was decarbonated and then titrated by NaOH 0.1 N solution until pH 7. The result was expressed in g l^{-1} tartaric acid.

RNA Extraction and Reverse Transcription (cDNA Synthesis)

Total RNAs extraction was performed using a commercial RNeasy kit (Qiagen) with slight modifications. After centrifugation, cells were added to the extraction buffer together with 600 µl of sterile glass beads (0.5 mm in diameter). The cells were then disrupted using the Precellys instrument (Bertin Technologies, France) at 6500 *g* for 30 s followed by chilling on ice for 30 s. This step was repeated six times. The extraction was then continued according to the manufacturer's instructions (Qiagen).

The extracted RNA was quantified by measuring absorbance at 260 nm using a bio-photometer (Eppendorf). The RNAs (2 µg of total RNA) were treated with 5 U of DNase (Fermentas/Thermo Fisher Scientific, France) following the protocol described by the manufacturer. As a quality control assay, the absence of contaminant genomic DNA in RNA preparations was checked before cDNA synthesis using RNA as a template in real-time PCR assays (RNA not reverse-transcribed to cDNA). cDNA was then synthesized from 1 µg of total RNA in 20 µl reaction mixture using the iScript cDNA synthesis kit (Bio-Rad, France). Each RNA extraction was performed in triplicate.

Primer Design

The primers for RT-PCR (target and housekeeping reference genes) given in **Table 1** were designed using the free online Primer3 0.4.0 software[1]. The primers were designed to have length about 18–22 bp, a G/C content of over 50%, and a Tm of about 60°C. The PCR product sizes ranged from 90 to 120 bp. Secondary structures and dimers formation were controlled with the Oligo Analyzer 1.0.3.0 software. Primer specificity and PCR product size were obtained *in silico* from the entire genome of the S288C strain[2].

PGK1 and *TDH2* genes (**Table 1**) were used as housekeeping reference genes because they were shown to be two genes whose expression remained stable and independent of growth conditions, as highlighted by (Vaudano et al., 2011).

Primers were purchased from Eurogentec, Belgium. In order to confirm the specificity of the primers only for *S. cerevisiae* genomic DNA in sequential culture samples, each couple of primers was tested in RT-qPCR using the genomic DNA of *S. cerevisiae* or *M. pulcherrima* as a template. No amplification was detected in the *M. pulcherrima* genomic DNA template (data not shown).

Quantitative Real-Time PCR

Real time PCR was performed in 96-well plates on a CFX-96™ Real Time system (Bio-Rad) using SYBR Green as fluorophore. Reactions were carried out in 25 µl of mix containing 12.5 µl of PCR master mix (Promega), 2.0 µl of primer mix (7 pM final concentration), 5.5 µl of DNase and RNase free H_2O, and 5 µl of cDNA. Positive (*S. cerevisiae* genomic DNA as template) and negative (water as template) controls were also incorporated in each assay. The thermocycling program consisted of one hold at 95°C for 3 min; 40 cycles of 10 s at 95°C, 30 s at 60°C and 30 s at 72°C and a final extension at 72°C for 5 min. After the completion of the thermocycling program, melting curve data were then collected to verify PCR specificity, contamination and the absence of primer dimers. The melting curve was

[1]http://frodo.wi.mit.edu/primer3/

[2]http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome

TABLE 1 | Genes and primers used in RT-qPCR.

Genes	NCBI Gene ID[a]	Description	Forward and reverse primers 5′ → 3′	Primer size	PCR product salt (bp)[b]
PDC1 (YLR044C)	850733	Pyruvate decarboxylase, isozyme 1	CTTACGCCGCTGATGGTTA GGCAATACCGTTCAAAGCAG	19 20	95
PDC5 (YLR134W)	850825	Pyruvate decarboxylase, isozyme 5	GGCTGATGCTTGTGCTTCTA GGGTGTTGTTCGTCAATAGC	20 20	120
ALD6 (YPL061W)	856044	Cytosolic aldehyde dehydrogenase, isozyme 6	TCTCTTCTGCCACCACTGAA CCTCTTTCTCTTGGGTCTTGG	20 21	100
ALD4 (YOR374W)	854556	Mitochondrial aldehyde dehydrogenase, isozyme 4	CGGGTTTGGTAAGATTGTGG TGCGGACTGGTAAATGTGTC	20 20	106
ACS2 (YLR153C)	850846	Acetyl-CoA synthase, isozyme 2	ATTGGTCCTTTCGCCTCAC GCTGTTCGGCTTCGTTAGA	19 19	118
ADH1 (YOL086C)	854068	Alcohol dehydrogenase, isozyme 1	GGTCACTGGGTTGCTATCTCC CCTTCACCACCGTCAATACC	21 20	107
ADH2 (YMR303C)	855349	Alcohol dehydrogenase, isozyme 2	TGCCCACGGTATCATCAAT GCAAACCAACCAAGACAACAG	19 21	98
CAT2 (YML042W)	854965	Carnitine acetyltransferase 2	CAAACTGATGACCCATGACG GGACTGCGATCCTTGGAATA	20 20	94
GPD1 (YDL022W)	851539	Glycerol-3-phosphate dehydrogenase isozyme 1	TTTTGCCCCGTATCTGTAGC TGGACACCTTTAGCACCAACT	20 21	100
PGK1 (YCR012W)	850370	3-Phosphoglycerate kinase, key enzyme in glycolysis and gluconeogenesis	GGTAACACCGTCATCATTGG AAGCACCACCACCAGTAGAGA	20 21	100
TDH2 (YJR009C)	853465	Glyceraldehyde-3-phosphate dehydrogenase, isozyme 2	AACATCATCCCATCCTCTACCG GGACTCTGAAAGCCATACCG	22 20	94

[a]*Identification number;* [b]*bases pairs.*

obtained by increasing the temperature from 60 to 95°C at 0.5°C/10 s.

The PCR efficiency of each primer pair (E) was evaluated by running a standard curve with serial dilution of cDNA. When $E = 100\%$, the amount of PCR product can double in each cycle. Efficiencies and threshold cycle (C_T) values were obtained by using the automated system software setting. The threshold cycle value was defined as the number of cycles required to reach a point in which the first fluorescent signal is recorded as statistically significant above background. In this study, the threshold fluorescence baseline was set manually at 100 relative fluorescence units (RFU).

The relative expression of a given gene was calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). The gene expression levels were given as a differential of the expression levels in *S. cerevisiae* in mixed culture conditions *versus* expression levels of *S. cerevisiae* in pure culture. The results were normalized by using two reference genes *PGK1* and *TDH2* (**Table 1**). The data were analyzed using the comparative critical threshold ($\Delta\Delta C_T$) in which the amount of sample target RNA was adjusted to a control target RNA, where:

- Control: target RNA of *S. cerevisiae* from pure culture conditions
- Sample: target RNA of *S. cerevisiae* from mixed culture conditions

$$\Delta C_T = C_T \text{ gene of interest} - C_T \text{ reference gene}$$
$$\Delta\Delta C_T = \Delta C_T \text{ of sample} - \Delta C_T \text{ of control}$$
$$\text{Relative expression level} = 2^{-\Delta\Delta C_T}$$

We considered that genes were significantly down- or over-expressed if their relative expression level was found to be at least twofolds lower or higher than the control conditions as previously described (Desroche et al., 2005).

Statistical Analysis

Metabolite concentrations were subjected to one-way analysis of variance (ANOVA) followed by a Tukey's (HSD) *post hoc* test (confidence interval 95%) to test for significance differences between the wines.

RESULTS

Fermentation Behavior of Pure and Sequential Cultures

Yeast growth dynamics and sugar consumption during must fermentation were monitored for single and sequential cultures (**Figure 2**). The fermentation kinetics of the control *S. cerevisiae* PB2023 pure culture indicated that the maximal population was reached after 3 days (1.4×10^8 viable cells ml^{-1}). This cell concentration was maintained until the end of fermentation (**Figure 2A**). *S. cerevisiae* completed the alcoholic fermentation in 8 days without remaining sugar. When the alcoholic fermentation was conducted with sequential culture of *M. pulcherrima* MCR-24 and *S. cerevisiae* PB2023 (inoculation ratio 10:1), the fermentation progressed to completion in 10 days (**Figure 2B**). The maximum population reached for *S. cerevisiae* was 3×10^8 viable cells ml^{-1} and 4×10^8 viable cells ml^{-1} for *M. pulcherrima*. The presence of *M. pulcherrima* did not affect the growth of the *S. cerevisiae* PB2023 strain.

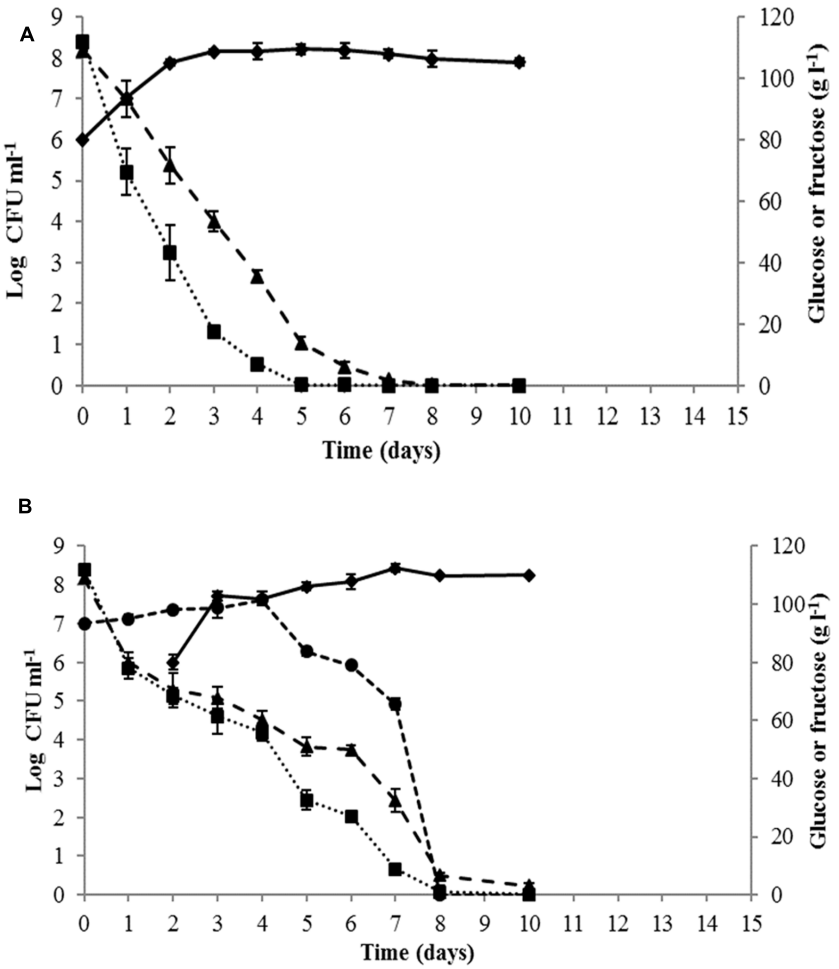

FIGURE 2 | Growth kinetics and sugar consumption during mono-culture and sequential culture fermentations: **(A)** *S. cerevisiae* PB2023 (–♦–), and **(B)** *M. pulcherrima* MCR 24 (–●–)/*S. cerevisiae* PB2023 (–♦–). Glucose (··■··) and fructose (–▲–). Data are representative of three independent trials.

However, *M. pulcherrima* MCR 24 population dropped after the inoculation of *S. cerevisiae* PB2023 and no viable cells were detected after 8 days.

The evolution of ethanol showed different kinetics in sequential and pure fermentations (**Figure 3A**). During the first 72 h of fermentation, as expected, the *S. cerevisiae* pure culture produced ethanol faster and in higher concentration than that produced by sequential culture, after which production was progressive and at a lower rate until the end of fermentation (10.58% v/v). *M. pulcherrima*/*S. cerevisiae* sequential culture showed a lower but regular trend for ethanol production until the end of fermentation (10.14% v/v). In both cases, the fermentation yields were slightly higher than usual [21 g l^{-1} sugars for 1% (v/v) ethanol instead 16.8 g l^{-1}]. These data were probably linked to winemaking trials in small volumes (350 ml).

Saccharomyces cerevisiae pure culture produced a higher amount of glycerol (4.97 g l^{-1}) in the first 4 days of fermentation compared to the sequential culture (3.52 g l^{-1}). After day 4, glycerol was produced gradually until the end of fermentation (5.67 g l^{-1}). Sequential culture exhibited lower concentrations of glycerol in the first 4 days of fermentation, but its concentration was higher at the end of the process (6.46 g l^{-1}) (**Figure 3B**).

The acetic acid production kinetics of pure and sequential cultures are shown in **Figure 3C**. Pure culture of *S. cerevisiae* produced significantly higher amounts of acetic acid (0.35 ± 0.01 g l^{-1}) compared to sequential culture (0.21 ± 0.03 g l^{-1}). For *S. cerevisiae* pure culture, 57% of the final amount was produced during the first 3 days of fermentation. Interestingly, the presence of *M. pulcherrima* in culture together with *S. cerevisiae* led to a reduction of acetic acid production from the beginning of fermentation.

Gene Expression during Alcoholic Fermentations

Previous data suggested that the metabolic pathways could be affected by interactions occurring between both yeasts during alcoholic fermentation. In this context, we studied the influence of *M. pulcherrima* MCR 24 growth on acetic acid and glycerol productions of *S. cerevisiae* evaluating Pdc, aldehyde dehydrogenase, Acs, and alcohol dehydrogenase gene

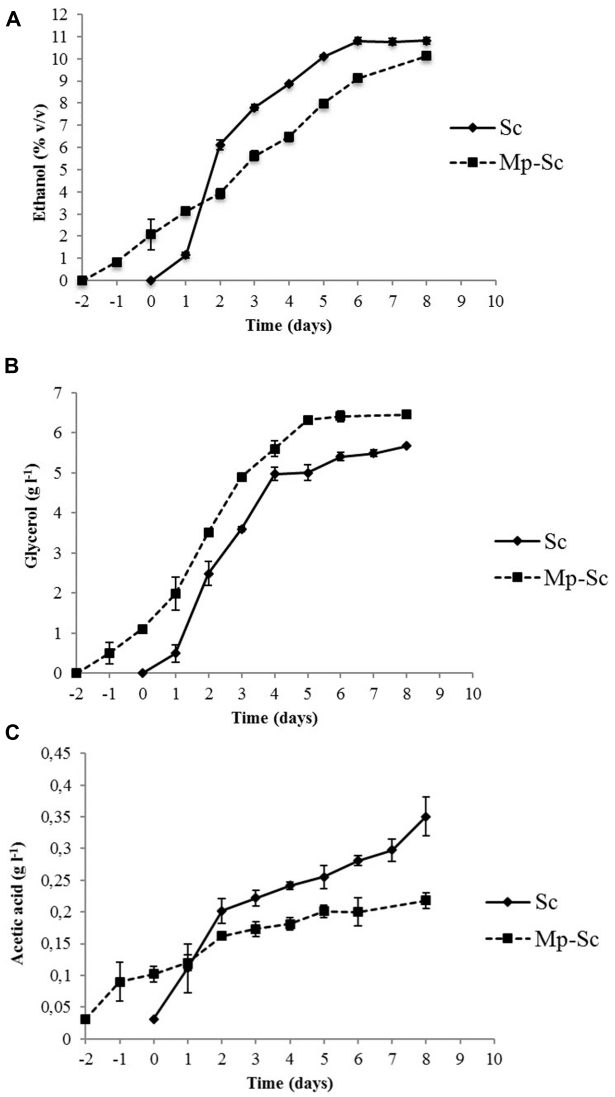

FIGURE 3 | Evolution of metabolites production during alcoholic fermentation carried out by *S. cerevisiae* PB2023 (Sc) and *M. pulcherrima* MCR 24/*S. cerevisiae* PB2023 (Mp-Sc). **(A)** Ethanol production; **(B)** glycerol production; **(C)** acetic acid production. Day 0 corresponds to the day of inoculation with *S. cerevisiae* PB2023. Data are representative of three independent trials.

expression during alcoholic fermentations. These enzymes are the key enzymes involved in the acetic acid production pathway. We have added the analysis of the expression of Gpd. Gene expression in *S. cerevisiae* was evaluated in sequential culture relative to the gene expression of *S. cerevisiae* in pure culture (control) (**Figure 4**). Time 0 corresponds to the day of inoculation of the *S. cerevisiae* PB2023 strain in the sequential culture.

Figure 4A shows the differential gene expression level of *GPD1* in sequential culture condition. Dihydroxyacetone phosphate is converted to glycerol-3-phosphate, an intermediate for glycerol formation, by a Gpd enzyme encoded by the gene *GPD1* (**Figure 1**). The *GPD1* gene was over-expressed at 24 h after inoculation of *S. cerevisiae*, then the transcriptional level dropped and remained stable until the end of fermentation. This observation can be linked to the increase in the quantity of glycerol at the first 24 h of fermentation and then a similar production rate should be observed for *S. cerevisiae* in both fermentation conditions (pure and sequential culture) but it is hazardous to correlate this hypothesis with the analytical data shown **Figure 3B**. Indeed, *M. pulcherrima* MCR 24 produced glycerol (approximately 1 g l^{-1}) before inoculation with *S. cerevisiae* and the levels measured after 48 h of fermentation may have resulted from the co-production of glycerol by both yeasts.

The differential of *PDC1* and *PDC5* gene expression levels during fermentation is shown in **Figure 4B**. The *PDC1* gene was slightly over-expressed at 24 and 48 h after inoculation. After that, gene expression decreased gradually until the end of fermentation. However, *PDC5* gene expression was not significantly affected by the sequential culture in the first 48 h but it was highly over-expressed at the 3rd day of fermentation (6.6-fold). Then, expression decreased gradually until the end of fermentation. Interestingly, we assume that Pdc encoding by both genes was not induced at the same time but alternately, confirming the hypothesis of their auto-regulation during alcoholic fermentation (Hohmann and Cederberg, 1990; Eberhardt et al., 1999). The alternate over-expression of the *PDC1* and *PDC5* genes was observed in the first 4 days of fermentation. After that, the transcriptional levels of both genes in sequential culture condition were identical to transcriptional levels of these genes in pure culture conditions. Furthermore, over-expression of these genes suggests that the sequential culture led to an increase in the production of acetaldehyde from pyruvate.

The differential expressions of genes directly involved in acetate production, i.e., *ALD6*, *ALD4*, *ACS2*, are presented in **Figure 4C**. The *ALD6* gene was over-expressed in the first 3 days of fermentation, reaching its maximum level of expression on the 2nd day (7.4-fold; **Figure 4B**). However, the mitochondrial *ALD4* gene was not over-expressed and remained stable during fermentation. This means that mitochondrial aldehyde dehydrogenase was not affected by the mixed culture condition, but cytosolic Ald6 activity could be privileged in order to regenerate the reduced co-enzyme NADPH (**Figure 1**). The *ACS2* gene encoding Acs did not present over-expression in the mixed culture condition. The *CAT2* gene encoding carnitine acetyltransferase was twofold lower expressed in sequential culture condition (**Figure 4E**).

The expression levels of genes *ADH1* and *ADH2* encoding alcohol dehydrogenase are shown in **Figure 4D**. No over-expression was observed in the *ADH1* gene during fermentation. In contrast, the *ADH2* gene was highly over-expressed 24 h after inoculation of *S. cerevisiae* (fourfold), which is involved in the conversion of ethanol into acetaldehyde (**Figure 1**). After 24 h, the *ADH2* gene expression level dropped rapidly and a down regulation of *ADH2* was observed from the 3rd to the 6th day of fermentation.

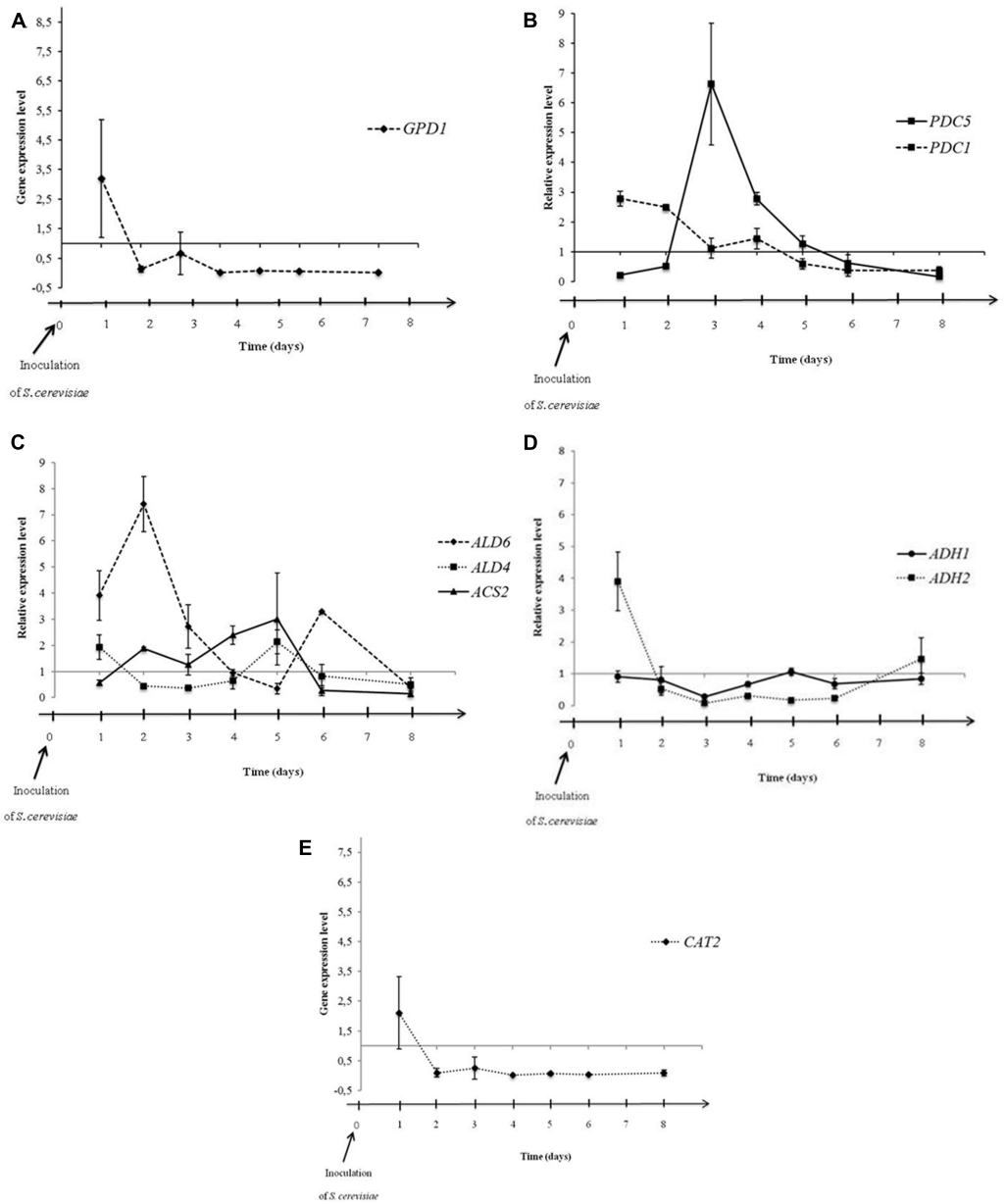

FIGURE 4 | Expression levels of *GPD1* **(A)**, *PDC1* and *PDC5* **(B)**, *ALD6*, *ALD4*, and *ACS2* **(C)**, *ADH1* and *ADH2* **(D)**, and *CAT2* **(E)** in *S. cerevisiae* PB2023 at different stages of sequential culture fermentation. Data are representative of three independent trials. The gene expression levels were given as a differential of the expression levels in *S. cerevisiae* in sequential culture conditions *versus* expression levels of *S. cerevisiae* in pure culture.

DISCUSSION

The early inoculation of *M. pulcherrima* MCR 24 did not compromise the growth of *S. cerevisiae* PB2023, preventing the risk of a sluggish or a stuck alcoholic fermentation. Moreover, the *M. pulcherrima* population dropped after the inoculation of *S. cerevisiae* and no viable cells were detected after 8 days (**Figure 2B**). Such an antagonistic effect has been reported previously (Jolly et al., 2003; Rodríguez et al., 2010; Comitini et al., 2011; Sadoudi et al., 2012). This result could not be linked to intolerance to ethanol concentration, since we previously demonstrated that the MCR 24 strain can produce approximately 10% v/v ethanol (Sadoudi et al., 2012). According to Nguyen and Panon (1998), the antagonistic effect could be attributed to killer toxins. Another explanation is the interaction occurring between both yeasts, mediated by the cell–cell contact mechanism (Nissen and Arneborg, 2003) or competition between yeasts for the nutrients available in the must. *S. cerevisiae* PB2023 grew faster than *M. pulcherrima* MCR 24 and thus it could impoverish the medium. Sequential inoculation did not affect the ethanol level in

the wine despite the death of *M. pulcherrima*. On the other hand, it induced a significant increase in glycerol content and a decrease in acetic acid concentration (**Figure 3**). These data confirm the benefits of using *M. pulcherrima* prior the inoculation of the *S. cerevisiae* starter, in accordance with previous results (Bely et al., 2008; Comitini et al., 2011), but they do not explain the positive impact of *M. pulcherrima* on *S. cerevisiae* metabolism.

All previous analytical data suggest that the metabolic pathways could be rerouted by interactions occurring between both yeasts during alcoholic fermentation. During the latter, acetic acid is produced *via* the cytosolic PDH bypass. In aerobic conditions, the PDH complex leads to the formation of acetyl-CoA in the mitochondria by oxidative decarboxylation of pyruvate. However, in fermentative conditions, the conversion of pyruvate to acetyl-CoA can occur *via* an indirect route, involving Pdc (which is also a key enzyme in alcoholic fermentation), Ald and Acs. This bypass route is the source in the cytosolic compartment of acetyl-CoA, which is used for lipid synthesis and acetate which can be precursor of volatile esters.

The production of glycerol involves the reduction of dihydroxyacetone phosphate derived from the glycolytic degradation of sugar. The NAD^+-dependent Gpd catalyzes the first step in glycerol production. This metabolism also permits the regeneration of reducing equivalents (NADH), more particularly at the beginning of *S. cerevisiae* growth in fermentative conditions.

The over-expression of *PDC1* and *PDC5* encoding two isoforms of Pdc and the *ALD6* gene encoding cytosolic aldehyde dehydrogenase (**Figures 4B,C**) leads to the assumption of an over production of acetic acid by-product, which appears inconsistent with the analytical data which shows that acetate was reduced in mixed culture condition. One explanation could be due to the conversion of acetate into acetyl-CoA used in other metabolic pathways such as lipid synthesis or esterification related to the production of esters. Indeed, we previously observed higher levels of acetate esters in Sauvignon wine from a *M. pulcherrima*/*S. cerevisiae* sequential culture (Sadoudi et al., 2012). However, it is clear that acetyl-CoA was not transported into mitochondria since the *CAT2* gene encoding carnitine acetyltransferase was under-expressed in sequential culture condition (**Figure 4E**).

The lower acetate production could not be due ethanol production since the ethanol contents are comparable under the two fermentation conditions. Another hypothesis that could explain our analytical data is that a part of dihydroxyacetone phosphate is used for glycerol production at the beginning of fermentation. Glycerol can be produced mostly at the beginning of fermentation in response to hyper osmotic conditions (high concentration in sugars). Moreover, anaerobic conditions require the production of endogenous electron acceptors and glycerol production can serve as a redox valve to eliminate excess reducing power in *S. cerevisiae* (Ansell et al., 1997). The *M. pulcherrima* strain MCR 24 may have depleted oxygen in the must during sequential culture, since it was inoculated 48 h before *S. cerevisiae*. Oxygen depletion (anaerobiotic conditions) could explain the modulation of glyceropyruvic fermentation and the orientation of metabolism to the PDH bypass, leading to the production of acetate and glycerol. These metabolism orientations are necessary to maintain the redox balance by regenerating NAD and NADH co-enzymes. Furthermore, increased glycerol formation requires an equimolar amount of cytoplasmic NADH. This requirement could be satisfied by a lower reduction of acetaldehyde to ethanol on the one hand and an increase in oxidation to acetate on the other (Blomberg and Adler, 1989; Nevoigt and Stahl, 1997). Therefore an increase in acetate production is usually accompanied by an increase in glycerol formation; however, a high levels of glycerol is not necessarily accompanied by high levels of acetic acid or acetaldehyde (Remize et al., 2000).

Independently of the expression of genes involved in acetate and glycerol production pathways, we hypothesized the possible consumption by *M. pulcherrima* MCR 24 of part of the acetate produced by *S. cerevisiae* in sequential culture fermentation. We performed a mono-culture with the *M. pulcherrima* MCR 24 strain using standardized grape juice supplemented with 1.5 g l^{-1} of acid acetic and observed the consumption of 0.57 g l^{-1} of the initial acetic acid during 8 days of fermentation (data not shown).

CONCLUSION

This work is the first attempt to investigate *M. pulcherrima* and *S. cerevisiae* yeast–yeast metabolic interaction, reflected by gene expression in the acetic acid and glycerol production pathway in *S. cerevisiae* during controlled sequential fermentation in winemaking. The environmental changes in must induced by the presence of *M. pulcherrima* induced the alteration of the entire acetic acid and glycerol metabolic pathway of *S. cerevisiae*.

Future accession to the *M. pulcherrima* genome may provide very interesting investigative leads on the nature of interactions occurring in sequential fermentations at the transcriptomic level.

AUTHOR CONTRIBUTIONS

MS designed the experiments, analyzed the data, and wrote the manuscript. SR analyzed the data and wrote the manuscript. VD analyzed the data. HA and RT-M supervised the study.

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Saccharomyces and non-*Saccharomyces* Competition during Microvinification under Different Sugar and Nitrogen Conditions

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The inoculation of wines with autochthonous yeast allows obtaining complex wines with a peculiar microbial footprint characteristic from a wine region. Mixed inoculation of non-*Saccharomyces* yeasts and *S. cerevisiae* is of interest for the wine industry for technological and sensory reasons. However, the interactions between these yeasts are not well understood, especially those regarding the availability of nutrients. The aim of the present study was to analyze the effect of nitrogen and sugar concentration on the evolution of mixed yeast populations on controlled laboratory-scale fermentations monitored by density, plate culturing, PCR-DGGE and sugar and nitrogen consumption. Furthermore, the effect of the time of inoculation of *Saccharomyces cerevisiae* respect the initial co-inoculation of three non-*Saccharomyces* yeasts was evaluated over the evolution of fermentation. Our results have shown that *S. cerevisiae* inoculation during the first 48 h conferred a stabilizing effect over the fermentations with non-*Saccharomyces* strains tested and, generally, reduced yeast diversity at the end of the fermentation. On the other hand, nitrogen limitation increased the time of fermentation and also the proportion of non-*Saccharomyces* yeasts at mid and final fermentation. High sugar concentration resulted in different proportions of the inoculated yeast depending on the time of *S. cerevisiae* inoculation. This work emphasizes the importance of the concentration of nutrients on the evolution of mixed fermentations and points to the optimal conditions for a stable fermentation in which the inoculated yeasts survived until the end.

Keywords: Torulaspora, Hanseniaspora, Starmarella, fermentation, wine

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INTRODUCTION

Wine is the result of alcoholic fermentation performed by yeasts during a complex process that transform the sugars present in the grape must into ethanol and carbon dioxide. During this alcoholic fermentation, a microbiological population evolves as a consequence of the chemical changes produced in the environment (Riberéau-Gayon et al., 2006). Many studies have established the yeast succession of non-*Saccharomyces* to *Saccharomyces* during spontaneous fermentation of

grape juice. These non-*Saccharomyces* yeasts are the predominant microbiota in grapes and the main responsible for starting spontaneous alcoholic fermentation and often, under uncontrolled fermentations, lead to sluggish or stuck fermentations. For that reason, winemakers tend to inoculate grape must with commercial yeasts to ensure the completion of the fermentation, but compromising the complexity or the particular microbial footprint of wines of a certain region. In recent years, good properties and contribution of the non-*Saccharomyces* yeasts to wine and fermentation process have been described (Pretorius, 2000; Fleet, 2008; Ciani and Comitini, 2011; Jolly et al., 2014; Padilla et al., 2016a). With the aim to obtain wines that reflect a certain *terroir*, a previous study part of the WILDWINE project (Mas et al., 2016) accomplished the isolation and the characterization of multiple yeast strains from Priorat region to better understand the winemaking process and also to determine the source of microorganisms that produce a particular microbial footprint (Padilla et al., 2016b). The contribution of non-*Saccharomyces* takes part mostly during beginning and mid fermentation (Fleet, 2008). Non-*Saccharomyces* yeasts are able to produce metabolites or hydrolyze aromatic precursors providing new wine styles and enhancing their complexity (Ciani et al., 2010; Viana et al., 2011; Andorrà et al., 2012; Jolly et al., 2014).

The possibility to obtain wines with differential characteristics due to the role of non-*Saccharomyces* yeasts explains the increasing interest of using mixed cultures. As we have mentioned, one of the objectives of the WILDWINE project is to mimic the natural microbiota of a vineyard by the use of mixed inocula to perform fermentations to fight the wine uniformity derived from the widespread use of commercial *S. cerevisiae* starter cultures (Mas et al., 2016). Besides, interaction between non-*Saccharomyces* and *S. cerevisiae* has not been extensively studied, however some positive metabolic interactions have been described (Ciani et al., 2010; Ciani and Comitini, 2015). In the present study, the most characteristic non-*Saccharomyces* yeast isolated during the WILDWINE project were subjected to mixed alcoholic fermentation under different nutrient conditions (Mas et al., 2016; Padilla et al., 2016b).

The main problems during mixed fermentations are related to the nutrient composition of the must and the competition between the different yeast strains involved (Andorrà et al., 2010; Wang et al., 2015, 2016). It has been demonstrated that the consumption of nitrogen at the beginning of the fermentation by non-*Saccharomyces* yeast can prevent the correct development of *S. cerevisiae*.

Sugar and nitrogen composition of the grape must are key factors for the evolution of the alcoholic fermentation and the development of the yeasts (Bell and Henschcke, 2005; Beltran et al., 2005; Martínez-Moreno et al., 2012).

During the last few years, sugar content in grape must has become an important aspect since its concentration is increasing as a consequence of climate change and some viticultural practices (Mira de Orduña, 2010; Webb et al., 2012). The higher sugar content in grapes and, consequently, in musts is a problem for yeast physiology and it creates an osmotic stress that can produce, among others, stuck fermentations or wines with higher alcohol content.

In case of nitrogen, a higher or lower content can be harmful on fermentation kinetics and it has been demonstrated that a nitrogen concentration of 140 mg/L is the minimum required for yeasts to complete alcoholic fermentation (Bell and Henschcke, 2005), although this value is dependent on the sugar concentration (Martínez-Moreno et al., 2012). The same as sugar concentration, many factors can influence the nitrogen content on grapes and, consequently, on must such as environmental conditions and cultural practices (Bell and Henschcke, 2005).

The aim of this study was to determine the yeast dynamics and nutrient consumption during mixed fermentations of *Saccharomyces* and non-*Saccharomyces* yeast under four different nutrient conditions and with sequential addition of *S. cerevisiae* at four different time points. The fermentations were followed by density, plate culturing, PCR-DGGE and sugar consumption. According to our results, we propose the most suitable inoculation strategy for mixed fermentations using four strains isolated from Priorat region under the different nutrient concentrations.

MATERIALS AND METHODS

Yeast Strains and Starter Cultures Preparation

Four different yeast strains frequently isolated from natural must from Priorat Appellation of Origin (Catalonia, Spain) were employed (Padilla et al., 2016b). These yeasts were identified by ITS sequencing and identified and deposited in the Spanish Type Culture Collection (CECT) as *Saccharomyces cerevisiae* CECT 13132, *Hanseniaspora uvarum* CECT 13130, *Candida zemplinina* CECT 13129 (synonym: *Starmerella bacillaris*, Duarte et al., 2012) and *Toluraspora delbrueckii* CECT 13135. The starter cultures were prepared by growing the yeasts strains separately in liquid YPD medium (2% glucose, 2% Bacto peptone, 1% yeast extract, 2% agar, w/v; Cultimed, Barcelona, Spain) at 28°C with a stirring rate of 150 rpm in an orbital shaker.

Mixed Inoculum Conditions

Fermentations were carried out in 250 mL of synthetic grape must (pH 3.3) as described by Riou et al. (1997), but with some modifications. The final concentration of sugars was either 200 or 240 g/L (denominated 200S or 240S, respectively) with a combination of glucose and fructose of 100 or 120 g/L each. The available nitrogen was either 100 or 300 mg/L (denominated 100N or 300N, respectively). Another variable was the time of the inoculation of *S. cerevisiae*: co-inoculation (0D), at 24 h (1D), at 48 h (2D) and at the 5th day (5D) after the inoculation of the non-*Saccharomyces*. Also, control fermentations were conducted for each nutrient condition with the sole inoculation of *S. cerevisiae*. Fermentations were considered finished when density was below 1000 g/L, or without variation for three consecutive days.

All the fermentations were performed in duplicate and inoculated at a concentration of $1.2 \cdot 10^6$ cells/mL of *H. uvarum*, $5 \cdot 10^5$ cells/mL of *S. bacillaris*, $1 \cdot 10^5$ cells/mL of *T. delbrueckii* and $2 \cdot 10^6$ cells/mL of *S. cerevisiae*. These concentrations resemble

yeast populations of natural musts from Priorat, where the non-*Saccharomyces* yeasts were isolated (Wang et al., 2015) and the practice of inoculating commercial *Saccharomyces* presentations.

Density, Acetic Acid, and Sugar Measurements

The fermentations were monitored daily by density with Densito 30PX Portable Density Meter (Mettler Toledo, Spain). Once the fermentations were finished (the density was under 1000 g/L or stable for 3 days), concentrations of glucose and fructose and the acetic acid concentration in the final fermentation samples were analyzed by Miura One Multianalyzer (TDI, Barcelona, Spain) using the enzymatic kit from Biosystems S. A. (Barcelona, Spain). Samples for plating, qPCR and PCR-DGGE were taken at the beginning (24 h after incubation started), in the middle (density approximately 1020–1030 g/L) and at the end of fermentation (density below 1000 g/L or stable for 3 days). Maximum fermentation rate (R) was calculated as maximum slope of the density measurements respect the time. Also, time to reach the 10, 50, and 75% of the final density (referred as t10, t50, and t75, respectively) were calculated as additional parameters of the fermentation kinetics (Table S1). Successful fermentations were considered when density was below 1000 and residual sugar was below 3 g/L.

Plate Culturing

Fresh samples were directly analyzed by culture-dependent techniques at each fermentation stage (beginning, middle and end of fermentation). The total yeast populations were enumerated on plates with YPD medium. The Wallerstein Laboratory nutrient agar (WL; Oxoid, England) is useful to quantify and identify wine microorganisms and was used to discriminate between the used yeast species by colony morphology and color (Pallmann et al., 2001).

DNA Extraction

Cell pellets from 1 mL of samples at each fermentation stage (beginning, middle and end of fermentation) were collected by centrifugation after washing with sterile water and kept at −80°C for further culture-independent analysis by and PCR-DGGE. DNA cell pellets were extracted according to Hierro et al. (2007). The concentration and purity of DNA was determined using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, U.S.A.).

PCR-DGGE Analysis

The PCR reactions were performed using a Gene Amp PCR System 2720 (Applied Biosystems, USA) with Primers $U1^{GC}$ and U2 (Meroth et al., 2003). The DGGE procedures followed the description in Andorrà et al. (2008) with a modified DGGE gel using a denaturing gradient from 35 to 55% urea and formamide. A marker prepared with the PCR products of each individual yeast species was included in the DGGE gels for migration comparison and yeasts identifications.

Statistical Analysis

Fermentation kinetics variables (residual sugar, acetic acid concentration, R, t10, t50, and t75) have been used to construct a dissimilarity matrix based on Euclidean distance between their values. All these variables have been used to construct a dissimilarity matrix based on the Euclidean distance between their values. ANOSIM (an analog of univariate ANOVA which tests for differences between groups of samples) was run in PRIMER v6 (Clarke and Gorley, 2006) to determine significant differences between the different fermentations among the main experimental factors (sugar and nitrogen content, residual sugar, *S. cerevisiae* inoculation time). Principal coordinate analysis (PCoA) was used to summarize and visualize the different fermentations under each Nitrogen condition respect the final residual sugar (as an estimator of fermentation success). Pearson correlation analysis were performed between the residual sugar and the rest of parameters.

RESULTS AND DISCUSSION

Effect of Nutrients Concentration on Fermentation Kinetics

Fermentations with optimal nitrogen concentration (300N-240S and 300N-200S) were all completed in 5–13 days, with the fermentations under excess of sugar (300N-240S) the slower ones (**Table 1**) (Figure S1). On the other hand, most of the fermentations performed under limiting nitrogen concentration (100N-240S and 100N-200S) got stuck (**Table 1**). From these results we observed that the nitrogen content had a stronger effect than the sugar concentration in yeast metabolism and affected the fermentation kinetics. Also, ANOSIM results showed that the fermentations under different nitrogen concentration (100N and 300N) were significantly different (**Table 2**), i.e., their kinetics parameters (R, t10, t50, t75, residual sugar and acetic acid) were different for each nitrogen condition. However, sugar concentration (200S and 240S) did not result in significant differences (**Table 2**).

It has been previously described that nitrogen concentration below 140 mg/L are limiting to growth and result in a decrease of the fermentation rate by *S. cerevisiae*, an increase the risk of sluggish and stuck fermentation as well as an increase in residual sugars (Bell and Henschcke, 2005; Martínez-Moreno et al., 2012; Tesnière et al., 2015). However, according to our results, both 100N control fermentations inoculated just with *S. cerevisiae* were able to be completed in 7–8 days (**Table 1**). This could be explained by the different nitrogen requirements of the selected *S. cerevisiae* strain, autochthonous yeast that was grown in YPD before its inoculation in the synthetic must, thus allowing inner nitrogen accumulation. Mixed fermentations with the four yeast species, with expected different nitrogen and sugar requirements, got generally stuck under 100N and it would be interesting to investigate the required addition of nitrogen to complete those fermentations (**Table 1**) (**Figure 1**). This could be due to the known higher nitrogen requirements of non-*Saccharomyces* yeast (Andorrà et al., 2010, 2012). The consumption of the available nitrogen by the non-*Saccharomyces* yeasts and the delay in *S.*

TABLE 1 | Evolution of the different fermentations (0D, co-inoculated fermentation; 1D, inoculation of *S. cerevisiae* at 24 h; 2D, inoculation of *S. cerevisiae* at 48 h; 5D, inoculation of *S. cerevisiae* at 5 days; and Control, only *S. cerevisiae*) under four nutrient conditions (300N-200S, 300N-240S, 100N-200S, and 100N-240S).

Nutrient condition	Inoculation time	MF (days)	EF (days)	BF (CFU/mL)	MF (CFU/mL)	EF (CFU/mL)	Residual sugar (g/L)
300N	0D	3	5	4.0 ± 0.04E+06	6.7 ± 0.08E+07	3.9 ± 0.05E+07	4.87 ± 0.21
	1D	5	7	3.2 ± 0.08E+06	2.7 ± 0.09E+07	2.0 ± 0.04E+07	0.01 ± 0.01
200S	2D	5	8	3.2 ± 0.05E+06	4.1 ± 0.01E+07	4.8 ± 0.03E+07	Nd
	5D	4	6	7.1 ± 0.02E+06	3.9 ± 0.03E+06	3.0 ± 0.03E+06	10.18 ± 0.37
	Control	3	5	5.6 ± 0.09E+06	7.5 ± 0.07E+07	2.5 ± 0.07E+07	0.01 ± 0.01
300N	0D	5	7	8.4 ± 0.02E+06	2.9 ± 0.04E+08	1.9 ± 0.06E+08	5.52 ± 0.37
	1D	5	9	5.3 ± 0.05E+06	5.0 ± 0.01E+07	3.2 ± 0.04E+07	2.80 ± 0.14
240S	2D	7	13	3.0 ± 0.03E+06	4.0 ± 0.06E+07	2.3 ± 0.05E+07	Nd
	5D	7	12	8.5 ± 0.08E+06	2.3 ± 0.06E+08	1.2 ± 0.08E+08	30.90 ± 0.71
	Control	3	5	2.0 ± 0.05E+06	1.0 ± 0.09E+07	2.5 ± 0.03E+08	0.19 ± 0.01
100N	0D	5	8	8.0 ± 0.05E+06	7.4 ± 0.07E+07	5.4 ± 0.05E+07	0.32 ± 0.01
	1D	6	–	4.8 ± 0.07E+06	1.8 ± 0.04E+07	1.4 ± 0.05E+07	43.80 ± 3.68
200S	2D	6	–	4.2 ± 0.08E+06	2.7 ± 0.05E+07	7.6 ± 0.04E+06	53.80 ± 4.38
	5D	6	–	3.1 ± 0.03E+06	7.2 ± 0.06E+06	3.8 ± 0.04E+06	57.50 ± 2.62
	Control	5	8	3.0 ± 0.08E+06	1.1 ± 0.06E+07	7.4 ± 0.03E+06	Nd
100N	0D	7	–	3.9 ± 0.05E+06	2.4 ± 0.04E+07	2.6 ± 0.02E+07	13.20 ± 0.57
	1D	7	–	3.4 ± 0.04E+06	3.0 ± 0.05E+07	9.9 ± 0.04E+06	51.10 ± 0.49
240S	2D	11	–	2.1 ± 0.04E+06	9.6 ± 0.02E+06	9.8 ± 0.02E+06	40.40 ± 3.25
	5D	11	–	2.4 ± 0.08E+06	1.7 ± 0.06E+07	2.0 ± 0.04E+07	64.40 ± 2.76
	Control	5	7	3.3 ± 0.03E+06	1.1 ± 0.04E+07	8.8 ± 0.05E+06	19.30 ± 0.92

Results expressed as days spent to reach the middle (MF) and the end of the fermentation (EF), population growth in YPD at the beginning (BF), middle (MF) and end of the fermentation (EF) and the residual sugar (glucose+fructose) measured at the end of the fermentation or, when density was stable for three consecutive days, the last point was considered.

cerevisiae inoculation could increase the risk of stuck and sluggish fermentations (Medina et al., 2012).

High-sugar must (240S) was indeed expected to result in longer fermentations since it has been previously described that high sugar concentration slows down yeasts growth and the progress of fermentation (Riberéau-Gayon et al., 2006). It has been suggested that the main stress factor under high sugar conditions would be the ethanol content and not the sugar osmotic pressure (Nishino et al., 1985; Mauricio and Salmon, 1992). Bisson and Butzke (2000) observed that a nitrogen supplementation could be appropriate in fermentations with *S. cerevisiae* under 240 g/L of sugar to complete the fermentation and Martínez-Moreno et al. (2012) suggested that 160 mg/L of nitrogen would be the minimum requirement at this sugar concentration. Conversely, other authors demonstrated in *S. cerevisiae* that the addition of nitrogen in high-sugar musts did not necessarily lead to complete fermentations even taking into account the nitrogen utilization requirements by different strains of *S. cerevisiae* (Martínez-Moreno et al., 2012; Childs et al., 2015). According to our results, a supplementation of 300 mg/L of nitrogen was enough to finish all the 240S fermentations.

Effect of Sequential Inoculation of *S. cerevisiae* over Fermentation Kinetics

The inoculation time of *S. cerevisiae* have a significant impact over the fermentation kinetics parameters (**Table 1**), especially within each nitrogen concentration (**Figures 2A,B**). Control fermentations performed just with *S. cerevisiae* were the fastest to complete (5–8 days) under any of the nutrient conditions and only matched by co-inoculation (0D) under optimal sugar concentrations (300N-200S and 100N-200S).

TABLE 2 | ANOSIM of the different factors effect on the fermentations based on a dissimilarity matrix calculated by the Euclidian distance of the kinetic parameters.

Samples	Factor	R	*P*
All	Nitrogen	**0.402**	**0.001**
All	Sugar	0.036	0.15
All	Inoculation time	**0.243**	**0.001**
All	Residual sugar	**0.864**	**0.001**
All	Succ. fermentation	**0.561**	**0.001**

Values of statistical significance (P) below 0.05 (bold values) indicate significantly different fermentations considering a certain factor. Successful fermentation was considered when the residual sugar was below 3 g/L.

Under optimal nitrogen concentration (300N), the sequential inoculation of *S. cerevisiae* from 24 h onward had different effect over the fermentation kinetics depending on the sugar concentration. However, the earlier inoculation of *S. cerevisiae* did not imply that fermentation finished faster (**Table 1**). For example, it is interesting to observe that fermentations where *S.*

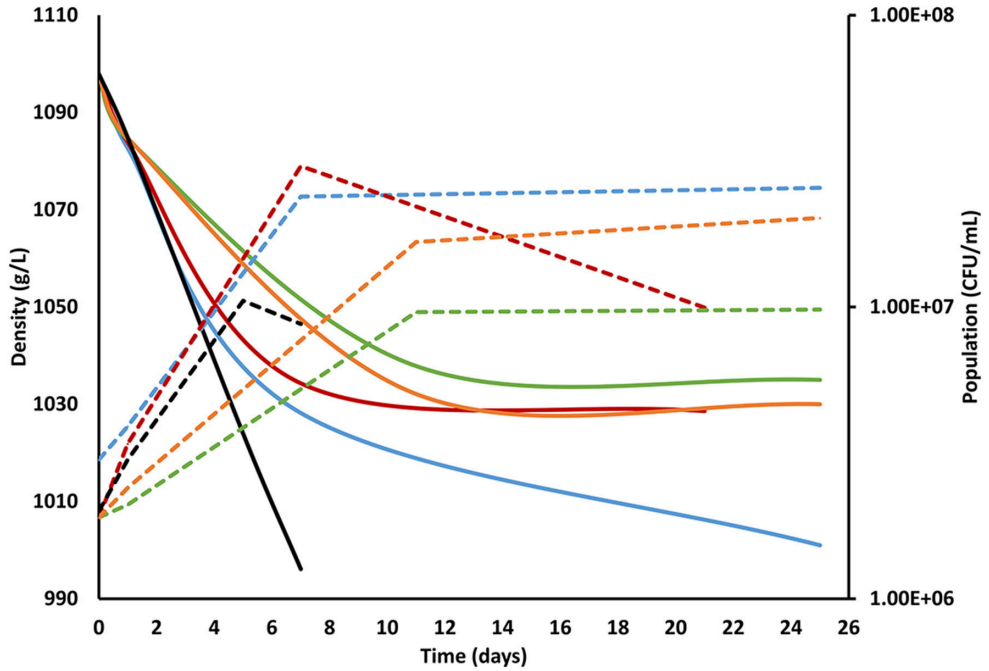

FIGURE 1 | Fermentation kinetics of the different inoculation strategies performed under 100N-240S nutrient conditions. The solid line shows the evolution of the fermentation measured by density (g/L) and the dotted line assessed by plate culturing in YPD (CFU/mL). The line color corresponds to each fermentation strategy: blue, co-inoculated fermentation; red, inoculation of *S. cerevisiae* at 24 h; green, inoculation of *S. cerevisiae* at 48 h; orange, inoculation of *S. cerevisiae* at 5 days, and black; control fermentation with only *S. cerevisiae*. Standard deviations were always lower than 10% and have been avoided in the figure for clarity.

cerevisiae was inoculated at 24–48 h (1D, 2D) under a nitrogen concentration of 300 mg/L took longer to finish than those where *S. cerevisiae* was added 5 days after the beginning of the fermentation (**Table 1**; Figure S1). This result was also reflected in the separation of these samples from the rest of the 300N samples as a consequence of the differences in the fermentation kinetics parameters (**Table 2**, **Figure 2B**). A possible explanation could be that at day 5, when *S. cerevisiae* was inoculated, half of the fermentation had already been spent and the viable non-*Saccharomyces* yeast were decreasing (**Table 1**, **Figure 3**) which meant less competition for nutrients by *S. cerevisiae*. Additionally, the death and the autolysis of non-*Saccharomyces* yeast could result in an extra nitrogen source for *S. cerevisiae* (Hernawan and Fleet, 1995).

Under limiting nitrogen concentration (100N), as stated in the previous section, most of the fermentations got stuck and have a high residual sugar (**Table 1**, **Figures 2A,C**). However, control fermentations were able to finish and, under optimal sugar conditions, the co-inoculation of *S. cerevisiae* and the three non-*Saccharomyces* allowed the fermentation to complete as well (**Table 1**). These results allowed the separation of these fermentations from the rest fermentations on the PCA analysis taking into account all the kinetics parameters (**Figure 2C**). Some authors have proved that co-inoculated fermentations with one or two non-*Saccharomyces* yeast species are a good strategy to ensure *S. cerevisiae* development and the fermentation process (Andorrà et al., 2010; Medina et al., 2012). According to our results, the time of *S. cerevisiae* inoculation acquired more importance under limiting nitrogen content as a consequence of nutrient consumption by the different yeasts species. Medina et al. (2012) demonstrated that an increase of the inoculum size of non-*Saccharomyces* yeasts or the inoculation of *S. cerevisiae* after 24 h decreases the growth of the latter and slowed the fermentation rate of the mixed fermentation as a consequence of the nutrient consumption by non-*Saccharomyces* yeasts.

Thus, a limiting nitrogen concentration together with a sequential inoculation of *S. cerevisiae* later than 48 h involves nitrogen consumption by non-*Saccharomyces* yeasts that limits *S. cerevisiae* development and the fermentation progress.

Yeast Dynamics by Plate Culturing and PCR-DGGE

Both culture dependent and independent techniques (plate culturing and PCR-DGGE) were used to follow yeast dynamics at each fermentation stage (beginning, the middle and the end of the fermentation). The differential morphology of the colonies on WL medium of the four selected yeast species allowed us to calculate the proportion of each cultivable yeast species at each fermentation stage (**Figure 3**). Moreover, to compare with molecular analysis results thus avoiding underestimation by the presence of viable but non cultivable (VBNC) yeast, we performed PCR-DGGE analysis of the extracted DNA at each fermentation stage using general yeast primers (Meroth et al., 2003).

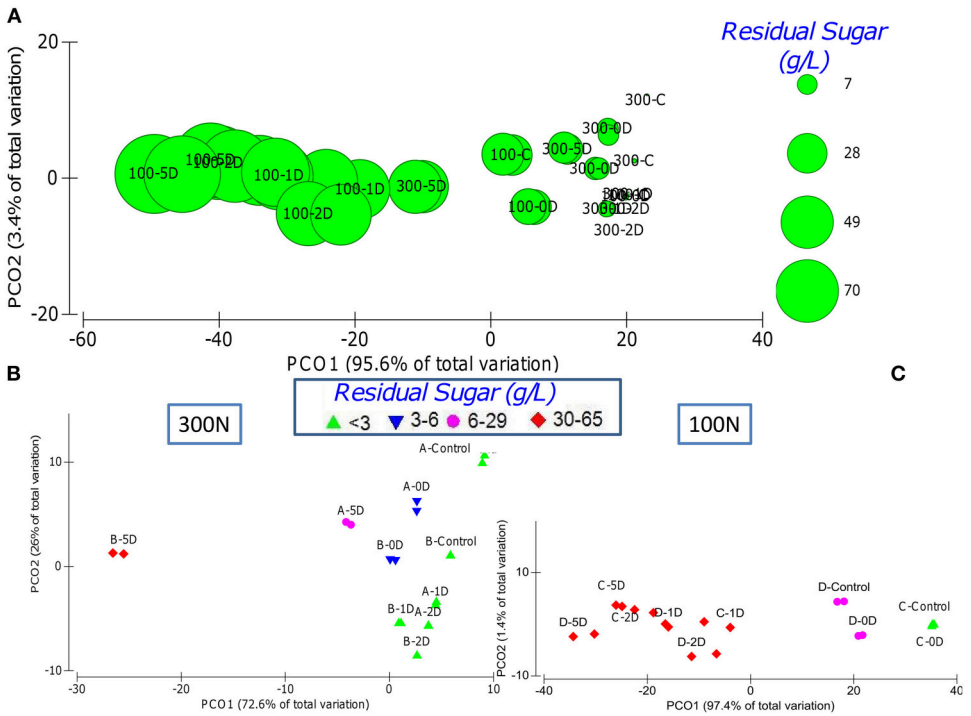

FIGURE 2 | PCA graphs displaying the dissimilarity between the different fermentations taking into account the kinetics parameters R, t10, t50, t75, acetic acid and residual sugar. (A) PCA representing all the fermentations respects the residual sugar content (proportional to the bubbles size) with the clustering of most of 100N fermentations at the left and most of the 300N fermentations at the right. PCA of the 300N **(B)** and 100N **(C)** fermentations respect the residual sugar where the initial sugar concentration is indicated by A or C (200S) and B or D (240S), Control represent the inoculations with only *S. cerevisiae* and the inoculation time of *S. cerevisiae* is indicated by 0D, 1D, 2D, and 5D.

Figure 3 and **Table 3** show that the results obtained by these two techniques were usually comparable. However, as previous studies have reported (Andorrà et al., 2008, 2010) plate culturing proved to be more sensitive than using PCR-DGGE when the proportion of a specific species was very low at some fermentation stages. For example, by DGGE we could not detect *S. bacillaris* and *T. delbrueckii* in most of the fermentation stages while a little proportion of these species was recovered by plate-culturing technique in almost all fermentation stages and conditions. However, under nutrient limiting and sugar excess conditions (100N-240S) the DGGE technique was more efficient and we were able to detect higher yeast diversity maybe as a consequence of the loss of yeast cultivability under these extreme conditions (**Table 3**).

The main yeast species at the beginning of the fermentation (24 h) in all cases was *H. uvarum* while, at the end of the fermentation *S. cerevisiae* took over. We used a higher inoculum of *H. uvarum* compared to the other non-*Saccharomyces,* as occurs on natural must from the Priorat DOQ region (Wang et al., 2016), and this would explain the *H. uvarum* high proportion at the beginning of the fermentation respect to *S. bacillaris* and *T. delbrueckii*. In this sense, our results are similar to those obtained in spontaneous grape fermentations where *H. uvarum* was in great proportion at the first stages of the fermentation in Priorat area (Constantí et al., 1998; Torija et al., 2001; Wang et al., 2016).

It is interesting that a low proportion of *S. cerevisiae* was recovered at the beginning of all the fermentations even when it was co-inoculated with the non-*Saccharomyces* even taking into account that its inoculum size was similar to that of *H. uvarum*. Previous studies have reported that the initial growth of *H. uvarum* retarded the growth of *S. cerevisiae* (Herraiz et al., 1990) which could be an explanation of this effect.

In the middle of the fermentation the yeast species proportion deeply varies depending on the nutrients and the time of inoculation of *S. cerevisiae* (**Figure 3**). For example, under optimal nutrient conditions (300N-200S) at the mid fermentation, the non-*Saccharomyces* yeasts overgrew *S. cerevisiae* that was just more abundant at inoculation 0D or 1D (37 and 44.4%, respectively). Medina et al. (2012) noticed a negative effect of non-*Saccharomyces* yeast on nutrient availability for *S. cerevisiae* reducing its ability for grow especially when it was sequentially inoculated. Interestingly, when they added nitrogen supplementation the fermentation rate and the proportion of *S. cerevisiae* increased, this effect was more prominent when they added a supplement of YAN and vitamin. This YAN consumption by non-*Saccharomyces* yeasts would explain the low imposition of *S. cerevisiae* over the different fermentations at the middle of the fermentation, specifically when *S. cerevisiae* was inoculated 24 h and after. However, under excess of sugar (300N-240S), *S. cerevisiae* was the most frequently recovered at 0D, 2D and 5D (52.6–66.6%) being in low

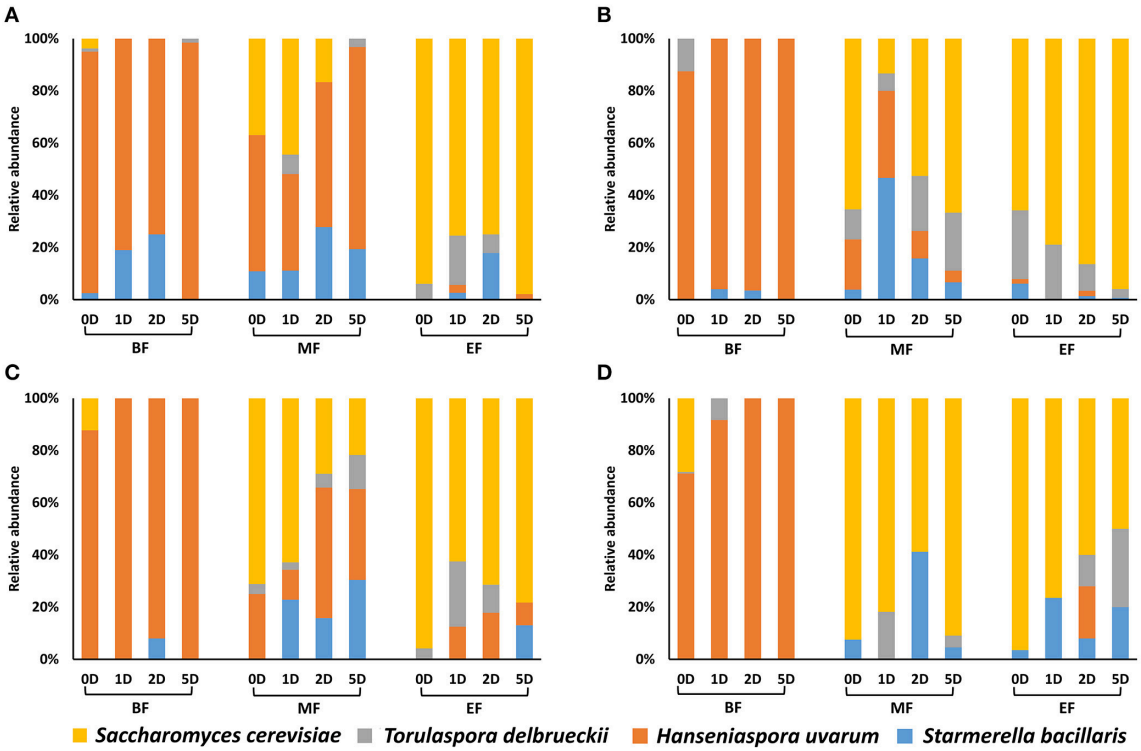

FIGURE 3 | Yeast population dynamics at the beginning (BF), middle (MF) and end of the fermentation (EF) under four different nutrient conditions, (A) 300N-200S, **(B)** 300N-240S, **(C)** 100N-200S, and **(D)** 100N-240S. The fermentations strategies were: 0D, co-inoculated fermentation; 1D, inoculation of *S. cerevisiae* at 24 h; 2D, inoculation of *S. cerevisiae* at 48 h; and 5D, inoculation of *S. cerevisiae* at 5 days.

TABLE 3 | Results of the DGGE-PCR for *H. uvarum* (Hu), *S. bacillaris* (Sb), *T. delbrueckii* (Td) and *S. cerevisiae* (Sc) expressed as "++" (the intensity of the band detected by DGGE gel was high), "+" (the intensity of the band detected by DGGE gel was weak) and "−" (no band was detected by DGGE gel).

Nutrient condition	Inoculation time	Beginning fermentation				Middle fermentation				End fermentation			
		Hu	Sb	Td	Sc	Hu	Sb	Td	Sc	Hu	Sb	Td	Sc
300N	0D	++	−	−	−	+	−	−	−	−	+	+	++
	1D	++	−	−	−	++	+	+	++	−	+	+	++
200S	2D	++	−	−	−	++	−	−	+	−	−	−	+
	5D	++	−	−	−	+	−	−	−	−	−	−	+
300N	0D	+	−	−	−	−	+	+	++	−	+	+	++
	1D	++	−	+	−	++	−	+	+	−	+	−	++
240S	2D	++	−	+	−	−	+	−	++	−	−	−	++
	5D	++	−	−	−	++	−	+	++	−	−	+	+
100N	0D	+	−	−	+	+	−	−	++	−	−	−	++
	1D	+	+	−	−	++	−	+	+	−	−	−	+
200S	2D	++	+	−	−	++	−	+	+	+	−	−	++
	5D	+	−	+	−	++	−	−	−	−	−	−	+
100N	0D	++	−	−	+	+	+	+	++	−	−	−	++
	1D	++	−	−	−	++	−	−	+	+	−	−	++
240S	2D	++	−	−	−	−	+	+	++	−	+	+	++
	5D	++	−	−	−	+	+	+	++	−	−	−	++

proportion at 1D when the non-*Saccharomyces* yeasts (mainly *S. bacilaris*) represented more than 80%. Thus, at 300N-240S *S. cerevisiae* was able to overtake non-*Saccharomyces* yeasts at the middle of the fermentation except when it was inoculated at 24 h although the non-*Saccharomyces* yeasts were present in the mid fermentation under any of the conditions contemplated in the present study. We also observed that the excess of sugar (240S) affected negatively to *H. uvarum* respect the 200S conditions. Under nitrogen limitation (100N-200S/240S), we recovered higher proportion of *S. cerevisiae* at the middle of the fermentation than under the respective 300N fermentations.

At the end of the fermentation, *S. cerevisiae* was the most abundant yeast under any of the analyzed conditions, though *S. bacillaris* and *T. delbrueckii* were also present and generally in higher proportion than *H. uvarum*. In a previous study, Ciani et al. (2006) proved the high persistence of *H. uvarum* in mixed fermentations with *S. cerevisiae* under excess of sugar (270 g/L) and low temperature (15°C), which is in accordance with our results. Wang et al. (2016) demonstrated that *T. delbrueckii* and *S. bacillaris* where able to maintain its cultivability longer than *H. uvarum* when they were inoculated with *S. cerevisiae*. Furthermore, many interactions between non-*Saccharomyces yeasts* and *S. cerevisiae* can occur in the mixed fermentations under the studied conditions: yeast-yeast cell contact, antimicrobial compounds release or competition for substrate (Ciani and Comitini, 2015). It has been described that *S. cerevisiae* produce metabolites that negatively affect non-*Saccharomyces* yeasts (Pretorius, 2000; Pérez-Nevado et al., 2006; Wang et al., 2016). So, the effect of these metabolites together with the chemical changes on the medium could provide an explanation for the decrease of *H. uvarum* and the persistence and increase of *T. delbrueckii* and *S. bacillaris* along the fermentation, because the sensibility to these antimicrobial compounds is species and strain specific (Wang et al., 2016).

Fermentation Products

Total residual sugars were evaluated at the end of the fermentation or, in the case of stuck fermentations, at the last considered point with stable density for three consecutive days, using an enzymatic kit as described in Section Density, Acetic Acid, and Sugar Measurements. Residual sugars were significantly correlated with all the kinetic parameters considered except with the initial sugar concentration (Table S2).

Successful fermentations with residual sugar below 3 g/L where just those performed under optimal nitrogen concentration inoculated with *S. cerevisiae* at 48 H or before and under limiting nitrogen concentration when *S. cerevisiae* was the only yeast inoculated or when the non-*Saccharomyces* yeasts where co-inoculated (**Figures 2B,C**). These successful fermentations had kinetics parameters statistically different from the rest of fermentations tested (**Table 2**).

Fermentations performed under suitable nitrogen content (300N-200S/240S) presented the lowest residual sugars when they were sequentially inoculated at 24 or 48 h (**Table 1**). Unexpectedly, co-inoculated fermentations had a final sugar content between 4 and 6 g/l which could be explained by the high persistence of non-*Saccharomyces* yeast (**Figure 3**) that have been described as low fermentative yeasts (Pretorius, 2000). Besides, when *S. cerevisiae* was added after 5 days, sugar content was quite high as a consequence of the *S. cerevisiae* nutrient deprivation by non-*Saccharomyces* yeasts, which compromised its development and metabolic capacities (Andorrà et al., 2010; Medina et al., 2012).

On the other hand, under nitrogen limiting conditions (100N-200S/240S) the residual sugar concentration was very high at all fermentation stages as a consequence of the stuck fermentations resulting from the nutrient limitation (Bell and Henschcke, 2005) and just the co-inoculated fermentations (100N 200S) that completed the fermentation showed a lower residual sugar (**Table 1**).

CONCLUSIONS

Nowadays, the use of mixed fermentations represents a powerful tool as a consequence of the combination of the positive abilities of non-*Saccharomyces* yeasts with *S. cerevisiae*. Despite this fact, nutrient must conditions and the time of the inoculation of *S. cerevisiae* can determine an adequate fermentation performance. We have demonstrated the negative impact of limiting nitrogen musts on mixed fermentation resulting in stuck fermentations with higher significance than sugar concentration. However, an excess of sugar must slowed down the fermentation rate. Furthermore, the best inoculation time of *S. cerevisiae*, under adequate nitrogen concentration would be before 48 h to ensure the completion of the fermentation due to the nitrogen consumption by non-*Saccharomyces*. However, inoculations before 24 h low the proportion of non-*Saccharomyces* yeasts that could contributed to the complexity of the wines. On the other hand, under nitrogen-limiting conditions, *S. cerevisiae* should be co-inoculated to ensure the fermentation process and the nitrogen availability for this yeast.

AUTHOR CONTRIBUTIONS

JL performed part of the experiments, analyzed the results and draft the manuscript. MM performed part of the experiments. AM and MP conceived the study and participated in its design and coordination and draft the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2016.01959/full#supplementary-material

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Yeast Monitoring of Wine Mixed or Sequential Fermentations Made by Native Strains from D.O. "Vinos de Madrid" Using Real-Time Quantitative PCR

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There is an increasing trend toward understanding the impact of non-*Saccharomyces* yeasts on the winemaking process. Although *Saccharomyces cerevisiae* is the predominant species at the end of fermentation, it has been recognized that the presence of non-*Saccharomyces* species during alcoholic fermentation can produce an improvement in the quality and complexity of the final wines. A previous work was developed for selecting the best combinations between *S. cerevisiae* and five non-*Saccharomyces* (*Torulaspora delbrueckii*, *Schizosaccharomyces pombe*, *Candida stellata*, *Metschnikowia pulcherrima,* and *Lachancea thermotolorans*) native yeast strains from D.O. "Vinos de Madrid" at the laboratory scale. The best inoculation strategies between *S. cerevisiae* and non-*Saccharomyces* strains were chosen to analyze, by real-time quantitative PCR (qPCR) combined with the use of specific primers, the dynamics of inoculated populations throughout the fermentation process at the pilot scale using the Malvar white grape variety. The efficiency of the qPCR system was verified independently of the samples matrix, founding the inoculated yeast species throughout alcoholic fermentation. Finally, we can validate the positive effect of selected co-cultures in the Malvar wine quality, highlighting the sequential cultures of *T. delbrueckii* CLI 918/*S. cerevisiae* CLI 889 and *C. stellata* CLI 920/*S. cerevisiae* CLI 889 and, mixed and sequential cultures of *L. thermotolerans* 9-6C combined with *S. cerevisiae* CLI 889.

Keywords: qPCR, native yeast, non-*Saccharomyces*, *Saccharomyces cerevisiae*, multi-starter fermentation, Malvar wine, sensorial analysis

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INTRODUCTION

Alcoholic fermentation is a complex ecological and biochemical process where a succession of yeasts of several genera and species are able to convert must sugars into ethanol and carbon dioxide, as well as into important secondary metabolites (Barata et al., 2012; Sun et al., 2014; Albergaria and Arneborg, 2016). Even though *Saccharomyces* species are present at a low frequency on the surface of healthy grapes, *Saccharomyces cerevisiae* is considered the primary microorganism in the fermentation process and it is widely used in oenology (Martini et al., 1996; Fleet, 2003). However,

during the last decade, non-*Saccharomyces* yeasts species have been proposed for winemaking as they could contribute to the improvement of wine quality (Ciani et al., 2014; Wang et al., 2015; Masneuf-Pomarede et al., 2016; Puertas et al., 2016). Thus, a new trend has emerged in winemaking using starter cultures composed by non-*Saccharomyces* yeasts, together with *S. cerevisiae* or for sequential fermentation with *S. cerevisiae*.

Molecular methods are showing useful results for detection and faster identification of microorganisms throughout the wine elaboration process (Ivey and Phister, 2011). Classical microbiological methods involving isolation coupled with the enumeration of microbes by plating can lead to misinterpretation of the real number of microorganisms since these methods fail to detect viable but non-culturable (VBNC) organisms (Divol and Lonvaud-Funel, 2005; Quirós et al., 2009; Salma et al., 2013; Wang et al., 2016) and minor populations present are difficult to detect on plates (Cocolin et al., 2013; David et al., 2014). Instead, molecular techniques, generally named culture-independent methods, are used for the identification of microorganism directly in the system through the study of their DNA or RNA without the need for isolation and cultivation, reducing detection time (Andorrà et al., 2008). Real-time quantitative PCR (qPCR) has been widely used in wine for microorganism detection during wine elaboration (Rawsthorne and Phister, 2006; Andorrà et al., 2008, 2010; Tofalo et al., 2012; Wang et al., 2014), providing significant advantages as the low detection level, the speed by which assays are performed, and the ability to quantify yeasts present following alcoholic fermentation.

In a previous work of García et al. (2017), small-scale fermentations were elaborated to study the oenological characterization of five non-*Saccharomyces* native yeast species under several co-culture conditions in combination with selected strain of *S. cerevisiae* CLI 889 to improve the organoleptic properties of the regional Malvar wines. There, the best inoculation process was selected depending on the non-*Saccharomyces* strain inoculated. Preferred sequential inoculations were elaborated with *S. cerevisiae* CLI 889 in combination with *Torulaspora delbrueckii* CLI 918 that produced wines with a higher fruity and floral aroma and lower ethanol content; with *Candida stellata* CLI 920 that increased the aroma complexity and glycerol content; and, with *Lachancea thermotolerans* 9-6C, produced an increase in acidity and floral and ripe fruit aroma. In the case of *Schizosaccharomyces pombe*, sequential fermentation was selected according to its fruity aroma score obtained after tasting. However, mixed cultures of *S. cerevisiae* with *Metschnikowia pulcherrima* CLI 457 and *L. thermotolerans* 9-6C was chosen due to a lower volatile acidity observed in final wines. Moreover, an increase of glycerol and ripe fruit aroma in the case of *M. pulcherrima* was observed, and for *L. thermotolerans* mixed culture the freshness, citric aroma, and full body were the main aspects to verify at the pilot scale.

Regarding these results, the aim of this work is to study yeast population evolution using real-time PCR during pilot winemaking trials under the best inoculation strategies. Moreover, validation of their positive effect on wine fermentation and wine quality was observed in the previous laboratory scale study (García et al., 2017) using sensory analysis.

MATERIALS AND METHODS

Yeast Strains

The non-*Saccharomyces* strains used in this study are *T. delbrueckii* CLI 918, *S. pombe* CLI 1079, *C. stellata* CLI 920, *M. pulcherrima* CLI 457 and *L. thermotolerans* 9-6C, and *S. cerevisiae* CLI 889 strain were previously isolated on the Madrid winegrowing region and selected and characterized in our laboratories based on some established and desirable oenological criteria (Arroyo, 2000; Cordero-Bueso et al., 2013, 2016).

Wine Fermentation and Sampling

The pilot winemaking (stainless steel tanks with 16 L of must) was performed at IMIDRA's experimental cellar is located in the Madrid winegrowing region, Spain (40°31′ N, 3°17′ W and 610 m altitude). Grapes were collected from Malvar (*Vitis vinifera* cv.) white grape variety to elaborate the wines, which were obtained in accordance with the cellar standard practices for harvest. Musts were racked, homogenized, and dislodged statically at 4°C to clarify and be sulfited (50 ppm). Musts obtained from two different vineyards, Must I and Must II, showed 1095 and 1099 g L^{-1} of density, pH values were 3.05 and 3.15, titratable acidity (expressed as g L^{-1} of tartaric acid) was 5.7 and 4.8, and yeast assimilable nitrogen (YAN) values were 218 and 100 mgN L^{-1}, respectively.

Triplicate fermentations were carried out in stainless steel tanks with 16 L of fresh Malvar must at a controlled temperature of 18°C without agitation and, the tanks were locked to maintain anaerobiosis throughout alcoholic fermentation (CO_2 was released through a sterile Müller valve with 96% H_2SO_4). Tanks were inoculated with a *pied de cuve* until a concentration of 10^6 cells mL^{-1} of each yeast strain. These inocula were achieved by an overnight culture of the different yeast strains in sterile must of the same variety prepared away from the cellar. Preselected combinations between *S. cerevisiae* CLI 889 and the different non-*Saccharomyces* species were the best results in García et al. (2017). We named mixed fermentation when both strains are inoculated at the same time, and in sequential fermentation, the non-*Saccharomyces* culture was inoculated at first and the addition of *S. cerevisiae* takes place when the wine contains 5% alcohol (*v*/*v*). The trials tested in must I, were: sequential culture of *T. delbrueckii* CLI 918 and *S. cerevisiae* CLI 889 strains (s-Td/ScI); mixed culture of *M. pulcherrima* CLI 457 and *S. cerevisiae* CLI 889 strains (m-Mp/ScI); and pure culture of *S. cerevisiae* CLI 889 (p-ScI), culture considered as control. The combinations in Malvar must II were: sequential culture of *S. pombe* CLI 1079 and *S. cerevisiae* CLI 889 (s-Sp/ScII); sequential culture of *C. stellata* CLI 920 and *S. cerevisiae* CLI 889 (s-Cs/ScII); mixed culture of *L. thermotolerans* 9-6C and *S. cerevisiae* CLI 889 (m-Lt/ScII);

sequential culture of *L. thermotolerans* 9-6C and *S. cerevisiae* CLI 889 (s-Lt/ScII); and pure culture of *S. cerevisiae* CLI 889 (p-ScII) as a control.

The fermentation process was monitored daily though density, °Baumé, and temperature measurements until constant density (lower than 1000 g L^{-1}). Samples were taken for every tank during the vinification process. Samples (1 mL) for qPCR analyses were centrifuged and pellets were immediately cryo-preserved. For total yeast counts, samples were spread on yeast extract peptone dextrose (YPD) plates and on lysine agar medium [0.25% L-Lysine monohydrochloride (Sigma–Aldrich, St. Louis, MO, United States), 1.17% yeast carbon base (Difco, Detroit, MI, United States), and 2% agar, w/v], a selective medium for the differentiation of non-*Saccharomyces* yeast populations which does not support the growth of *S. cerevisiae* (Walters and Thiselton, 1953). One week after fermentation finished, the wines were bottled after racking and adding 50 ppm SO_2.

Oligonucleotides

Specific-species primers were designed in this work from conserved sequences of the variable D1/D2 domains of the 26S rDNA gene. Generated sequences were aligned with sequences of strains of the same species (**Table 1**) available at the National Centre for Biotechnology Information (NCBI)[1] using Clustal W multiple-sequence alignment (Thompson et al., 1994). The primer design was performed using the Primer3Plus program[2]. Furthermore, the properties of each primer were verified by

[1]http://www.ncbi.nlm.nih.gov
[2]http://www.primer3plus.com

TABLE 1 | List of the accession numbers from GenBank of the sequences used for primer design.

Yeast species	Strain/isolate number	Accession number	Primer[b]					
			SC	Tods	SP1	CS1	MP2	LTH2
Saccharomyces cerevisiae	CLI 889[a]	MF001376	+	−	−	−	−	−
	GS1-3	FJ912839						
	N9323	EU268657						
	cs56	JX129910						
	CBS 2811	KY109393						
Torulaspora delbrueckii	CLI 918[a]	JQ707782	−	+	−	−	−	−
	t15-CTR-7	HQ845012						
	BBMV3FA5	KF735113						
Schizosaccharomyces pombe	CLI 1079[a]	MF001377	−	−	+	−	−	−
	CLI 1085	JQ804983						
	ATCC 16979	KF278469						
	NCYC 3748	JF951752						
Candida stellata	CLI 920[a]	JQ707776	−	−	−	+	−	−
	CBS 2843	EF452199						
	NX8A	EF564405						
Metschnikowia pulcherrima	CLI 457[a]	MF001378	−	−	−	−	+	−
	cs51	JX129913						
	N213	EU268661						
Lachancea thermotolerans	9-6C[a]	MF001379	−	−	−	−	−	+
	CLI 1219	JQ707778						
	cs240	JX129903						
Pickia kudriavzevii	CLI 1216	JQ707777						
	cs280	JX129897						
	cs336	JX129895						
Candida zemplinina	cs271	JX129898						
Candida apicola	cs15	JX129912						
Hanseniaspora uvarum	cs247	JX129900						
	B-1-7	FJ842088						
Hanseniaspora guilliermondii	A11-1-5	EU386752						
	CEC 13A2	KR069091						
Issatchenkia terricola	cs212	JX129906						
Zygosaccharomyces bailii	N2314	EU268642						
Zygosaccharomyces rouxii	CECT 10425	KX539237						

Species and strain designations were included. [a]*These sequences belong to the yeast strains used in this work.* [b]*Results by conventional PCR with primers used in this work (+, presence of PCR product; −, absence of PCR product).*

TABLE 2 | Primer sequences used for real-time quantitative PCR analysis.

Yeast species	Primer name	Sequence 5′–3′	Reference
Saccharomyces cerevisiae	SC1	GAAAACTCCACAGTGTGTTG	Zott et al., 2010
	SC2	GCTTAAGTGCGCGGTCTTG	
Torulaspora delbrueckii	Tods L2	CAAAGTCATCCAAGCCAGC	Zott et al., 2010
	Tods R2	TTCTCAAACAATCATGTTTGGTAG	
Schizosaccharomyces pombe	SP1-F	AGTGAAGCGGGAAAAGCTCA	This work
	SP1-R	ATCGACCAAAGACGGGGTTC	
Candida stellata	CS1-F	AGTAACGGCGAGTGAACAGG	This work
	CS1-R	GGCTATCACCCTCTATGGCG	
Metschnikowia pulcherrima	MP2-F	AGACACTTAACTGGGCCAGC	This work
	MP2-R	GGGGTGGTGTGGAAGTAAGG	
Lachancea thermotolerans	LTH2-F	CGCTCCTTGTGGGTGGGGAT	This work
	LTH2-R	CTGGGCTATAACGCTTCTCC	

The microorganisms' targets for each couple of primers are included.

NIST Primer Tools[3]. Primers used in this study (**Table 2**) were synthetized by TIB MOLBIOL (Berlin, Germany). Moreover, conventional and real-time PCR were carried out using a range of yeast species to verify the specificity of each primer set.

DNA Extraction and Real-Time PCR Assays

Yeast cell pellets were washed with sterile distilled water, and the pellets were resuspended in 700 μL of AP1 buffer (DNeasy Plant Mini Kit; QIAGEN, Valencia, CA, United States) and transferred in a 2-mL microcentrifuge tube containing 1 g of 0.5 mm-diameter glass beads. The tubes were shaken in a mixer mill (Retsch GmbH, Haan, Germany) for 3 min at the maximum rate and then centrifuged at 10,000 rpm for 1 min. Then, the supernatant was transferred to a sterile tube and purified using DNeasy Plant Mini Kit (QIAGEN) according to the manufacturer's instructions.

qPCR was performed on an Applied Biosystems Prism 7500 sequence detection system (Applied Biosystems, Carlsbad, CA, United States). PCR amplification was conducted in optical-grade 96-well plates (Applied Biosystems) and each 25 μL reaction mixture containing 5 μL of DNA, 0.7 μM of each respective primer, and 12.5 μL of SYBR Green Master Mix (Roche Diagnostics GmbH, Mannheim, Germany). Each reaction was made in triplicate. The reaction conditions were an initial step at 95°C for 10 min and 40 cycles of 95°C for 15 s, 60°C for 1 min and 72°C for 30 s. The C_T was determined automatically by the instrument. The coefficients of efficiency (E) were calculated using the formula $E = (10^{-1/\text{slope}}) - 1$ (Higuchi et al., 1993).

Standard Curves

Standard curves for each yeast strain were created by plotting the cycle threshold (C_T) values of the qPCR performed with dilution series of yeast cells (10^7 to 10^3 cells mL^{-1}) against the log input cell mL^{-1} (ABI PRISM 7500 sequence detection system, Applied Biosystems). Standard curves were created for the six yeast strains used in this work.

[3]https://www-s.nist.gov/dnaAnalysis/primerToolsPage.do

Artificial Contamination of Wines

Commercial Tempranillo red wine and Malvar white wine, previously sterilized by filtration, and YPD liquid medium were artificially contaminated with *T. delbrueckii* CLI 918, at known concentrations (10^6 to 10^2 cells mL^{-1}). After incubation of 24 h at 20°C, DNA was isolated as indicated before for qPCR analysis. Standard curves for quantification of samples and determination of amplification efficiency were constructed. These dilutions were also plated on YPD agar and incubated 1 week at 28°C to obtain the number of CFU per milliliter using an easySpiral® plater (Interscience, St. Nom, France).

Study of *Saccharomyces cerevisiae* at the Strain Level

Microsatellite multiplex PCR analysis was used to check the presence of *S. cerevisiae* CLI 889 in the different types of elaboration, using the highly polymorphic loci SC8132X, YOR267C, and SCPTSY7 (Vaudano and Garcia-Moruno, 2008). The analysis was performed according to Cordero-Bueso et al. (2011) and Tello et al. (2012).

Analytical Determination

Oenological parameters as alcohol degree, pH, volatile acidity, total acidity, reducing sugars, glycerol, malic acid, and lactic acid were measured by Fourier transform infrared spectroscopy in the laboratories of Liec Agroalimentaria S.L. (Manzanares, Spain). An accredited laboratory for physico-chemical analysis in wines to conform to UNE-EN ISO/IEC 17025:2005 rules. YAN was determined in must by the formol titration method (Gump et al., 2002).

Quantification of major volatile compounds was carried out in a GC Agilent 6850 with a FID detector equipped with a column DB-Wax (60 m × 0.32 mm × 0.5 μm film thickness) from J&W Scientific (Folsom, CA, United States). Analyses were done according to Gil et al. (2006) and Balboa-Lagunero et al. (2013).

Sensorial Analysis

The final wines were subjected to two sensory analyses, triangle tests (ISO 4120:2007) and descriptive analysis by a trained

panel of seven skilled judges from the IMIDRA Institute. Using triangle tests, the judges determine if a sensory difference exists between the wines tested. Sensory descriptive analysis was based on the description of attributes of the wines though 15 aroma and taste descriptors, and the panelists were asked about their preferences. These attributes were estimated on basis a scale from 1 (low intensity) to 10 (high intensity) and total scores were obtained as the mean and standard deviation of seven evaluations (Arroyo et al., 2009; Balboa-Lagunero et al., 2013).

Statistical Analysis

Analysis of variance was carried out by an ANOVA Tukey test to determine significant differences ($\alpha = 0.05$) between the samples with their respective fermentation control. PCA analysis was performed to identify the most influential oenological parameters and volatile compounds in the different types of cultures. The data were analyzed with SPSS Statistics 21.0 Software for Windows (SPSS, Inc., Chicago, IL, United States).

RESULTS

Primer Design, Specificity and Sensitivity of qPCR

Primers proposed in this work were designed on the variable D1/D2 domains of 26S rDNA gene, amplifying products between 100 and 150 bp in length. Primers for the quantification of *T. delbrueckii* and *S. cerevisiae* strains were designed by Zott et al. (2010) from the region of internal transcribed spacers (ITSs) of the ribosomal DNA region. The other primers used were designed for this work according to those described in the material and methods sections. Sequences for all primers are listed on the **Table 2**.

Each pair of primers exhibited *in silico* specific homology to only species for which were designed. Additionally, conventional PCR was performed using purified DNA from the yeast species used in this study and different strains belonging to the yeasts species *Candida vini*, *Wickerhamomyces anomalus*, *Zygosaccharomyces bailii*, *Meyerozyma guilliermondii*, *Pichia membranifaciens*, *Priceomyces carsonii*, and *Lachancea fermentati*, the most usual species isolated during spontaneous fermentation of Malvar must in the experimental cellar of IMIDRA (Cordero-Bueso et al., 2013), which are also included in the IMIDRA Institute Collection. Amplifications were observed only for those species which the primers that were specifically developed (**Table 1**).

To determine the standard curves qPCR, YPD cultures of each strain containing 10^7 cells mL^{-1} were serially diluted 10-fold until 10^3 cells mL^{-1} and DNA were extracted from 1 ml of each dilution. The DNA was then amplified by qPCR and standard curves were constructed. The slope, intersection, correlation coefficient (R^2), and efficiency of the standard curves obtained are shown in **Table 3**. The assays were linear over five orders of magnitude and, the detection limit for all yeast species was 10^3 cells mL^{-1}.

Quantification in Artificially Contaminated Wines

To study the influence of the wine matrix on the efficiency of the real-time PCR system, standard curves using artificial contaminated wines with *T. delbrueckii* CLI 918 strain were obtained from white (Malvar) and red (Tempranillo) wines, and YPD (control) cultures (**Figure 1**). *T. delbrueckii* CLI 918 strain was used to study this influence. Detection limits for all curves were 10^2 cells mL^{-1} being linear over five orders of magnitude. The correlation coefficients, slopes, and efficiencies of the amplification of standard curves are shown in **Figure 1**. It could be possible to observe that the efficiency of qPCR in red wine is lower than white wine and YPD medium, however the differences observed were not statistically significant ($p < 0.05$). This type of analysis was also done for other yeast species used in this study (data not shown) and the results agreed with the *T. delbrueckii* trial.

Yeast Inoculated Population Analysis by qPCR during Alcoholic Fermentation

qPCR analysis was used to analyze the dynamics of five non-*Saccharomyces* yeasts inoculated, revealing that they were present throughout the alcoholic fermentation. A culture-dependent technique on YPD plates were used to follow the evolution of total cultivable yeasts (**Figure 2**).

Pure cultures of *S. cerevisiae* CLI 889 (p-ScI and p-ScII) used as controls in the fermentations with must I and must II presented different population dynamics. The control p-ScI slowly started to ferment, achieving the highest population at day 9, its fermentation finished with a population of 2.5×10^6 cells mL^{-1} after 32 days (**Figure 2A**). Instead, p-ScII culture reached the greatest population on the second day of fermentation, finishing with 2.7×10^4 cells mL^{-1} after 40 days (**Figure 2D**). The amount of sugar daily transformed in these pure cultures when 50% of the sugar content had been consumed (V_{50}) was higher in p-ScII (V_{50}: 16.23) than p-ScI (V_{50}: 13.30); finally, the p-ScI culture ended the fermentation with 9.86 g L^{-1} of reducing sugars and 13.5% (*v*/*v*) of ethanol, while p-ScII was able to consume the sugars present in the grape must and finished with 13.0% (*v*/*v*) of ethanol (Supplementary Table S1).

TABLE 3 | Standard curves performed for each yeast species.

Yeast	Slope	Intersection	R^2	Efficiency (%)
S. cerevisiae	−3.17 ± 0.04	37.20 ± 0.26	0.997 ± 0.00	106.7 ± 1.97
T. delbrueckii	−3.27 ± 0.13	38.15 ± 0.61	0.996 ± 0.00	102.2 ± 5.62
S. pombe	−3.12 ± 0.05	37.58 ± 0.45	0.999 ± 0.00	108.9 ± 1.06
C. stellata	−3.19 ± 0.21	37.53 ± 0.81	0.998 ± 0.00	105.9 ± 8.72
M. pulcherrima	−3.29 ± 0.01	39.06 ± 0.04	0.992 ± 0.00	101.3 ± 0.39
L. thermotolerans	−3.11 ± 0.18	37.97 ± 0.41	0.993 ± 0.00	109.4 ± 3.29

The slope, intersection, correlation coefficient (R^2), and efficiency of standard curves of S. cerevisiae, T. delbrueckii, S. pombe, C. stellata, M. pulcherrima, and L. thermotolerans were determined by qPCR analysis. Mean ± standard deviation of triplicate qPCR amplifications are shown. Efficiency was estimated by the formula $E = (10^{-1/slope}) - 1$.

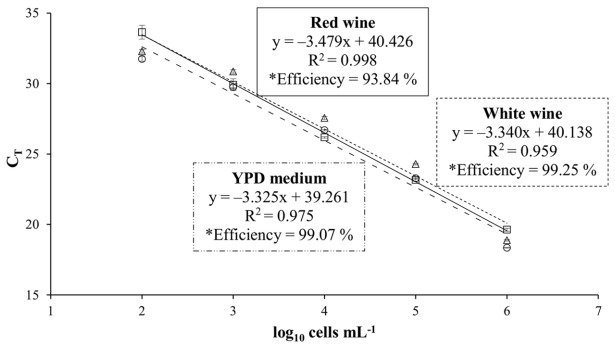

FIGURE 1 | Standard curves obtained for *Torulaspora delbrueckii* CLI 918 from YPD culture (○, –..–), white wine (△, - - - -), and red wine (□, —). C_T values of standard curves from YPD medium, white wine and red wine are the averages of three individual repetitions. *Efficiency was estimated by the formula $E = (10^{-1/slope}) - 1$.

Regarding to mixed cultures (**Figure 2C** for *M. pulcherrima* and 2G for *L. thermotolerans*), on **Figure 2C** it could be possible to observe a small increase of *S. cerevisiae* population until day 9, after that a decrease and a maintenance in its population were observed. In contrast, *M. pulcherrima* population decreased from the beginning of the fermentation, finishing with three orders of magnitude lower than its control (p-ScI) at the end of fermentation after 32 days of vinification. The density values decreased to day 16, when the slow decrease of density coincided in time with the population stabilization of *M. pulcherrima* and *S. cerevisiae*. In the case of *L. thermotolerans* mixed fermentation (**Figure 2G**), there was an increase of this yeast population at the beginning, and after 6 days, a decrease was observed. In the whole fermentation process, the *S. cerevisiae* population was higher than *L. thermotolerans* population. The growth profile of *S. cerevisiae* in this mixed culture (**Figure 2G**) shows a high similarity with its control p-ScII (**Figure 2D**). Both cases on mixed fermentations, the fermentation takes the same time to reduce the density than the controls, and the residual sugars in final wines were also similar to their respective controls.

For sequential cultures, *S. cerevisiae* CLI 889 strain was inoculated at day 13 (represented by the asterisk in the graphics). It is worth noting that the native *S. cerevisiae* population increased between four and five orders of magnitude during the beginning of sequential fermentations, however, an improvement of the fermentation rate has been observed after *S. cerevisiae* inoculation (**Figures 2B,E,F,H**). After microsatellites multiplex PCR analysis to check the presence of *S. cerevisiae* CLI 889 strain from its day of inoculation (day 13) over another *S. cerevisiae* presented in the cellar environment, we found that the microsatellite pattern of the strain inoculated was exhibited by all the isolates analyzed. In **Figure 2B** it is possible to observe that the highest concentration of *T. delbrueckii* CLI 918 was achieved after 5 days, remaining at this level during the alcoholic fermentation, and finishing with the greatest final concentration in comparison with the other non-*Saccharomyces* tested in the sequential cultures. Although this fermentation takes the same length that its control, they need 32 days to reduce the density to lower than 1000 g L^{-1}, the amount of residual sugars is different, showing lower concentrations for the sequential inoculation than its control (Supplementary Table S1). In the *S. pombe*/*S. cerevisiae* sequential culture (**Figure 2E**), an increment of *S. cerevisiae* population after *S. cerevisiae* CLI 889 inoculation can be observed. The *S. pombe* CLI 1079 population is maintained high during the fermentation even after *S. cerevisiae* is added. At the end of vinification, this non-*Saccharomyces* strain finished with approximately one order of magnitude less than *S. cerevisiae* population. *C. stellata* CLI 920 which seemed to be less competitive in this type of inoculation, presented a number of cells two orders of magnitude lower than *S. cerevisiae* from the day 9 (**Figure 2F**). This strain in sequential fermentation (**Figure 2F**) presented its higher counts after the first 24 h, then started to decrease until the end of fermentation (day 40). In this case, however, the inoculation of the *S. cerevisiae* strain produces an improvement of the fermentation rate, showing on **Figure 2F** a high reduction on the density, but the amount of *S. cerevisiae* was not changed. *L. thermotolerans* in sequential culture (**Figure 2H**) remained at high and relatively stable cell levels until day 15 when its population decreased more quickly, ending with three orders of magnitude less than *S. cerevisiae* at the end of fermentation, probably due to *S. cerevisiae* CLI 889 inoculation at day 13, which also produced a decrease of density.

Analytical Determination of Wines

The main oenological parameters analyzed are listed in Supplementary Table S1, which shows that sequential fermentations produced wines so different to their control. Most of the cases the differences involve three or more parameters, while on mixed fermentations the differences with respect to the controls are reduced to a few parameters. Although the differences observed in the ethanol produced among the different fermentations exhibited a significant difference, these differences are lower than 0.5% (*v*/*v*), having no consideration for establish differences due to this parameter. However, the differences with respect to the control can be observed using other parameters, such as glycerol or malic acid. Volatile compounds analyzed (Supplementary Table S2) do not show significant differences on single compounds, but they have been observed when clusters of compounds have been conducted.

To confirm the differences among pure cultures of *S. cerevisiae* (p-ScI and p-ScII, considered as controls) and co-culture-fermented wines, a principal component analysis (PCA) was elaborated (**Figure 3**) from all data obtained from the analysis of oenological parameters and volatile compounds (Supplementary Tables S1, S2). The first two principal components, PC1 and PC2 accounted for 72.23% of total variance (**Figure 3**). PC2, which is mostly formed by volatile compounds (the impact of each parameter on the component is indicated in brackets) as ethyl isovalerate (0.971), ethyl-3-hydroxybutyrate (0.949), 1-butanol (0.914), isoamyl acetate (0.750), ethyl butyrate (0.614), and ethyl hexanoate (0.606), allowed us to differentiate the different types of culture with non-*Saccharomyces* species in combination with the *S. cerevisiae* strain, while the main parameters for PC1 were hexanoic acid (0.989), octanoic acid (0.982), 1-hexanol

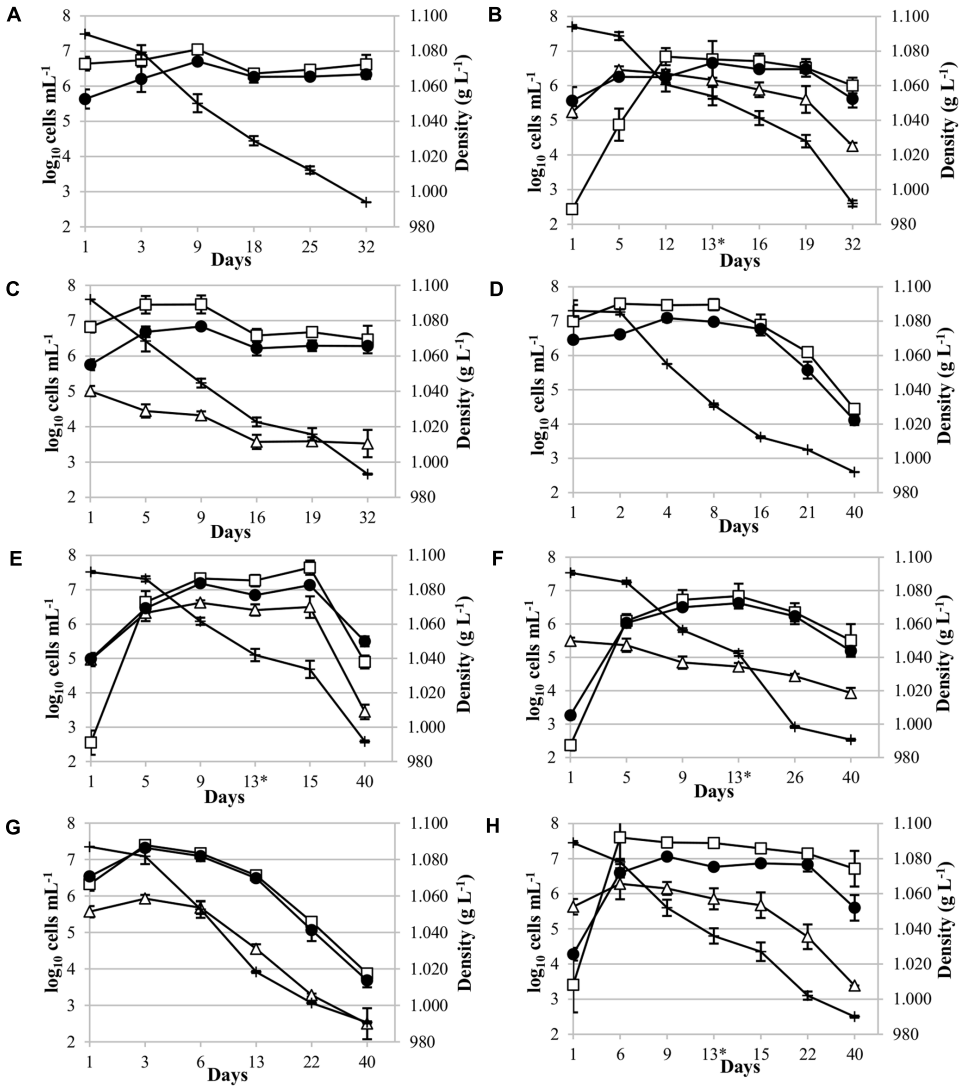

FIGURE 2 | Yeast population dynamics during Malvar fermentation. Results, expressed as $\log_{10}$ cells mL^{-1}, were obtained using YPD culture media (●) and qPCR analysis [(□) for *Saccharomyces cerevisiae*; (△) for non-*Saccharomyces* strains] and, density values (+) expressed in g L^{-1}. Species population analyzed: **(A)** Pure culture of *S. cerevisiae* CLI 889, control p-Scl; **(B)** sequential culture of *T. delbrueckii* CLI 918 and *S. cerevisiae* CLI 889 (s-Td/Scl); **(C)** mixed culture of *Metschnikowia pulcherrima* CLI 457 and *S. cerevisiae* CLI 889 (m-Mp/Scl); **(D)** pure culture of *S. cerevisiae*, control p-Scll; **(E)** sequential culture of *Schizosaccharomyces pombe* CLI 1079 and *S. cerevisiae* CLI 889 (s-Sp/Scll); **(F)** sequential culture of *Candida stellata* CLI 920 and *S. cerevisiae* CLI 889 (s-Cs/Scll); **(G)** mixed culture of *Lachancea thermotolerans* 9-6C and *S. cerevisiae* CLI 889 (m-Lt/Scll); and **(H)** sequential culture of *L. thermotolerans* 9-6C and *S. cerevisiae* CLI 889 (s-Lt/Scll). Asterisk in graphics indicates the day of inoculation of *S. cerevisiae* strain in sequential cultures.

(0.979), isovaleric acid (0.965), diacetyle (0.961), isoamyl alcohol (0.929), β-phenylethyl alcohol (0.927), isobutanol (0.901), and pH (0.876), differentiating the cultures elaborated with Malvar must I and must II. This PCA confirmed the evidence given by the analytical assays, making it possible to confirm a higher similarity between mixed cultures and their respective controls in contrast with the greater differences found in sequential cultures (**Figure 3**).

Sensory Profile of the Produced Wines

Wines elaborated were tested by skilled judges from the IMIDRA Institute as the sensorial panel. For fermentations conducted with must I, all panelists were able to distinguish sequential culture of *T. delbrueckii* from the control with a 0.1% significance level by triangle tests. In the case of mixed culture of *M. pulcherrima*/*S. cerevisiae*, tasters differentiated this type of inoculation with respect to the control with a 5% significance level (data not shown). Most panelists considered the sequential culture of *T. delbrueckii* as the best one wine due to its higher aroma intensity, overall quality, and its fruity and floral aroma; also, they denoted its bitter taste (**Figure 4A**). The mixed culture of *M. pulcherrima*/*S. cerevisiae* was described by tasters for its acid and alcoholic character (**Figure 4A**), but also residual sugars in this fermentation (Supplementary Table S1) were detected. The

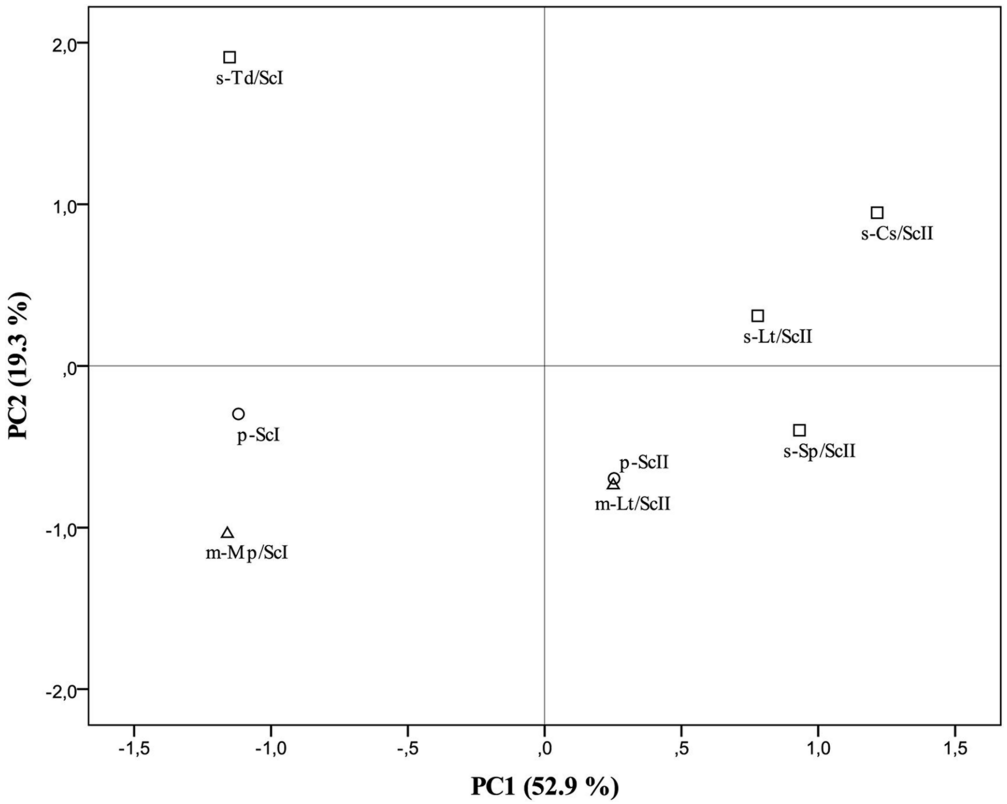

FIGURE 3 | Results of the principal component analysis (PCA) performed on the oenological parameters and volatile compound data. Mean of triplicates wine samples derived from pure cultures of *S. cerevisiae* (p-ScI and p-ScII), sequential culture of *T. delbrueckii* and *S. cerevisiae* (s-Td/ScI), mixed culture of *M. pulcherrima* and *S. cerevisiae* (m-Mp/ScI), sequential culture of *S. pombe* and *S. cerevisiae* (s-Sp/ScII), sequential culture of *C. stellata* and *S. cerevisiae* (s-Cs/ScII), mixed (m-Lt/ScII) and sequential (s-Lt/ScII) cultures of *L. thermotolerans* and *S. cerevisiae* in the plane formed by the two first principal components. Pure cultures represented by circles (○), mixed cultures by triangles (△), and sequential cultures by squares (□).

aroma was described by tasters as ripe fruit and banana but, in general, this wine was described as not intense and its lower concentration of volatile compounds compared to the rest of wines can also be seen (Supplementary Table S2).

Furthermore, on fermentations with must II, tasters were able to differentiate the sequential culture of *S. pombe*/*S. cerevisiae* (s-Sp/ScII), the sequential culture of *C. stellata*/*S. cerevisiae* (s-Cs/ScII), and the mixed culture of *L. thermotolerans*/*S. cerevisiae* (s-Lt/ScII) from the control with a 5% significance level by triangle tests; and, the sequential culture of *L. thermotolerans*/*S. cerevisiae* was differentiated with a 1% significance level through the same tests (data not shown). However, there was no clear preference on sensorial analysis; three of the seven panelists preferred the sequential culture of *C. stellata*/*S. cerevisiae*, and two of them chose the mixed culture of *L. thermotolerans*/*S. cerevisiae*, while the other two preferred the sequential culture of *L. thermotolerans* by descriptive analysis.

Sequential culture of *C. stellata* was described by tasters as a wine with a pleasant fruity (green apple, grapefruit) and floral aroma; it was denoted as fresh and full-bodied on the palate (**Figure 4B**).

Lachancea thermotolerans in sequential and mixed cultures were well-accepted by tasters (**Figure 4B**). The mixed culture was noted for an intense flavor, balanced acidity, and alcohol with slight sweetness and full body. Its aroma was described as lemon, apple, and nut notes and high aroma intensity. Instead, the sequential culture of *L. thermotolerans* presented the highest acidity of all wines (**Figure 4B**) due to its higher lactic acid content (Supplementary Table S1). Tasters highlighted its fruity (ripe fruit) and floral aroma and freshness on the palate.

Finally, tasters noted that sequential culture of *S. pombe*/*S. cerevisiae* did not improve the organoleptic characteristics to Malvar wines (**Figure 4B**). This wine was described as acid and bitter, low aromatic intensity with citric notes probably due to ethyl octanoate, and ethyl hexanoate volatile compounds (Supplementary Table S2). Additionally, microbiological aroma was detected by tasters in this culture.

DISCUSSION

In this study, we quantified the evolution of inoculated non-*Saccharomyces* and *Saccharomyces* populations during alcoholic fermentation in different combinations between strains of different species in a natural must of a white grape Malvar variety. A rapid culture-independent qPCR method for detection

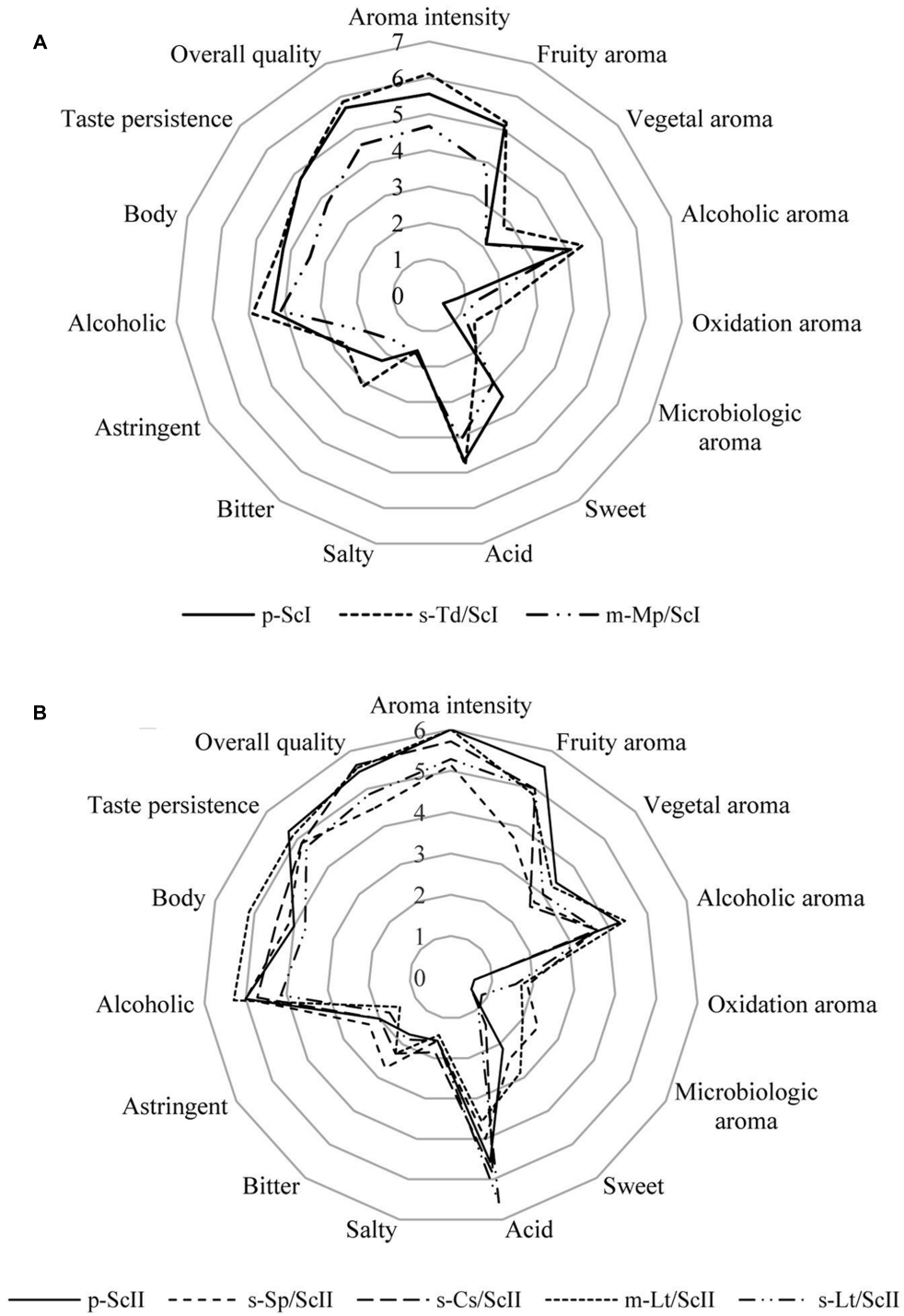

FIGURE 4 | Cobweb diagrams of mean sensory scores of wines made with *Saccharomyces* and non-*Saccharomyces* combinations. **(A)** Cobweb graph of wines: p-ScI, s-Td/ScI, and m-Mp/ScI; and **(B)** cobweb graph of wines: p-ScII, s-Sp/ScII, s-Cs/ScII, m-Lt/ScII, and s-Lt/ScII. Abbreviations related with the type of culture employed and the yeast strains are explained in **Figure 2**.

and enumeration of different yeasts was applied in Malvar wine fermentations. Four pairs of primers were designed in this work into the variable D1/D2 domains of the 26S ribosomal DNA gene to the strains *S. pombe* CLI 1079, *C. stellata* CLI 920, *M. pulcherrima* CLI 457, and *L. thermotolerans* 9-6C; this region has previously been used to develop qPCR methods for several yeasts (Andorrà et al., 2010; Albertin et al., 2014). Two other pair of primers were designed by Zott et al. (2010)

to the ITS region of rDNA, and this region is widely used in yeast species identification due to the high degree of interspecies sequence variations (Esteve-Zarzoso et al., 1999; Schoch et al., 2012). These qPCR species-specific primers showed an excellent specificity with all wine yeasts tested and did not amplify other representative wine species. Moreover, standard curves elaborated with the different yeast strains presented high efficiencies, and good detection limits, we enumerated the concentration of 10^3 cells mL^{-1}, and the trials were linear over five orders of magnitude.

The *T. delbrueckii* CLI 918 strain has been utilized to study the matrix influence in the efficiency of qPCR system. Our results were able to show that the matrix of red wine influences on the PCR amplification or on the DNA extraction and purification, due presumably to its much higher proportion of polyphenols. It is known that wine is a complex matrix that presents various PCR inhibitors (Zoecklein et al., 1999; Phister and Mills, 2003), such as major compounds as polyphenols, tannins, and polysaccharides. The efficiency obtained on qPCR analysis from red wine is lower than from white wine and YPD medium, although these values are similar in all cases and without statistical significance. Some authors have reported problems of amplification with DNA isolated directly from wine (Phister and Mills, 2003; Martorell et al., 2005). The assay performed here helped to check that the wine matrix did not significantly influence in the efficiency of the qPCR analysis. According to our results the construction of standard curves in different matrices do not substantially modify the results, and any matrix can be used to quantify the yeast populations from wine fermentation.

It had long been considered that the non-*Saccharomyces* yeasts are present at the beginning of alcoholic fermentation, being replaced by *S. cerevisiae* which has a high capacity to take over the process. In this work, the dynamics of five non-*Saccharomyces* yeasts in co-inoculation with *S. cerevisiae* have been analyzed, revealing that these non-*Saccharomyces* species were present throughout the fermentation process. If they are present during fermentation we expected contribution to the chemical and sensory attributes of the final wines. However, even though these five non-*Saccharomyces* strains were present during fermentation, *S. cerevisiae* was the most abundant yeast under any of the co-cultures tested at the end of the fermentations. Different mechanisms have been described to explain the dominance of *S. cerevisiae* over other competitors during wine fermentation, i.e., cell-to-cell contact (Nissen et al., 2003); competition for nutrients (Taillandier et al., 2014; Kemsawasd et al., 2015b; Lleixà et al., 2016); secretion of toxic compounds (Pérez-Nevado et al., 2006; Branco et al., 2015; Ramírez et al., 2015; Wang et al., 2016), or changes in the medium (Goddard, 2008; Salvadó et al., 2011). These effects caused by *S. cerevisiae* metabolite production and changes in the medium could provide an explanation for the decrease of *M. pulcherrima* and *C. stellata* and the increase and persistence of *T. delbrueckii*, *S. pombe*, and *L. thermotolerans* belong to the fermentation, due to their higher fermentative power (García et al., 2017) in relation to the amount of alcohol produced by the yeast species (Lopes et al., 2006) and, therefore, related to their alcohol tolerance (Ciani et al., 2016). In the case of *L. thermotolerans*, the enhancement of total acidity produced by this species can also influence in the growth of *S. cerevisiae* and other yeast species. However, the sensibility to these toxic compounds has been described as species- and strain-specific (Wang et al., 2016).

The multi-starter fermentations, combining both non-*Saccharomyces* yeasts and *S. cerevisiae* species able to complete the fermentation, are being studied in depth. All these yeast interaction studies have been increased to explain yeast–yeast interactions and their underlying mechanisms in the increasing use of controlled mixed cultures (Ciani et al., 2010; Ciani and Comitini, 2015). These studies have also been driven by the presence of viable and non-culturable microorganisms in wine samples (Millet and Lonvaud-Funel, 2000; Divol and Lonvaud-Funel, 2005), and may have a false idea about the number of non-*Saccharomyces* species on microbiological methods based on plating (Serpaggi et al., 2012; Wang et al., 2016). In this study, the counts obtained by qPCR were contrasted with plating in YPD non-selective medium and LYS medium (data not shown), a selective medium for non-*Saccharomyces* yeasts. Generally, the yeast populations observed in LYS agar were higher than those obtained by qPCR. This greater growth on LYS medium, could be explained by the growth of other non-*Saccharomyces* yeasts present in the non-sterile Malvar must. This fact is in agreement with the results obtained by Phister and Mills (2003) in a *Dekkera bruxellensis* study.

Differences on the evolution of *Saccharomyces* and non-*Saccharomyces* yeasts have been observed depending on the type of inoculation. In the mixed culture of *M. pulcherrima*/*S. cerevisiae*, the *M. pulcherrima* CLI 457 population started to decrease at 24 h in contrast with the increase of *S. cerevisiae* counts studied by qPCR. The antagonist effect of *M. pulcherrima* on several yeasts, including *S. cerevisiae*, which leads to delays in the fermentation, has been studied (Nguyen and Panon, 1998; Türkel and Ener, 2009). This phenomenon was due to a killer effect linked to pulcherrimin pigment produced by *M. pulcherrima* strains, Türkel and Ener (2009) found three strains of *M. pulcherrima* (UMY12, UMY14, and UMY15) that produce the same amount of the pigment pulcherrimin, but their antimicrobial activities showed important variations. Different distinct biotypes within the *M. pulcherrima* species with respect the pulcherrimin production were identified by Pallmann et al. (2001). However, it has recently been described a difficulty in classifying *Metschnikowia fructicola* species since this species is not distinguishable from *Metschnikowia andauensis* and other species of the *M. pulcherrima* clade because of a possible heterogeneity of rRNA repeats (Cordero-Bueso et al., 2017). For this reason, we keep the original designation for this yeast strain, keeping the same yeast species name described on the published document by Arroyo et al. (2010). However, the variable D1/D2 domain of this strain was sequenced by Macrogen to be identified with 99% of sequence identity as *M. pulcherrima* and its sequence included in GenBank Database (accession number MF001378). Our results showed that the mixed culture of *M. pulcherrima*/*S. cerevisiae* finished with a high level of reducing sugars, in the same way that happened in co-cultures with these strains in laboratory scale fermentations (García et al., 2017), so it could be possible that the *M. pulcherrima* CLI 457

strain had a negative effect on the fermentative capacity of the *S. cerevisiae* CLI 889 strain. Instead, sequential fermentation with *T. delbrueckii* and *S. cerevisiae* finished with sugar values lower than 4 g L^{-1} as at laboratory level (García et al., 2017). Therefore, the fermentative capacity of *T. delbrueckii* in the first days seems to influence in the low sugar content of final wines, independently of the scale of fermentations, which is in agreement with results obtained by Puertas et al. (2016).

Some authors have reported the competition mechanisms between *L. thermotolerans* and *S. cerevisiae* in mixed culture. Hansen et al. (2001) found that oxygen increases the competitiveness between *L. thermotolerans* CBS 2803 and *S. cerevisiae* Saint Georges S101 strains in mixed culture. In the same way, Nissen et al. (2004) concluded that *S. cerevisiae* Saint Georges S101 is able to grow and ferment more efficiently under oxygen-limited conditions present during wine fermentation in comparison with *L. thermotolerans* CBS 2803 and *T. delbrueckii* CBS 3085. Although other previous studies (Nissen and Arneborg, 2003; Nissen et al., 2003) showed that the death of *L. thermotolerans* in mixed culture with *S. cerevisiae* was induced by a cell-to-cell contact mediated mechanism with the same strains used by Nissen et al. (2004). Finally, Kemsawasd et al. (2015a) concluded that cell-to-cell contact and antimicrobial peptides play a combined role in the death of *L. thermotolerans* CBS 2803 in mixed fermentation with *S. cerevisiae* Saint Georges S101 strain. Our strain of *L. thermotolerans* in mixed culture showed a loss of viability most pronounced, although both populations decreased during fermentation process from day 3.

In sequential cultures, the *S. cerevisiae* population found in Malvar wine in the first 24 h of fermentation were low, between 10^2 and 10^3 cells mL^{-1}. It can be seen that native *Saccharomyces* yeasts of the cellar environmental started to grow on the following days, but when *S. cerevisiae* CLI 889 was inoculated (day 13), this strain causes a progressive fall in the density until the end of sequential fermentations. It is well-known that *S. cerevisiae* yeasts are very competitive and normally dominates the fermentation due to its fast growth, efficient glucose competition, good ability to produce ethanol, and a higher tolerance to environmental stresses (Piškur et al., 2006). In this study, the growth of *S. cerevisiae* CLI 889 after its inoculation may have been affected by environmental factors, such as a low controlled temperature (18°C) during the fermentation process, a different availability of nutrients in the musts, and a wine elaboration without the addition of nutrients. After microsatellites multiplex analysis, the presence of the inoculated *S. cerevisiae* CLI 889 strain at the end of fermentation together with other *S. cerevisiae* strains could be confirmed; although in sequential culture, *S. cerevisiae* CLI 889 was found in lower percentage than in mixed cultures at the end of fermentation.

Nutrient content of the musts can modulate the yeast populations, the time of fermentation and secondary metabolites produced during alcoholic fermentation (Beltran et al., 2005; Andorrà et al., 2012; Kemsawasd et al., 2015b). In grape must, nitrogen is considered the main limiting nutrient for optimized growth and good fermentation performance (Bisson, 1999). We could observe when Malvar must II was used in the elaboration of wines, the fermentation length was increased in the cultures (40 days) compared to the elaborations with must I that finished in 32 days; the higher YAN content of must I (218 mgN L^{-1}) than must II (100 mgN L^{-1}) could have influence in the fermentation rate in agreement with other studies (Bely et al., 1990; Monteiro and Bisson, 1992; Beltran et al., 2005). Medina et al. (2012) noticed a negative effect of non-*Saccharomyces* yeasts on nutrient availability for *S. cerevisiae* reducing its ability to grow, especially when it was sequentially inoculated. In the tested sequential fermentations, it could be possible that the YAN consumption by non-*Saccharomyces* would explain the slow growth of *S. cerevisiae* CLI 889, although *S. cerevisiae* population was eventually greater at final of fermentation in all cases, since it is well-known that *S. cerevisiae* strains show a favorable adaptation to the nitrogen-limited wine fermentation environment (Marsit et al., 2015). Additionally, a higher alcohols production (isobutanol, isoamyl alcohol, metionol, and β-phenylethyl alcohol) has been noted in fermentations elaborated with Malvar must II. This is related to the nitrogen concentration, the less nitrogen there is available in the fermentation medium, the more higher alcohols are produced (Beltran et al., 2005; Andorrà et al., 2012). The higher alcohols, along with glycerol, are the end-products of reductive pathway alternatives to the ethanol products. However, we did not detect in all co-cultures a significant decrease in the ethanol content with regard to their controls. Other volatile compounds as acetates, ethyl esters, and 1-propanol have also presented positive correlation with the level of nitrogen in the fermentation process (Rapp and Versini, 1995), this correlation can be observed for most of these compounds when the wines were elaborated with must I.

In terms of glycerol content, we can confirm the use of the tested non-*Saccharomyces* strains provides an enhancement of glycerol both at laboratory scale and at the pilot scale with the exception of *L. thermotolerans* 9-6C that did not produce high concentrations with respect to its controls at both scales. It is well-known that several non-*Saccharomyces* yeasts can considerably increase the glycerol concentrations in wine (Soden et al., 2000; Cominiti et al., 2011; Englezos et al., 2015; Benito et al., 2016b). Glycerol is one of the major compounds produced during wine fermentation, and it is important in yeast metabolism for regulating the redox potential in the cell (Prior et al., 2000). This compound contributes to mouth-feel, sweetness, and complexity in wines (Ciani and Maccarelli, 1998), but its production is usually linked to increased acetic acid production (Prior et al., 2000). In our results, the volatile acidity values measured as grams per liter of acetic acid, were kept low, especially at the pilot scale, with a particular decline in volatile acidity produced by *T. delbrueckii* CLI 918 in sequential culture.

In respect of the oenological parameters studied, the behavior of the yeast strains and the wine styles were similar regardless of the scale of fermentation tested. However, due to the type of vinification being different, some parameters changed at the pilot scale. Most of the wines can be considered as dry since their sugar content was less than 4 g L^{-1} at final of fermentation (Belitz and Grosch, 1999), with the exception of pure culture of *S. cerevisiae* p-ScI and mixed culture of *M. pulcherrima*/*S. cerevisiae* (m-Mp/ScI) (Supplementary Table S1). Generally, volatile acidity values are lower for all co-cultures in this work.

Sequential culture of *T. delbrueckii*/*S. cerevisiae* (s-Td/ScI), in comparison with its control (p-ScI), was distinguished for a significant decrease in volatile acidity (0.34 g L^{-1}) and an increase of glycerol content (Supplementary Table S1). In relation with aromatic compounds, sequential culture of *T. delbrueckii* presented higher concentration of β-phenylethyl alcohol, and esters, such as ethyl butyrate, ethyl isovalerate, isoamyl acetate, ethyl hexanoate, and 2-phenylethyl acetate (Supplementary Table S2) associated with the fruity and floral character of this wine.

In relation with cultures elaborated with *L. thermotolerans* 9-6C and *S. cerevisiae* CLI 889, the effect of *L. thermotolerans* on oenological and sensorial properties of wines (increase of lactic acid, glycerol, and β-phenylethyl alcohol) depends on the way of inoculation with *S. cerevisiae* (Kapsopoulou et al., 2007; Gobbi et al., 2013). We observed a higher lactic acid and β-phenylethyl alcohol content in sequential culture due to *L. thermotolerans* 9-6C growth before *S. cerevisiae* CLI 889 inoculation. *L. thermotolerans* seems to be dominant over *S. cerevisiae* due to the significant enhancement in total acidity and, consequently, a decrease of pH. In contrast, this behavior appears to be softened in mixed culture. This result contrasts with other studies (Gobbi et al., 2013; Benito et al., 2016a) that also observed this pattern of competitiveness in the different inoculation strategies with *L. thermotolerans* and *S. cerevisiae*.

Our results showed that *C. stellata* CLI 920, along with *L. thermotolerans* 9-6C, are strains that produce lactic acid and, therefore, they increase the total acidity, both at the laboratory scale using sterile Malvar must and at the pilot scale. This production could be related with the higher concentration of ethyl lactate observed in both sequential inoculations since this ester is produced by esterification from acid lactic and ethanol (Inaba et al., 2009; Delgado et al., 2010). Higher concentrations of ethyl lactate after the use of co-cultures with *L. thermotolerans* and *S. cerevisiae* have been documented by other authors (Cominiti et al., 2011; Gobbi et al., 2013; Benito et al., 2015, 2016a).

In relation to the *S. pombe* strain, we tested in this work the *S. pombe* CLI 1079 yeast strain instead of the CLI 1085 strain used at laboratory scale due to the low growth capacity of this latest strain, that did impossible a successful *pied de cuve* at the pilot scale. The *S. pombe* CLI 1079 in sequential culture were able to finish the fermentation with residual sugars less than 4 g L^{-1}; this strain presented a low consumption of the malic acid at the pilot scale, ending the fermentation with 1.00 g L^{-1} of malic acid, a value slightly lower than its control (p-ScII). Additionally, glycerol content was higher than the control. This culture presented an elevated concentration of β-phenylethyl alcohol and the highest values of alcohols. Volatile compounds associated with cheese and butter aromas were higher in sequential culture of *S. pombe* than the control p-ScII.

CONCLUSION

We can confirm that the inoculation strategies conducted at the laboratory scale produce a notable improvement in the quality of regional Malvar wines at the pilot scale also. Tasters were able to distinguish the different elaborations with respect the controls and most appreciated wines by tasting panel were those elaborated in sequential cultures with *T. delbrueckii* CLI 918/*S. cerevisiae* CLI 889 and *C. stellata* CLI 920/*S. cerevisiae* CLI 889 and, mixed and sequential cultures with *L. thermotolerans* 9-6C in combination with the *S. cerevisiae* CLI 889 strain. Sequential cultures have produced more different wines with respect to the controls, providing organoleptic properties associated with the non-*Saccharomyces* strains, but more studies need to be carried out varying the moment of inoculation of *S. cerevisiae* strain in these cultures to prevent native *S. cerevisiae* growth on musts, and the reduction of the fermentation time. This work provides the basis for the implementation of new biotechnological strategies for improving Malvar wine quality and it can be tested in commercial wineries.

AUTHOR CONTRIBUTIONS

MG, TA, and BE-Z designed the experiments, analyzed the results, discussion of the results and wrote the manuscript. MG, JC, and JMC performed experiments and analyzed results.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2017.02520/full#supplementary-material

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Genetic Polymorphism in Wine Yeasts: Mechanisms and Methods for Its Detection

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The processes of yeast selection for using as wine fermentation starters have revealed a great phenotypic diversity both at interspecific and intraspecific level, which is explained by a corresponding genetic variation among different yeast isolates. Thus, the mechanisms involved in promoting these genetic changes are the main engine generating yeast biodiversity. Currently, an important task to understand biodiversity, population structure and evolutionary history of wine yeasts is the study of the molecular mechanisms involved in yeast adaptation to wine fermentation, and on remodeling the genomic features of wine yeast, unconsciously selected since the advent of winemaking. Moreover, the availability of rapid and simple molecular techniques that show genetic polymorphisms at species and strain levels have enabled the study of yeast diversity during wine fermentation. This review will summarize the mechanisms involved in generating genetic polymorphisms in yeasts, the molecular methods used to unveil genetic variation, and the utility of these polymorphisms to differentiate strains, populations, and species in order to infer the evolutionary history and the adaptive evolution of wine yeasts, and to identify their influence on their biotechnological and sensorial properties.

Keywords: SNP, insertions, deletions, ploidy changes, interspecific hybridization, gene horizontal transfer, PCR-based methods, NGS

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INTRODUCTION

During the advent of agriculture, humans learnt to put to good use spoiled fruit juices that spontaneously fermented in order to produce alcoholic beverages (Mortimer, 2000), of which grape wine is one of the oldest (McGovern et al., 1997). Alcoholic fermentation of grape must to wine is a complex process that involves the sequential development of microorganisms, mainly yeasts, but also filamentous fungi, lactic acid bacteria and acetic acid bacteria (Pretorius, 2000). Several dozens of yeast species may be present in early wine fermentation stages. However, the yeast population progressively becomes dominated by yeasts that belong to the *Saccharomyces* genus, mainly *Saccharomyces cerevisiae* as alcohol concentration increases (Fleet and Heard, 1993).

Yeasts from the *Saccharomyces* genus exhibit distinctive physiological properties that are not found in other yeasts (Vaughan-Martini and Martini, 2011). The most important is their excellent ability to ferment sugars vigorously to produce alcohol under both aerobic and anaerobic conditions (Piškur et al., 2006; Dashko et al., 2014). This aptitude allows them to quickly colonize sugar-rich substrates and outcompete other yeasts that are much less tolerant to the ethanol and heat produced during fermentation (Goddard, 2008; Salvadó et al., 2011). Consequently, wine

S. cerevisiae strains are highly specialized organisms that have evolved to utilize the different environments or ecological niches provided by human activity. This process can be described as "unaware domestication" and is responsible for the peculiar genetic characteristics of these yeasts (Fay and Benavides, 2005; Liti et al., 2009; Almeida et al., 2015). *S. cerevisiae* strains that exhibit high ethanol tolerance and excellent fermentative ability are extensively used in winemaking as starter cultures. However, a side-effect of the widespread use of these commercial starter cultures is the elimination of native microbiota, which might result in wines with similar analytical and sensory properties, depriving them from the variability and diversity that define the typicality of a wine. Nonetheless, a way of balancing control and yeast population diversity during wine fermentation is the selection of non-*Saccharomyces* yeasts with optimal oenological traits.

Thus, in recent years, other wine yeast species attracted much interest for their potential application to solve new challenges in the winemaking industry generated by consumer demands of aromatic wines with lower ethanol contents, or due to the modification of the composition and properties of grape must because of climate change (Jones et al., 2005). New yeast starters from other *Saccharomyces* species, and from non-*Saccharomyces* species, are being developed to be used in mixed cultures or in sequential inoculations in order to direct fermentations to obtain wines with higher glycerol concentration and aroma intensity, and lower ethanol and acetic acid, contents. In this way, alternative *Saccharomyces* species, such as *S. uvarum* and *S. kudriavzevii*, and their hybrids with *S. cerevisiae*, exhibit good fermentative capabilities at low temperature, and produce wines with lower alcohol concentration, higher glycerol amounts, and excellent aromatic profiles (González et al., 2007; Gamero et al., 2013; Peris et al., 2016), properties of great interest for the wine industry. Additionally the use of non-*Saccharomyces* species, such as *Metschnikowia pulcherrima*, in co-cultures with *S. cerevisiae* has been suggested as an enological practice to reduce ethanol contents in wine (Contreras et al., 2014; Morales et al., 2015). The use of *Candida zemplinina, Hanseniaspora vineae,* and *Torulaspora delbrueckii* yeasts has been proposed to improve the organoleptic properties of wines (Renault et al., 2009; Medina et al., 2013; Jolly et al., 2014).

The study of natural yeast isolates, both at interspecific and intraspecific level, has revealed a great phenotypic diversity, which is explained by a corresponding genetic variation. Thus, the mechanisms involved in promoting these genetic changes are the main engine driving yeast biodiversity. Currently, an important task to understand biodiversity, population structure and evolutionary history of wine yeasts is the study of the molecular mechanisms involved in yeast adaptation to the industrial process, and on remodeling the genomic features of wine yeast, unconsciously selected since the advent of winemaking (Barrio et al., 2006; Marsit and Dequin, 2015). Genetic variation is the ultimate source of heritable variation, acted upon by evolutionary forces such as selection and genetic drift. The neo-Darwinian theory of evolution by natural selection was founded on the notion that natural populations hold abundant genetic polymorphisms to respond to selection. This genetic variability is due to the occurrence of different alleles originated by mutation and homologous recombination. Adaptation is then the result of the gradual accumulation of minor changes in allele frequencies due to the action of natural selection. Different molecular approaches have shown that mutations include not only the generation of new alleles by nucleotide changes, but also the acquisition of new genes or the formation of radically different alleles originated by other mechanisms.

This article reviews the mechanisms involved in generating genetic polymorphisms in yeasts, the molecular methods used to unveil genetic variation, and the utility of these polymorphisms to differentiate strains, populations, and species in order to infer the evolutionary history and the adaptive evolution of wine yeasts, and to identify their influence on their biotechnological and sensorial properties.

MECHANISMS INVOLVED IN THE GENERATION OF YEAST GENETIC POLYMORPHISMS

Yeast genomes are exposed to dynamic mechanisms generating genetic polymorphisms with different evolutionary consequences. These mechanisms can be classified in single nucleotide polymorphisms (SNPs), short sequence insertions or deletions, recombination and gene conversion, short tandem duplications, gene and segmental duplications, gross chromosomal rearrangements (GCRs), ploidy changes and interspecific hybridization (**Figure 1**).

Single Nucleotide Polymorphisms

Single nucleotide polymorphisms are single nucleotide positions in DNA at which different sequence alternatives (alleles) exist in individuals of the same population or species (Brookes, 1999). More broadly speaking, they correspond to the single nucleotide substitutions or small nucleotide insertion–deletions (indels) generated by point mutation due to errors in DNA replication or DNA repair. Although SNPs are less variable than microsatellites (see below), they represent the most widespread type of sequence variation in genomes. SNPs are presently inferred mainly from single gene, multilocus, and genome sequence comparisons (Ben Ari et al., 2005; Fay and Benavides, 2005; Liti et al., 2009; Hyma and Fay, 2013).

Nucleotide polymorphisms have also emerged as valuable genetic markers to reveal the evolutionary history of populations. In this way, SNPs from genome sequence analyses have been used to determine phylogenetic relationships among *S. cerevisiae* strains (Liti et al., 2009; Almeida et al., 2015; Gallone et al., 2016) and other *Saccharomyces* species (Almeida et al., 2014; Peris et al., 2014).

Nucleotide polymorphisms in coding regions or regulatory sequences may change protein structure and function or modify gene expression. Therefore, sequence analyses can also be useful to unveil adaptive evolution in yeasts. In their study, Aa et al. (2006) also reported the presence of a greater replacement polymorphism in gene *SSU1*, which provided evidence for

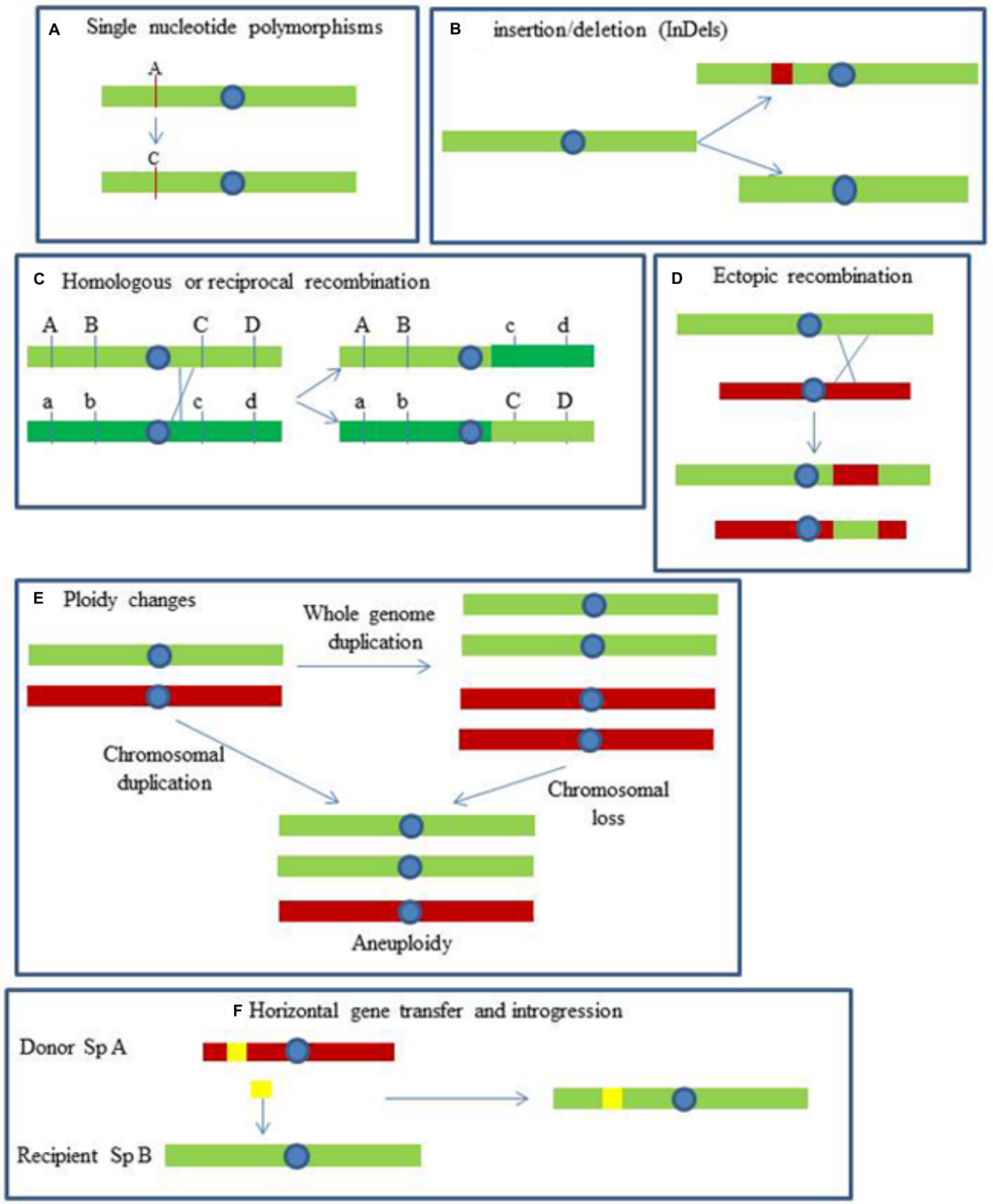


FIGURE 1 | Mechanisms involved in the generation of yeast genetic polymorphism. (A) Single nucleotide polymorphisms: changes in single nucleotides. **(B)** InDels: insertion and deletion events of relatively short pieces of DNA. **(C)** Homologous or reciprocal recombination: gene conversion by crossing-over between homologous chromosomes. **(D)** Ectopic recombination: unequal crossing-over between non-homologous loci. **(E)** Ploidy changes: the whole genome, or large parts, is duplicated or lost. **(F)** Horizontal gene transfer: transfer of genes by alternative means to sexual reproduction (adapted from Steensels et al., 2014).

diversifying selection by acting on its protein product, a sulfite exporter involved in sulfite resistance, as a response to the selective pressure imposed by employing sulfite in winemaking as a bactericide.

Nucleotide divergences in promoter regions may have major effects on gene expression levels, which can also be affected by nucleotide changes in the coding regions of transcription factor genes. In a comparative genome analysis searching for promoters with divergent function, Engle and Fay (2012) identified changes in both the coding and upstream non-coding sequences of yeast transcription factor gene *FZF1,* which resulted in differences to confer sulfite resistance. Non-coding changes affected transcription factor expression, whereas coding changes affected the expression of *SSU1*, the sulfite pump.

In some cases, polymorphisms have been demonstrated as being involved in generating phenotypic variation in yeast properties of biotechnological interest. By way of example, Marullo et al. (2007) studied the genetic basis for variations in acetic acid production in wine strains by quantitative trait loci (QTL) mapping. They showed that this variation was due to a non-synonymous single-nucleotide polymorphism in *ASP1*. The corresponding amino acid replacement abolished the catalytic activity of encoded asparaginase type I, which affected nitrogen assimilation, the CO_2 production rate and acetic acid production. Guillaume et al. (2007) also described nucleotide substitutions in gene *HXT3*, which encodes one of the hexose transporters, that resulted in improved fructose assimilation during wine fermentation. Oliveira et al. (2014) observed that non-synonymous nucleotide divergences between *GPD1* genes from *S. kudriavzevii* and *S. cerevisiae* could explain differences in the V_{max} of glycerol-3-phosphate dehydrogenases, responsible for higher glycerol production in *S. kudriavzevii* (Arroyo-López et al., 2010).

In some extraordinary cases, missense and nonsense mutations can take an adaptive value. Will et al. (2010) showed that independent loss in *S. cerevisiae* strains of paralogous *AQY1* and *AQY2* genes, which encode the water-transporter aquaporins involved in freeze-thaw tolerance, provided a major fitness advantage in highly sugar-rich environments.

Microsatellites

Microsatellites, simple sequence repeats (SSR) or short tandem repeats (STR) are direct duplications of short motifs of nucleotides arranged in tandem, which display variation in the number of repeats. The high polymorphism of microsatellites is due to the relatively high motif insertion/deletion (InDels) rates generated by slipped-strand mispairing between contiguous repeats during replication, and by unequal crossover between motifs.

The sequence that surrounds the repeat region is usually conserved, and allows the design of PCR primers to amplify the repeat region. Differences in the number of repeats are identified as length polymorphisms in PCR products by using high-resolution electrophoresis, including automatic DNA sequencers. Microsatellite codominant polymorphisms have proven very useful for strain discrimination (González-Techera et al., 2001; Pérez et al., 2001; Legras et al., 2005; Masneuf-Pomarède et al., 2007), for the genetic analysis of yeast populations (Ayoub et al., 2006; Legras et al., 2007), and to also determine levels of heterozygosity and ploidy (Bradbury et al., 2006; Katz Ezov et al., 2006).

Microsatellites variation may affect phenotypic traits only when located in regulatory and coding regions. With yeasts, the most important source of gene elongation is the presence of codon repeats generated by trinucleotide expansions. No correlation between these expansions and variation in yeast traits of industrial interest has yet been demonstrated. However, a higher frequency of codon repeats in transcription factors and protein kinases has been described in yeasts (Richard and Dujon, 1997; Albà et al., 1999). Changes in the length of repeats in such cellular components of the cell signaling system could alter their biochemical properties, and therefore readjust their interactions with regulatory DNA regions or with other transcription factors, which could provide evolutionary divergence (Albà et al., 1999; Malpertuy et al., 2003).

Meiotic, Mitotic Recombination, and Levels of Heterozygosity

Homologous or reciprocal recombination and gene conversion due to equal crossing-over between homologous chromosomes are the main mechanisms that generate new combinations of mutations. A non-reciprocal recombination due to unequal crossing-over is the source of the duplications, deletions, and translocations that may be involved in the generation of novelties, as reported in the following sections.

In diploid *Saccharomyces* yeasts, the frequency and nature of recombination during sexual, and also asexual, reproduction have an important impact on their patterns of variability. Recombination occurs during both meiosis and mitosis, although meiotic recombination is about 1000 times more frequent. The analysis of recombination rates and linkage disequilibrium using molecular markers provides interesting information about sexual reproduction frequency in yeasts (Koufopanou et al., 2006; Kuehne et al., 2007; Magwene et al., 2011; Gallone et al., 2016).

Mortimer (2000) observed that natural populations *S. cerevisiae* from wine fermentations and vineyards were diploid, homothallic and showed a low genetic diversity correlated with their high fertility. These observations led the authors to propose a mechanism of evolution for these wine yeasts, named as "genome renewal". This mechanism is based on the ability of homothallic haploid *S. cerevisiae* cells to switch their mating type during mitosis, followed by a mother–daughter mating. This way, strains of *S. cerevisiae*, accumulating heterozygous recessive mutations during long periods of asexual reproduction, can change to completely homozygous diploids, except for the *MAT* locus, after a single sexual cycle followed by a homothallic switching of the haploid spores. This process, called haploselfing or autodiploidization, promotes the action of selection, by removing recessive deleterious genes and fixing recessive beneficial alleles, thereby enabling yeasts to adapt efficiently to changing environmental conditions. However, mitotic recombination or gene conversion during vegetative growth (Puig et al., 2000) as well as break-induced replication

(Pâques and Haber, 1999) also promote loss of heterozygosity (LOH) in diploid wine *S. cerevisiae* cells (Ramírez et al., 2004). The direction of the LOH is asymmetrical in heterozygous yeasts due to the mechanisms involved, but the speed of the process increases as a consequence of the higher viability of the new homozygous yeasts with respect to the original heterozygous cells, which promotes a rapid asymmetric evolution in wine yeasts (Ambrona et al., 2005).

Ruderfer et al. (2006) developed a method to estimate the outcrossing rate in *S. cerevisiae* from whole-genome sequences from three strains and one of their sibling species, *S. paradoxus*. Based on recombination patterns, they estimated that the outcrossing rate was very low in yeasts as it occurred only once every 50000 divisions, which suggested that sex in yeast primarily involves inbreeding via intratetrad mating or haploselfing.

Many population genomic studies (Liti et al., 2009; Almeida et al., 2015; Strope et al., 2015) were based on homozygous strains derived from monosporic cultures, which make impossible to characterize the genome heterozygosity. Nonetheless, the presence of clinical and industrial mosaic strains suggested a significant admixture between *S. cerevisiae* lineages.

Sequencing of new clinical, environmental, and industrial isolates of *S. cerevisiae* unveiled a high number of heterozygous positions across the genomes of clinical and industrial yeasts (Argueso et al., 2009; Akao et al., 2011; Borneman et al., 2011; Magwene et al., 2011; Gallone et al., 2016) in contrast to *S. cerevisiae* isolated from wild environments such as oak forests from North America and Asia, which show very low levels of heterozygosity (Kuehne et al., 2007; Wang et al., 2012). Magwene et al. (2011) proposed that the high levels of heterozygosity observed in clinical and industrial strains most likely resulted from outcrossing between genetically diverse lineages, mediated by unaware strain trafficking due human activity. In addition to the presence of mosaic monosporic strains (Liti et al., 2009), this is also supported by the observation of two populations of *S. cerevisiae*, native and introduced wine strains, coexisting and interbreeding in Cachaça fermentations (Badotti et al., 2014).

Yeast outcrossing likely occurs in natural environments because sexual reproduction has not been observed in fermentation environments (Puig et al., 2000), and several authors (Pulvirenti et al., 2002; Stefanini et al., 2016b) showed that the insect gut provides the appropriate conditions for sporulation, germination, and mating of *Saccharomyces* strains.

Magwene et al. (2011) also proposed that these high levels of heterozygosity coupled with clonal expansion and selfing during rare sexual cycles generate a very large number of new homozygous allelic combinations facilitating rapid adaptation to the novel environments created by human activity. The lower levels of heterozygosity in wine yeasts compared to other industrial yeasts, such as brewing yeasts, suggest that these rare sexual cycles, favored by nutrient depletion, seem to be more frequent in wine yeasts (Borneman et al., 2016; Gallone et al., 2016). However, Ambrona and Ramírez (2007) observed after sporulation of wine yeasts that the frequency of mating between cells from the same ascus, favored by physical proximity, was higher than haploselfing and than mating between germinated haploid cells from different tetrads. This mating restriction slowed down the LOH process of the wine yeast population, maintaining the heterozygosity lower than would be expected by outcrossing but higher than expected under the Mortimer genome renewal model.

Gene and Segmental Duplications

Gene duplication is the most important source of new genes in eukaryotes. Paralogs are redundant gene copies generated by duplication. Paralogs are unrestricted to preserve their original function and, therefore, can undergo divergent evolution resulting in novel gene functions.

Gene duplications can be produced by different mechanisms to result in the duplication of a single gene or group of adjacent genes (Koszul et al., 2006) in chromosome duplication, called aneuploidy (Hughes et al., 2000), or in the duplication of the whole genome content, called polyploidy (Wolfe and Shields, 1997).

In some cases, redundant genes can be retained if there is an evolutionary advantage to having extra dose repetitions. In others, one duplicate is free to accumulate mutations because only one of the duplicates is under purifying selection due to constraints to preserve the ancestral function. The classical Dobzhansky–Muller model, of generation of novel genes by duplication, postulates that a pair of paralogs is preserved if one of the copies gains a new function while the other maintains the original role. Nevertheless, this process, called neo-functionalization, is expected to be particularly unusual because beneficial mutations resulting in a new function are very rare comparing to loss-of-function mutations, which can be neutrally fixed in the unrestricted copy. As a result, the redundant duplicate finally becomes a non-functional gene, a process known as non-functionalization. According to the classical model, the presence of paralogous genes in the genome would be rare in the long term, however, the sequencing of complete yeast genomes showed that the preservation of duplicates is quite frequent (Wagner, 1998).

Force et al. (1999) suggested an alternative mechanism to explain the retention of paralogous genes. This process, called sub-functionalization, requires an ancestral gene with more than one function, which are independent lost in the paralogous genes by complementary degenerative mutations. This model requires that both duplicates complement their preserved subfunctions to produce the full patterns of activity of the ancestral gene. Subsequently, the adaptive evolution can promote the subfunctional specialization of each paralogous gene.

One of the best known examples of subfunctionalization in yeasts is the *GAL1-GAL3* paralogous pair, present in *Saccharomyces* species (Hittinger and Carroll, 2007). The *GAL1* gene codes for a galactokinase that catalyzes the phosphorylation of α-D-galactose to α-D-galactose-1-phosphate in the first step of galactose catabolism, while the galactose-inducible *GAL3* gene encodes a transcriptional regulator involved in activation of the *GAL* genes, including *GAL1*, in response to galactose. *Kluyveromyces lactis* possesses one single *GAL1* gene coding for a protein with both functions, transcriptional regulator and galactokinase. The phylogenetic analysis of their sequences indicates that *Saccharomyces GAL1–GAL3* genes duplicated

after the divergence of *K. lactis GAL1*, and subsequently, each paralogous gene specialized by subfunctionalization in one of the original functions.

The most frequent events of gene duplications are those that involve a single gene or group of adjacent genes, called segmental duplication. Different mechanisms have been postulated to explain the origin of single-gene and segmental tandem duplications. The critical step lies in the origin of first tandem duplication, which requires the presence of similar nucleotide sequences to flank the duplicated region. These similar sequences may also be provided by transposable elements. An ectopic recombination between homologous chromosomes, or an unequal sister chromatide exchange at similar sequences, also results in genome region duplications. Subsequent duplications can occur by unequal non-homologous recombination between paralogous repeats (Zhao et al., 2014), which gives rise to tandemly repeated multigene families.

Yeast genomes encode hundreds of multigene families with three or more duplicated genes, which indicate that successive single gene or segmental duplications should have occurred. A comparative genome analysis (Dujon et al., 2004) reveled that tandem gene duplications are very frequent, and have occurred during the evolution of hemiascomycetous yeasts.

Different examples of segmental duplications are dispersed throughout the genome. One of them is the *CUP*1 tandem cluster, located on chromosome VIII, that encodes a copper metallothionein involved in cupper resistance (Welch et al., 1983). Gene copy number variations were generated by unequal non-homologous recombination (Zhao et al., 2014), and are clearly associated with cupper resistance differences (Warringer et al., 2011).

Other gene families are in the subtelomeric regions located nearby chromosome telomeres. Most subtelomeric gene families encode proteins involved in cell membrane and cell wall components, such as lectin-like proteins (*FLO* genes), sugar transporters (*HXT*), genes related to cell–cell fusion (*PRM*), and the assimilation and utilization of nutrients (*GAL*, *MAL*, *SUC*, and *PHO*), etc. (Carlson and Botstein, 1983; Ness and Aigle, 1995; Liti and Louis, 2005; Voordeckers et al., 2012).

Although these genes are not essential, they can be important for yeast adaptation to new environmental conditions. This way, genomic churning due to an ectopic recombination between repeated subtelomeric regions plays a key role in rapidly creating phenotypic diversity over evolutionary time, which favors the rapid adaptation of yeasts to industrial environments (Brown et al., 2010; Christiaens et al., 2012; Voordeckers et al., 2012).

Chromosomal Rearrangements

The analysis of chromosomal DNA by pulse field gel electrophoresis (PFGE) has revealed important chromosome length polymorphisms in yeasts (Bidenne et al., 1992; Querol et al., 1992; Schütz and Gafner, 1994; Nadal et al., 1999). These polymorphisms are due to GCRs, such as translocations, inversions, duplications, and deletions of large chromosomal regions.

The comparative analysis of chromosomes and genomes (Fischer et al., 2000; Infante et al., 2003; Kellis et al., 2003; Dunn et al., 2005) has shown that duplicated genes, transposable elements and dispersed tRNA-encoding genes are found at chromosomal breakpoints, which supports unequal non-homologous recombination as the mechanism implicated in the origin of GCR. Actually, Ty elements or δ-LTRs are well known as favoring genome instability by ectopic recombination in yeasts (Rachidi et al., 1999; Infante et al., 2003). Unequal non-homologous recombination between sequences of high similarity present in non-homologous genes, between duplicated genes, or between Ty retrotransposons could generate evolutionary novelties, such as new chimerical genes with a modified function or with changes in their regulation (Christiaens et al., 2012; Marsit et al., 2015).

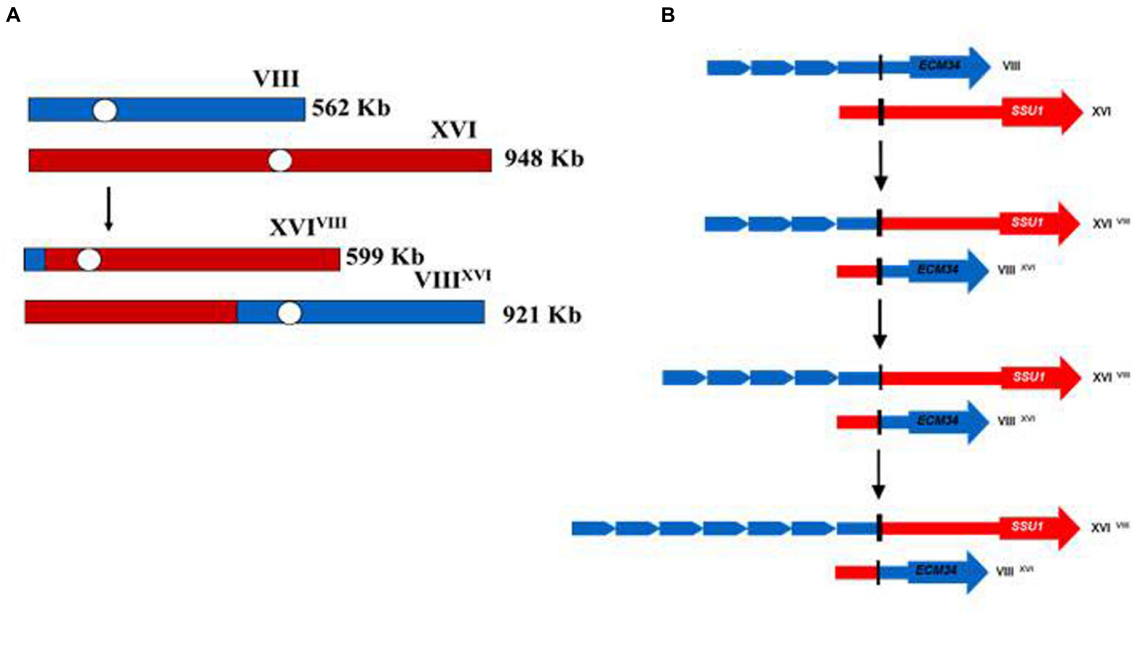
Industrial yeasts exhibit GCR associated to differences in physiological properties of industrial importance (Codón and Benítez, 1995), which is indicative of their potential role in the adaptation of yeasts to industrial environments. As examples, the fact that the same translocation in a region adjacent to *CIT1*, involved in tricarboxylic acid cycle regulation, repeats in different strains that have evolved under growth in glucose-limited chemostats is indicative of its adaptive value (Dunham et al., 2002). Competition experiments between *S. cerevisiae* strains with artificial translocations under different physiological conditions (Colson et al., 2004) have shown that translocated strains consistently outcompete the reference strain with no translocation.

Pérez-Ortín et al. (2002) demonstrated that the translocation between *S. cerevisiae* chromosomes VIII and XVI, found frequently in wine strains, was generated by an ectopic recombination between genes *ECM34*, a gene of unknown function, and *SSU1*, a gene encoding a sulfite pump, and resulted in a chimerical gene that confers greater resistance to sulfite, a preservative used during winemaking (**Figure 2A**). This recombination resulted in a new *SSU1* promoter that contained four repeats of a 76-bp sequence with putative binding sites for the transcription activator Fzf1p (**Figure 2B**). This translocation produced an enhanced expression for *SSU1*. These authors reported a perfect association between the sulfite resistance and the number of 76-bp repeated regions in the *SSU1* promoter (**Figure 2C**). In a recent QTL analysis study (Zimmer et al., 2014), another translocation between chromosomes XV and XVI has been related with a higher *SSU1* expression. This translocation is due to an ectopic recombination between the promoter regions of the genes *ADH1* and *SSU1,* and also produces an increase in the expression of *SSU1* during the first hours of alcoholic fermentation.

Chromosomal rearrangements are also involved in the postzygotic reproductive isolation between *Saccharomyces* species (Ryu et al., 1998). Although translocations may contribute to isolation (Delneri et al., 2003), they do not account by themselves for the isolation levels observed among *Saccharomyces* species (Fischer et al., 2000; Liti et al., 2006).

Ploidy Changes

Aneuploidy, i.e., change in chromosome copy numbers, is originated by chromosomal non-disjunction during meiosis or



C

Strain	Genotype	76-bp repeats in the *SSU1* recombinant promoter	Sulphite concentration (mM)				
			0	1	3	6	8
S228c	SSU1 XVI / SSU1 XVI	0 (non-recomb)	+	+	-	-	-
T73	SSU1 XVI / SSU1 XVI^VIII	4	+	+	+	-	-
CECT 10233	SSU1 XVI / SSU1 XVI^VIII	6	+	+	+	±	-
CECT 10557	SSU1 XVI^VIII / SSU1 XVI^VIII	2 x 3	+	+	+	±	-
Y9	SSU1 XVI^VIII / SSU1 XVI^VIII	2 x 4	+	+	+	+	-

FIGURE 2 | Mechanism involved in wine yeast tolerance to sulfite: the gene *SSU1*, a paradigm of adaptive genome rearrangement. (A) Reciprocal translocation between chromosomes VIII and XVI observed in wine yeast strains. **(B)** Organization of the *ECM34* and *SSU1* non-recombinant alleles and their corresponding recombinant variants obtained by an illegitimate crossing-over. **(C)** Sulfite tolerance of yeast strains exhibiting different numbers of repeats of a 76-bp region in the recombinant *SSU1* promoter (adapted from Pérez-Ortín et al., 2002).

mitosis, and generate a disproportion of gene products and the disruption of their interactions. Although it is one of the causes of their low sporulation levels, aneuploidy is, in general, tolerated by industrial yeasts and has been seen as an advantageous trait in yeasts because a higher number of gene copies may allow them to adapt to changing environments (Bakalinsky and Snow, 1990; Guijo et al., 1997).

Aneuploidies were detected originally by classical genetic analyses. Although most laboratory *Saccharomyces* strains appeared as diploid, higher aneuploidy levels have been described for certain industrial strains (Bakalinsky and Snow, 1990; Martínez et al., 1995; Guijo et al., 1997; Gallone et al., 2016). However, the development of array karyotyping (aCGH) and genome sequencing easily allowed the detection of whole chromosome aneuploidies in yeasts with contrasting results for wine strains. In this way, Infante et al. (2003) showed that *flor* yeasts were aneuploid for a few different chromosomes. However, in a similar study, Dunn et al. (2005) observed no aneuploidies in several commercial wine strains, including two of those previously described as aneuploid (Bakalinsky and Snow, 1990). Flow cytometry and microsatellite analyses of commercial wine yeasts have shown that most are diploid or almost diploid (Ayoub et al., 2006; Bradbury et al., 2006; Legras et al., 2007). This new evidence suggests that aneuploidy in wine strains is much

less frequent than in other industrial strains such as brewing yeasts (Gallone et al., 2016).

The sequencing of the first *S. cerevisiae* complete genome revealed the presence of 376 duplicated genes in 55 large regions, which led Wolfe and Shields (1997) to postulate an ancient whole genome duplication event occurred in an ancestor of *S. cerevisiae* after its divergence from *K. lactis*, about 100–200 million years ago.

Genome duplication, or polyploidization, in yeasts can theoretically occur by several mechanisms (Morales and Dujon, 2012), classified as autopolyploidization when the result is a polyploid yeast, with four allelic copies of each chromosome from one single species, or as allopolyploidization (also called amphidiploidization) when the resulting polyploidy yeast contains several copies of chromosomes from two different species. Autopolyploidization can be generated by (i) non-disjunction during one of the meiotic divisions generates diploid spores, which can subsequently conjugate with other diploid or haploid spores to form tetra- or triploid cells; (ii) a non-disjunction during mitosis in unicellular organisms also produces tetraploid cells; (iii) a rare-mating event between two diploid cells or a diploid and a haploid cells from the same species, these diploid cells become mating-competent by a gene conversion at the MAT locus. Allotetraploidization can be generated by (i) interspecific hybridization by spore-to-spore conjugation, and subsequent genome duplication by non-disjunction either during mitosis or during meiosis; (ii) interspecific rare-mating between diploid cells or between diploid and haploid cells from different species.

The analysis of complete genomes sequences from species of the *Saccharomyces* complex confirmed that the whole genome duplication event encompassed the entire genome and was produced by allotetraploidization due to an ancient hybridization event (Marcet-Houben and Gabaldón, 2015).

The most important consequences of the whole genome duplication event were the sudden acquisition of extra copies of each gene, with slight differences due to the chimeric origin of the duplicated genome, and the provision of new gene functions that have profoundly impacted the evolution of the *Saccharomyces* lineage, particularly the adaptation of these species to highly efficient fermentation performance under anaerobic conditions and the development of efficient glucose-sensing and glucose-repression pathways (Piškur and Langkjær, 2004; Wolfe, 2004). The allotetraploidization event provided the basis for the evolution of new gene functions involved in the improvement of the fermentative performance and fast growth of the ancestors of *Saccharomyces* yeasts, which allow to their descendant industrial yeasts to become, under the selective pressures unconsciously imposed to improve controlled fermentation processes, today's highly efficient mono- and oligosaccharide fermenters (Piškur et al., 2006).

Interspecific Hybridization

In wine *Saccharomyces*, another remarkable mechanism of adaptation to fermentation environments is interspecific hybridization. Reproductive isolation among *Saccharomyces* species is mainly postzygotic, therefore, interspecific spore-to-spore or rare-mating crosses are possible. Although these interspecific hybrids are sterile, they are viable and can reproduce asexually by budding (Naumov, 1996; Sipiczki, 2008).

A well known example of interspecific hybrids are the lager yeasts *S. pastorianus* (syn. *S. carlsbergensis*) (Kodama et al., 2005), which are partial allotetraploid hybrids between *S. cerevisiae* and *S. eubayanus* (Libkind et al., 2011).

Natural hybrids also appear in wine fermentation, *S. uvarum* × *S. cerevisiae* hybrids have been isolated in wines from Italy (Masneuf et al., 1998); Alsace, France (Demuyter et al., 2004; Le Jeune et al., 2007) and Tokaj, Hungary (Antunovics et al., 2005). Other type of hybrids between *S. cerevisiae* and *S. kudriavzevii* are also present in wine fermentations of European regions with Continental and Oceanic climates (González et al., 2006; Lopandic et al., 2007; Erny et al., 2012; Peris et al., 2012a). González et al. (2006) also found a *S. bayanus* × *S. cerevisiae* × *S. kudriavzevii* hybrid isolated from Swiss wine.

By combining the phylogenetic analysis of gene sequences with all the information available on the genetic and genomic characterization of *S. cerevisiae* × *S. kudriavzevii* hybrids, seven potential hybridization events have been predicted as the origin of *S. kudriavzevii* wine hybrids (Peris et al., 2012b). These hybrids appear to have generated by rare-mating crosses between a diploid cell of wine *S. cerevisiae* strains and a haploid spore or cell of European *S. kudriavzevii* strains, because most hybrids contain triploid chimerical genomes (Erny et al., 2012; Peris et al., 2012c).

All *S. cerevisiae* × *S. kudriavzevii* natural hybrids analyzed so far predominantly maintained a *S. kudriavzevii* mitochondrial genome. The only exception is the commercial wine strain AMH, which has lost 69% of the nuclear genes of *S. kudriavzevii* coding for proteins involved in mitochondrial functions. Contrastingly, artificial hybrids obtained under non-selective pressures, inherited their mitochondrial genome from either one or the other parental species randomly (Solieri et al., 2008; Pérez-Través et al., 2014a). This discrepancy has been associated in other hybrids to adaptation to low temperatures (Rainieri et al., 2008), the influence of respiration levels (Solieri et al., 2008; Albertin et al., 2013) or to nuclear-mitochondrial incompatibilities (Lee et al., 2008).

Interestingly, some of these *S. cerevisiae* × *S. kudriavzevii* hybrids showed introgressions between both parental mtDNAs due to recombination in the mitochondrial *COX2* gene (Peris et al., 2012a), gene that has been used to determine mitochondrial inheritance in hybrids (González et al., 2006). Similar introgressions were also found in other hybrids (Pérez-Través et al., 2014b; Peris et al., 2014), and a recent study (Peris et al., 2017) demonstrated that these introgressions are very common among *Saccharomyces* species, which suggests extensive ancestral hybridization events during their evolutionary history.

Genome sequencing and comparative genome hybridization demonstrated that *S. cerevisiae* × *S. kudriavzevii* hybrid strains contain aneuploidy differences and chimerical chromosomes that result from the recombination between "homeologous" chromosomes of different parental origin (Belloch et al., 2009; Borneman et al., 2012; Peris et al., 2012c) (**Figure 3**), promoting

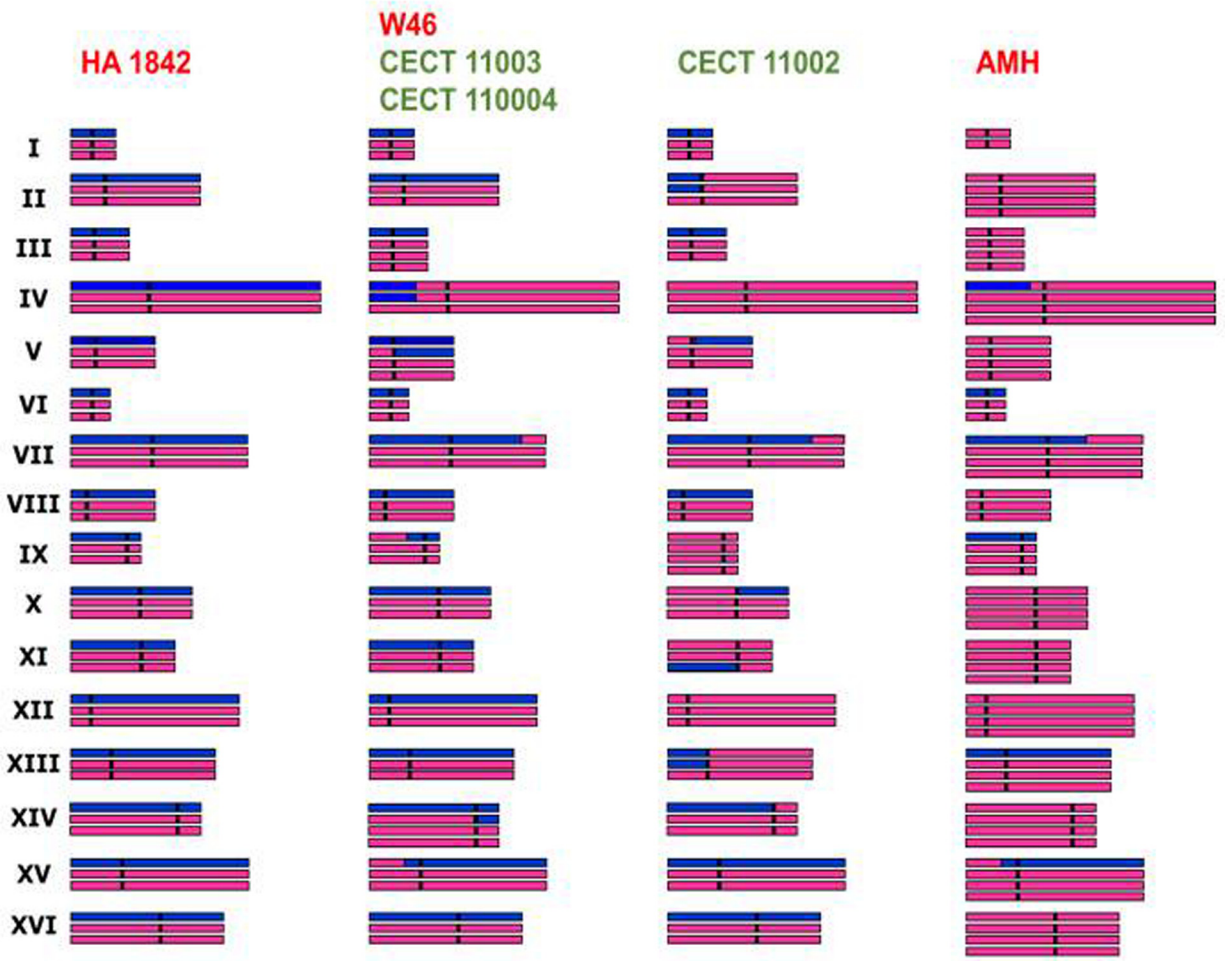


FIGURE 3 | Genome composition of natural *S. cerevisiae* × *S. kudriavzevii* hybrid representatives deduced from aCGH analysis, ploidy estimates and absence/presence of parental genes by RFLP analysis (Peris et al., 2012c). Purple and blue bars are used to represent the *S. cerevisiae* and *S. kudriavzevii* genome fractions, respectively.

the loss of variable segments of the parental subgenomes. The evolution of hybrid genomes under stressful environmental conditions could make hybrid genomes to preserve chromosome rearrangements of selective value (Dunn et al., 2013). Therefore, interactions between both parental genomes, as well as between nuclear and mitochondrial genomes, together with the harsh environmental conditions present during fermentation, determine the final composition of hybrid genomes, which in the case of *S. cerevisiae* × *S. kudriavzevii* hybrids is characterized by the preservation of the *S. cerevisiae* subgenome and a progressive reduction of the *S. kudriavzevii* fraction (Peris et al., 2012c).

The enological characterization of natural hybrid strains *S. cerevisiae* × *S. uvarum* and *S. cerevisiae* × *S. kudriavzevii* has demonstrated that hybrids are well-adapted to ferment at low and intermediate temperatures, produce moderate or higher glycerol levels, and less acetic acid and more aromas (higher alcohols and esters) compared to *S. cerevisiae* and *S. kudriavzevii* reference strains (González et al., 2007; Gamero et al., 2013). The advantages of these hybrids can be correlated with their genome composition (Combina et al., 2012; Gamero et al., 2014; Pérez-Torrado et al., 2015).

Horizontal Gene Transfer and Introgression

The comparative analysis of yeast genomes has shown the occurrence of genes present in a single yeast species or lineage for which the closest homologs are in bacteria (Hall et al., 2005). These genes, most of which encode metabolic enzymes, are rare in yeast genomes (<1%), but actually appear. By way of example, Gojkovic et al. (2004) demonstrated that a horizontal gene transfer (HGT) of a dihydroorotate dehydrogenase, from *Lactococcus lactis* to an ancestor of yeasts *Lachancea* and *Saccharomyces*, conferred them their capability to grow under anaerobic conditions.

Another example is the reacquisition of the biotin biosynthesis pathway in *Saccharomyces* yeasts. This pathway was lost in an ancestor of *S. cerevisiae*, but was later rebuilt by HGT

from bacteria and subsequent gene neofunctionalization after duplication (Hall and Dietrich, 2007).

The genome of *S. cerevisiae* wine yeast EC1118 (Novo et al., 2009) showed the presence of three chromosomal segments acquired through independent HGT events from other yeast species. The donors of two of these regions were later identified. Region B, which comes from *Zygosaccharomyces bailii*, was inserted by means of a circular DNA (Galeote et al., 2011). A recent study (Marsit et al., 2015) has demonstrated that Region C, the largest one, derives from a recent transfer from *Torulaspora microellipsoides*. Marsit et al. (2015) demonstrated that the presence of *FOT* genes in this Region C, which facilitate the transport of the oligopeptides present in grape must, results in improved fermentation efficiency. Borneman et al. (2011) also observed a horizontally acquired cluster of five conserved ORFs that was present in most of the wine strains, encoding two potential transcription factors (one zinc-cluster, one C_6 type), a cell surface flocullin, a nicotinic acid permease and a 5-oxo-L-prolinase.

For eukaryote-to-eukaryote HGT, unstable interspecific hybridization seems the most probable mechanism (Marinoni et al., 1999), although the unidirectional transfer of DNA from one nucleus to another in a newly formed hybrid prior to karyogamy has also been suggested (Morales and Dujon, 2012).

Such unstable interspecific hybridization can also explain the different events of introgression observed among *Saccharomyces* species (Liti et al., 2006; Dunn et al., 2012; Almeida et al., 2014). Some of these introgressed regions contain genes of adaptive value. Almeida et al. (2014) found in *S. uvarum* strains introgressed genome regions from *S. eubayanus*. These introgressed regions contain genes of the nitrogen metabolism, that might be advantageous in wine fermentation, in which nitrogen contents are limiting. Several *S. uvarum* strains isolated from New Zealand wines also contain introgressed regions from *S. eubayanus*. One of these regions comprises gene *FZF1,* encoding a transcription factor involved in the regulation of *SSU1*, the sulfite efflux pump gene. The presence of the *S. eubayanus FZF1* confers a higher tolerance to sulfite to these *S. uvarum* strains (Zhang et al., 2015). Recently, introgressions of the *SSU1* and *FZF1* genes from *S. paradoxus* to a wild Mediterranean population of *S. cerevisiae* have also been described (Almeida et al., 2017), which supports the adaptive value of introgressions.

METHODS TO DETECT GENETIC POLYMORPHISM

Traditionally, yeasts have been identified and classified by morphological and physiological traits (Kurtzman et al., 2011). These methods are laborious and time-consuming, and these characteristics have been influenced by culture conditions and can provide uncertain results (Yamamoto et al., 1991). Simplified biochemical methods have also been developed based on fermentation and assimilation characteristics (Rohm, 1990). Other methods have been based on the analysis of total proteins in the cell (Van Vuuren and Meer, 1987; Vancanneyt et al., 1991), isoenzymic patterns (Duarte et al., 1999), fatty acid analysis using gas chromatography (Cottrell et al., 1986; Tredoux et al., 1987; Moreira da Silva et al., 1994) or, more recently, the application of matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) for yeast differentiation (Blättel et al., 2013; Agustini et al., 2014), especially in the domain of medical sciences for the identification of pathogenic microorganisms (Stevenson et al., 2010; van Veen et al., 2010). However, DNA-based methods are currently the most widely used techniques for yeast differentiation. These techniques have the advantage of being independent of gene expression (Las Heras-Vazquez et al., 2003). Many molecular techniques have been developed to identify and characterize yeasts, such as DNA–DNA hybridization, electrophoretic karyotyping, restriction fragment length polymorphism (RFLP) and PCR-based methods. However, the irruption of next-generation sequencing (NGS) is revolutionizing the way for detecting genetic polymorphisms between organisms. NGS, also known as high-throughput sequencing, is the catch-all term used to describe a number of different modern sequencing technologies, which allow us to sequence DNA and RNA much more quickly and cheaply than Sanger sequencing.

Most studies into wine microbial ecology have invariably been conducted after culturing different microorganisms in distinct media. Nowadays, we witness a new era of microbiology due to the development of molecular biology techniques that allow us to identify and enumerate microorganisms using culture-independent methods. Avoiding the selective cultivation and isolation of microorganisms from natural samples is justified considering the biases related to traditional culture-dependent methods (Rantsiou et al., 2005). Presence of viable, but non-culturable, microorganisms has been described in wine samples (Millet and Lonvaud-Funel, 2000; Divol and Lonvaud-Funel, 2005). These microorganisms are unable to grow in plates, which may justify the differences reported by various authors between isolated and naturally occurring species in wine samples (Cocolin and Mills, 2003; Hierro et al., 2006b).

In this section, we discuss the most recent techniques for detecting genetic polymorphisms in wine yeasts. In wine microbial diversity studies, these techniques have been used mainly for *Saccharomyces* strains and have been used much less for non-*Saccharomyces* discrimination. Depending on the degree of polymorphism provided by the various molecular markers, they are more suitable for interspecific or for intraspecific discrimination. Therefore, we divided the molecular techniques into two main groups: those that can discriminate up to the species level and those that can discriminate up to the strain level.

Methods for Monitoring Yeast Species Diversity during Winemaking

One of the most successful methods for yeast identification thanks to its rapidity and simplicity consists in the PCR amplification of ribosomal genes and the later restriction of the

amplified fragment (PCR-RFLP). This technique is characterized by its easy execution and reproducibility. Guillamón et al. (1998) firstly adapted this technique to identify wine yeasts isolated from grape and wine fermentation processes. Later the restriction patterns of 191 yeast species were provided for the easy and reproducible identification of yeast isolated from food and fermentation processes (Esteve-Zarzoso et al., 1999; Fernández-Espinar et al., 2000; de Llanos-Frutos et al., 2004). To date, this method has been applied by numerous authors to study yeast biodiversity in grapes and wines (Torija et al., 2001; Beltrán et al., 2002; Hierro et al., 2006b; Ocón et al., 2010; Tello et al., 2012; Bezerra-Bussoli et al., 2013; Díaz et al., 2013).

Other independent-culture and PCR-based methods have also been applied for studying yeast species diversity during winemaking processes. This is the case of DGGE and real-time quantitative PCR. DGGE is a semi-quantitative technique based on the sequence-specific separation of PCR-derived rRNA gene amplicons in polyacrylamide gels that contain a linearly increasing concentration of denaturant (urea and formamide), as described by Muyzer (1999). Several authors have shown that DGGE is a well-suited technique for studying yeast population dynamics during wine fermentation (Cocolin et al., 2000; Prakitchaiwattana et al., 2004; Di Maro et al., 2007; Renouf et al., 2007; Stringini et al., 2009; Pérez-Martín et al., 2014), as well as the impact of different viticultural and enological techniques in this diversity (Nisiotou and Nychas, 2007; Andorrà et al., 2008; Milanović et al., 2013). A related technique is temperature gradient gel electrophoresis (TGGE), based on a linear temperature gradient for separating DNA molecules. TGGE has also been applied to the characterization of wine yeasts (Hernán-Gómez et al., 2000; Manzano et al., 2005). However, these methods have their drawbacks: they cannot discriminate between live and dead microorganisms and minor microorganisms go undetected when they co-exist with overwhelming species populations (Andorrà et al., 2008). A modification to the DGGE method has been recently proposed by Takahashi et al. (2014) to identify low-abundant eukaryotic microorganisms. These authors modified the co-amplification at lower denaturation temperature PCR (COLD-PCR) method used to detect minor SNPs that co-exist with an overwhelming majority of wild-type (WT) sequences, as proposed by Li et al. (2008). By combining this modified COLD-PCR with DGGE (mCOLD-PCR-DGGE), these authors detected low-abundant microorganisms more efficiently, even when a specific microorganism represented an overwhelming majority of the sample. *Schizosaccharomyces pombe* was detected in a model sample that co-existed with 10000 times as many *S. cerevisiae*. When mCOLD-PCR-DGGE was applied in a microbiota analysis of a fermenting white wine, *Candida* sp. and *Cladosporium* sp. were detected that were not detected by conventional PCR-DGGE.

Real-time PCR offers numerous advantages compared to other identification techniques. It is worth stressing its high specificity and sensitivity, its ability to quantify and the fact that no analysis after PCR is necessary (electrophoresis). qPCR can even be multiplexed to detect a number of organisms in one assay (Selma et al., 2009). This technique has been developed to detect and quantify total yeasts (Hierro et al., 2006a), *Brettanomyces* (Phister and Mills, 2003; Delaherche et al., 2004; Tofalo et al., 2012; Willenburg and Divol, 2012; Vendrame et al., 2014), *Hanseniaspora* (Hierro et al., 2007; Phister et al., 2007), *Saccharomyces* (Martorell et al., 2005b; Hierro et al., 2007; Salinas et al., 2009), and *Zygosaccharomyces* (Rawsthorne and Phister, 2006) in wine and other fermentation processes. The main disadvantage other than cost and personnel training lies in the method's inability to differentiate viable and non-viable microbes (Ivey and Phister, 2011). Several possible solutions have been indicated to overcome the detection of non-viable microorganisms; e.g., using RNA as a target for PCR amplification (Bleve et al., 2003; Hierro et al., 2006a) because, in theory, RNA is much more unstable than DNA, and is considered an indicator of viability; or using a fluorescent photoaffinity label which covalently couples to nucleic acids upon exposure to light, such as EMA and PMA (Andorrà et al., 2010). These dyes can only enter cells with compromised cell walls and cell membranes, and thus remove DNA from dead cells and then quantify only live microorganisms. However, this and other techniques are being replaced with the power of NGS techniques.

The determination and comparison of the nucleotide sequences of different yeast genome regions is a very useful tool for identifying and inferring phylogenetic relationships between different yeast species. The two most commonly used regions are those that correspond to domains D1 and D2 located at the 5′ end of gene 26S (Kurtzman and Robnett, 1998) and gene 18S (James et al., 1997). The availability of these sequences in databases, especially for the D1/D2 region of gene 26S, makes this technique very useful for assigning an unknown yeast to a specific species when the percentage of homology of its sequences is over or similar to 99% (Kurtzman and Robnett, 1998). However, some authors have advocated the use of multilocus sequence analyses (MLSA) for yeast identification (Kurtzman and Robnett, 2003; Tavanti et al., 2005). These sequences were obtained by the Sanger method. However, since 2005, the NGS methods have emerged and replaced previous techniques because the sequence data generated from a single experiment are immensely more numerous. NGS tools enable the sensitive profiling of microbial communities on an unprecedented scale by the massively parallel sequencing of short (100- to 600-bp) DNA fragments amplified by PCR. The large number of sequences delivered by a single NGS run (10^4 to 10^8 reads) allows a more sensitive description of diverse microbial communities and greater multiplexing, which means a greater per-run sequencing capacity (Bokulich et al., 2014). This technology has been recently applied to study microbial diversity in grapes and wine by metagenomics approaches. Metagenomic surveillances have revealed higher diversity than other community fingerprinting methods and culture-based methods (David et al., 2014; Taylor et al., 2014). In fact, Taylor et al. (2014) suggested that culture-based methods might miss up to approximately 95% of the community in some samples. Consequently, these methods are increasingly becoming the preferred tool to evaluate grape microbial community structures. Bokulich et al. (2014) comprehensively examined the communities of both bacteria and fungi in crushed Chardonnay

and Cabernet Sauvignon fruit in California by Illumina amplicon sequencing approaches and showed that microbiomes not only differed by region, but were also conditioned by climate, year, and cultivar. Similarly, Taylor et al. (2014) demonstrated regional distinction in fungal communities in vineyards across New Zealand. The diversity of yeasts associated with grapes and present in grape must have been shown to resemble that present on leaves (Bokulich et al., 2014; Pinto et al., 2014), and community composition to be influenced by chemical treatments, agronomic practices, and climatic conditions (Setati et al., 2012, 2015; Bokulich and Mills, 2013; David et al., 2014; Pinto et al., 2014). Setati et al. (2015) compared the mycobiome associated with South African (SA) Cabernet Sauvignon grapes in three neighboring vineyards that employed different agronomic approaches by a sequence-based metagenomic approach. The data revealed approximately 10-fold more fungal diversity than what is typically retrieved from culture-based studies.

Similar studies are reported in the literature about monitoring yeast biodiversity in must and during alcoholic fermentation. Pinto et al. (2015) characterized the wine microbiome from six Portuguese wine appellations. The wine fermentation process revealed a stronger impact on yeast populations compared with bacterial communities, and fermentation evolution clearly caused loss of environmental microorganisms. Interestingly, a biogeographical correlation for both yeast and bacterial communities was identified between wine appellations, which suggests that each wine region contains specific embedded microbial communities that might contribute to the uniqueness of regional wines. In a similar metagenomics study conducted during the spontaneous fermentation of "Vino Santo Trentino," Stefanini et al. (2016a) also suggested the existence of a highly winery-specific "microbial-terroir" during fermentation that could contribute significantly to the final product rather than a regional "terroir." This indication was extended to human-related environments through the observation already made in wild environments; namely microbial populations are influenced more by microevolution in their ecological niche than by their geographical location (Morrison-Whittle and Goddard, 2015).

It is noteworthy that two recent studies compared pyrosequencing technology with some of the above-mentioned methods for studying yeast diversity during winemaking, PCR-RFLP, quantitative PCR and DGGE (David et al., 2014; Wang et al., 2015). David et al. (2014) evidenced the power of NGS technology and the drawback of the former techniques for monitoring microbial diversity. DGGE proved unsuitable for the quantification of biodiversity and its use for species detection was limited by the initial abundance of each species. The isolates identified by PCR-RFLP were not fully representative of the true population. For population dynamics, high-throughput sequencing technology yielded results that differed in some respects from those obtained by other approaches. Wang et al. (2015) reached similar conclusions; massive sequencing was more appropriate for understanding the fungal community in grape must after crushing than the other techniques used in this study. They also concluded that the "*terroir*" characteristics of the fungus population related more to vineyard location than to grape variety.

Methods for Fingerprinting Yeast Strain Diversity during Winemaking

Fingerprinting generally examines the whole genome of an organism by often creating a banding pattern by digesting or amplifying genome regions that can be compared between organisms (Ivey and Phister, 2011). Fingerprinting methods are characterized because they present a sufficient degree of genetic polymorphism to differentiate between strains of the same yeast species. As not all the strains of a species present the same industrial traits, availability of techniques that can discriminate at the inter- and intraspecific levels is important. As mentioned for species-differentiation techniques, new genotyping by sequencing (GBS) methods is seen as future strain genotyping. However, many studies that have compared strains still rely on some type of fingerprinting as they provide rapid and less expensive alternatives (Ivey and Phister, 2011). Although many molecular methods have been developed for yeast strain typing, most have been exclusively applied to *Saccharomyces* strains, although the literature offers some non *Saccharomyces* typing examples.

Restriction Analysis of Mitochondrial DNA

The mtDNA of *S. cerevisiae* is a small molecule of between 65 and 80 kb, whose degree of variability can be shown by restriction. The high degree of polymorphism revealed by this technique among *S. cerevisiae* strains means that it is one of the most commonly applied techniques to characterize reference and commercial wine yeast strains (Querol et al., 1992; Guillamón et al., 1996; Fernández-Espinar et al., 2001; Esteve-Zarzoso et al., 2004; Schuller et al., 2004). This technique has been much more limited in typing strains that belong to other species, but some applications can be found in the literature to differentiate *Candida* spp. (Romano et al., 1996; Sabate et al., 2002), *Zygosaccharomyces* (Guillamón et al., 1997; Esteve-Zarzoso et al., 2003), *D. bruxellensis* and *P. guilliermondii* (Martorell et al., 2006) and *Kluyveromyces* (Belloch et al., 1997) strains. So although RFLP mtDNA analyses have shown narrow variability and limited usefulness for some species, it is an efficient technique for differentiating at the strain level in many other yeast species. At present, the *S. cerevisiae* mtDNA variability can also be analyzed by NGS methods (Wolters et al., 2015).

PCR Technique-Based Methods

The PCR technique has made available rapid methods to discriminate wine yeast strains. These methods detect the genetic polymorphism by amplifying different yeast genome regions. Amplified fragments are further separated in an agarose gel to obtain an exclusive banding pattern for each strain.

The Randomly Amplified Polymorphic DNA (RAPD-PCR) fingerprint amplifies genomic DNA with a single primer of arbitrary sequence, 9 or 10 bases in length, which hybridize with sufficient affinity to chromosomal DNA sequences at low annealing temperatures. The result is a pattern of amplified products of different molecular weights that can be characteristic of either the species or the different strains or isolates within the same species (Bruns et al., 1991; Paffetti et al., 1995). This technique has been successfully applied to differentiate wine yeast

strains belonging to different species (Quesada and Cenis, 1995; Baleiras Couto et al., 1996; Romano et al., 1996; Tornai-Lehoczki and Dlauchy, 2000; Čadež et al., 2002; Martínez et al., 2004; Gallego et al., 2005; Martorell et al., 2006; Urso et al., 2008; Pfliegler et al., 2014).

Although yeast genomes are not very rich in repetitive sequences compared with higher eukaryotes, the recent sequencing of entire yeast genomes has revealed the presence of different repetitive regions. The use of primers based on conserved sequences of these repeated regions has proven most useful for strain differentiation by PCR. Microsatellites are short (usually less than 10-bp) sequence repeats that have been shown to exhibit a substantial level of polymorphism in a number of eukaryotic genomes (Hennequin et al., 2001). The variability found in these regions can be shown by PCR amplification using specific oligonucleotides, such as $(GTG)_5$, $(GAG)_5$, $(GACA)_4$ or M13. The ability of these oligonucleotides to reveal polymorphisms among *S. cerevisiae* strains has been demonstrated by Lieckfeldt et al. (1993) by hybridization techniques. These same authors were the first to use these sequences as primers in a PCR reaction, and proved the usefulness of this technique for characterization at the strain level. It has later been used by other authors for typing *Saccharomyces* (Baleiras Couto et al., 1996; Pérez et al., 2001; Howell et al., 2004; Schuller et al., 2004; Masneuf-Pomarède et al., 2007), non-*Saccharomyces* (Capece et al., 2003), *Brettanomyces* (Miot-Sertier and Lonvaud-Funel, 2007), *Hanseniaspora* (Caruso et al., 2002), and *Zygosaccharomyces* (Martorell et al., 2005a) strains.

Delta (δ) sequences are elements which measure the 0.3 kb that flank retrotransposons Ty1 (Cameron et al., 1979). Around 100 δ copies are present in the yeast genome of *S. cerevisiae* as part of retrotransposons Ty1 or as isolated elements. The number and localization of these elements demonstrate certain intraspecific variability, which Ness et al. (1993) took advantage of to develop specific primers (δ_1 and δ_2) that are useful to differentiate strains of *S. cerevisiae*. Later Legras and Karst (2003) optimized the technique by designing two new primers (δ_{12} and δ_{21}) located very near δ_1 and δ_2. The use of either δ_{12} and δ_{21} or δ_{12} with δ_2 revealed a greater polymorphism as reflected by the appearance of more bands. Consequently, new primers were able to differentiate more strains, and 53 commercial strains were unequivocally differentiated (Legras and Karst, 2003). Schuller et al. (2004) confirmed this later by showing that the δ_2 and δ_{12} combination could identify twice as many strains as the set of primers designed by Ness et al. (1993).

Approximately 5% of *S. cerevisiae* genes possess introns. These introns are spliced from pre-mRNA to form functional mature mRNAs during a process that requires the spliceosome, a large ribonucleoprotein complex. A conserved sequence is present in the intron structure for spliceosome recognition. De Barros Lopes et al. (1996) designed primers based on these conserved sequences, known as intron splice sites (ISS). The use of these primers has enabled the differentiation of a large number of commercial wine strains. ISS primers can also be used with non-*Saccharomyces* strains because ISS are conserved in all the yeasts that have been studied to date. Hierro et al. (2004) used these primers to identify wine strains that belong to 15 different species. This technique has also been applied to genotype *B. bruxellensis* strains (Oelofse et al., 2009; Vigentini et al., 2012).

Amplified Fragment Length Polymorphism (AFLP) is a technique that involves the restriction of genomic DNA, followed by binding adapters to the obtained fragments and their selective PCR amplification. The adapter sequence and restriction sites are used as target primers for PCR amplification. Amplified fragments are separated in polyacrylamide gels and different genotypes display an exclusive banding pattern (Vos et al., 1995). AFLP is a useful technique for discriminating between wine yeasts at the strain level, as shown by de Barros Lopes et al. (1999) and other authors (Azumi and Goto-Yamamoto, 2001; Boekhout et al., 2001; Lopandic et al., 2007). However, its drawback is that it is very laborious, requires automatic sequencers, highly sophisticated for the wine industry, and the results obtained are also difficult to interpret. To overcome this drawback, Esteve-Zarzoso et al. (2010) developed a simplified AFLP method that allowed gel electrophoresis analyses and considerably reduced equipment requirements. Another remarkable improvement was to use non-labeled primers that reduces analysis costs. This simplified method was applied to the reference strains and colonies isolated from the spontaneous fermentation of species *H. uvarum*, *H. vinae*, *C. zemplinina*, and *S. cerevisiae*. Recently, this technique has been used also to characterize genetic variability within the *H. uvarum* species (Albertin et al., 2015).

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CRISPR/Cas9 System as a Valuable Genome Editing Tool for Wine Yeasts with Application to Decrease Urea Production

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An extensive repertoire of molecular tools is available for genetic analysis in laboratory strains of *S. cerevisiae*. Although this has widely contributed to the interpretation of gene functionality within haploid laboratory isolates, the genetics of metabolism in commercially-relevant polyploid yeast strains is still poorly understood. Genetic engineering in industrial yeasts is undergoing major changes due to Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR-associated protein (Cas) engineering approaches. Here we apply the CRISPR/Cas9 system to two commercial "starter" strains of *S. cerevisiae* (EC1118, AWRI796), eliminating the *CAN1* arginine permease pathway to generate strains with reduced urea production (18.5 and 35.5% for EC1118 and AWRI796, respectively). In a wine-model environment based on two grape musts obtained from Chardonnay and Cabernet Sauvignon cultivars, both *S. cerevisiae* starter strains and *CAN1* mutants completed the must fermentation in 8–12 days. However, recombinant strains carrying the *can1* mutation failed to produce urea, suggesting that the genetic modification successfully impaired the arginine metabolism. In conclusion, the reduction of urea production in a wine-model environment confirms that the CRISPR/Cas9 system has been successfully established in *S. cerevisiae* wine yeasts.

Keywords: CRISPR/Cas9 system, saccharomyces cerevisiae, wine, arginine degradation pathway, urea, ethyl carbamate

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INTRODUCTION

While for laboratory strains of *Saccharomyces cerevisiae* several molecular methods have allowed extensive interpretation of gene functionality, industrial and wild yeast strains are still poorly studied; indeed, the genetic manipulation of latter yeasts can be time consuming because of they are usually recalcitrant to some molecular techniques and they are characterized by complex genomes (i.e., diploid and polyploid species). For this reason, the development of a rapid and efficient gene-targeting system based on the type II bacterial Clustered Regularly Interspaced Short Palindromic Repeats and CRISPR associated protein (CRISPR-Cas9) system is gaining attention in several industrial fields. Taking advantage of the high efficiency of homologous recombination (HR)

in yeast, this system allows for double strand breaks and simultaneous gene editing of all copies of the target sequence (Gratz et al., 2013).

The CRISPR/Cas system, first discovered in *Escherichia coli*, is present in many eubacteria and archaea where it can provide resistance to bacteriophage or conjugative plasmids (Barrangou et al., 2007; Hryhorowicz et al., 2017). Foreign invading genetic material that is incorporated between CRISPR repeat sequences is transcribed and processed into CRISPR RNAs (crRNAs) that correspond to both foreign and CRISPR repeat DNA. The crRNAs hybridize with transactivating CRISPR RNAs (tracrRNAs) and the resulting crRNA/tracrRNA complex acts as a guide for the endonuclease Cas9, which cleaves invading nucleic acid sequences (Brouns, 2012; DiCarlo et al., 2013).

The main elements of the CRISPR/Cas9 system we used are a bacterial CRISPR-associated protein nuclease (Cas9), from *Streptococcus pyogenes*, and a short RNA guide. This latter element combines with Cas9 to target a specific DNA *locus* composed by 20 nucleotides and a NGG sequence, called protospacer adjacent motif (PAM), where the cleavage occurs in the nuclease domains RuvC and HNH (Mahfouz et al., 2014). The gRNA-Cas9 complex generates DSBs immediately before the PAM site on the target DNA (Ryan and Cate, 2014). Finally, the DSBs in the chromosomal DNA are repaired with knockouts/deletions or knock-ins/insertion by NHEJ (non-homologous end joining) and HR (homologous recombination) (Gratz et al., 2013).

Aside from the molecular advantage of producing quick genome changes by using a unique gene- editing approach, the CRISPR/Cas9 system has the potential to soon become the gold standard technique for the production of novel microorganisms suitable for the food industry. The system produces marker-free mutants and thus limits the environmental risk of using genetically modified microorganisms. Indeed, the system has been applied in many eukaryotic organisms (Komor et al., 2017) such as mammalian cell lines (Lee et al., 2015), insects (Gratz et al., 2013), and yeasts (DiCarlo et al., 2013; Ryan and Cate, 2014; Jakočiunas et al., 2015). It has also been applied in plants where genetic modifications introduced by genome editing can be indistinguishable from those introduced by conventional breeding, such that the plants might be classified different from traditional GMO (genetically modified organism) with environmental risk equivalent to that of conventionally-bred organisms (Bortesi and Fischer, 2015). Winemakers might also benefit application of this new approach to grapes and to yeasts, enabling better understanding of the connections between wine features and wine yeast genetics.

In wine, urea is a major precursor of ethyl carbamate (EC), the ethyl ester of carbamic acid (Weber and Sharypov, 2009). Urea is the metabolic intermediate in the arginine degradation pathway in *S. cerevisiae*, and accumulation of urea in wines generates EC via a reaction between ethanol and the carbamyl group of carbamic acid during wine storage. EC is found in fermented foods such as grape wine, sake, distillated spirits, bread, kimchi, yogurt (Lee, 2013). Stevens and Ough (1993) studied EC formation under different storage condition; the EC is usually found in significant amounts (0.01–0.025 mg/L) in wine and it increases dramatically at high temperature with a logarithmic increase when urea concentration decreases (Xue et al., 2015). EC is a carcinogenic compound in a number of mammalian species and it has been classified in March 2007 by the International Agency for Research on Cancer (IARC) in group 2A (probably carcinogenic to humans) from group 2B (possibly carcinogenic) (Lee, 2013). Several countries have limitations of the amount of EC in fermented food; for example, in Europe the determination of EC in foods is under study by EFSA. The determination of EC is difficult because of lack of physicochemical properties; gas chromatography with mass spectrometry and high- performance liquid chromatography with MS or FLD are methods for EC determination (Lu et al., 2015). Two methods have been developed for reducing EC levels in food; one is based on the monitoring of all steps of the production chain starting from the nitrogen fertilization of vineyards and the other one is based on the use of controlled temperature during storage (Weber and Sharypov, 2009). However, these two strategies are costly and often unworkable for small-scale wine producers.

To the best of our knowledge, there are no studies available that describe the use of the CRISPR/Cas9 approach in the wine field. In this study, we adopt a strategy to modify wine yeasts with the purpose of testing the robustness of this new molecular tool and offering a new engineering pipeline for further gene editing in specific metabolic pathways relevant for wine production. In this study, two commercial *S. cerevisiae* strains have been genetically engineered to eliminate the arginine permease encoded by *CAN1*, leading to strains with reduced urea production in laboratory and wine environments.

MATERIALS AND METHODS

Yeast Strains and Maintenance

Escherichia coli TOP10 served as plasmid host (Invitrogen, CA, USA). For plasmid-selective growth, the TOP10 strain was grown on LB [1% (w/v) Tryptone, 0.5% (w/v) Yeast Extract, 1% (w/v) NaCl] and 100 μg/L ampicillin. For solid media 2% (w/v) agar was included. Yeasts used in this work are two commercial wine strains of *S. cerevisiae*: AWRI796 (Maurivin, South Africa) and Lalvin EC1118 (Lallemand Inc, France). Cells were stored in YPD medium (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose, 5.5 pH) supplemented with 20% (v/v) glycerol at −80°C. Yeast pre-cultures were produced by inoculating glycerol stocks at 1% (v/v) in YPD broth at 30°C for 3 days.

Drug Sensitivity Test by Spot Tests

Saccharomyces cerevisiae (AWRI796, EC1118) wine yeast strains were tested for their sensitivity to Geneticin (G418), Nourseothricin (Nat), Hygromycin B (Hyg), which is often used for the selection of transformed cells and Canavanine (Can), which is used, e.g., to select against diploid cells in the Synthetic Genetic Array method (Tong and Boone, 2006). Since possible interaction between drug and nitrogen source contained in a medium can occur (Cheng et al., 2000), the capability of strains to growth under the presence of G418, Nat, Hyg and Can was tested in two types of media both based on YNB without amino acids and ammonium sulfate (Sigma Aldrich,

Germany) and 2% (w/v) glucose: (i) YNBA, contained 5 g/L of ammonium sulfate, and (ii) YNBG was added with 1 g/L of L-glutamic, as nitrogen sources. All drugs were prepared as stock solution in distilled water, sterilized by filtration on 0.22 μm filters and added to liquid or solid media after their sterilization in autoclave. Yeast pre-cultures were obtained in each medium after incubation in aerobic condition at 30°C for 3–5 days. After OD_{600nm} determination, yeast cultures were diluted to 0.1 OD_{600nm} in sterile water. Then, 1 mL of culture was centrifuged (10,000 g, 5 min) and pellet was washed once in 1 mL of sterile water. Five μL of cells were spotted on Petri dishes containing the corresponding solid media with 2% (w/v) agar and supplemented with different concentration of drug. In particular, cell sensitivity to antibiotics was assayed with: (i) G418 at 0.2, 0.4, 0.6, 0.8 g/mL, (ii) Nat at 0.1, 0.2, 0.3 g/mL, (iii) Hyg at 0.2, 0.4, 0.6, 0.8 g/mL, and (iv) Can at 0.1, 0.2, 0.4, 0.6 g/mL. Cellular growth was detected after 7 day at 30°C. Yeast growth in absence of any drug concentration was used as positive control. Spot tests to determine the drug sensitivity were carried out starting from two independent yeast cultures and in duplicate. The full capability of the investigated strain to grow under the tested condition was expressed by the sign "+"; the symbol "−" was assigned when no isolated colonies were detectable; "±" indicated that a slight inhibition cell growth was observed for single isolated colonies; the sign "−" meant that cells are sensitive to the tested concentration of drug.

DNA Manipulation

Plasmid DNA was prepared from *E. coli* (Sambrook et al., 1989). All restriction reactions were carried out according to manufacturer's instruction (New England Biolabs, MA, USA). PCRs were performed in a 25 μL reaction mixture composed of Phusion 2x master mix (Invitrogen, Carlsbad, CA, USA), 10 μM of forward and reverse primers and 80–100 ng DNA. The amplification cycle was an initial denaturation at 95°C for 5 min, 45 s at the annealing temperature (3°C lower than melting temperature) and 1.5 min at 72°C for the extension. Final extension took 10 min at 72°C. Amplicons were separated on 1% (w/v) agarose gel prepared in TBE buffer (0.09 M Tris, 0.09 M Boric acid, 2 mM EDTA) with 0.05 μg/L ethidium bromide and bands were UV visualized (Geldoc 1000 System, Bio-Rad Laboratories, California). Bands were extracted from gel, eluted in 50 μL of mQ water using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany), and quantified by Qubit® dsDNA BR (Broad-Range) Assay Kits (Invitrogen). All ligation reactions were performed using Rapid DNA Ligation Kit (Thermo Fisher Scientific, MA, USA) according to operating instructions.

Yeast Transformations

The wine *S. cerevisiae* strains (AWRI796, EC1118) were subjected to two sequential transformations. Each transformation was completed in duplicate. For the first transformation, cells were treated with a lithium/acetate protocol according to the procedure described by Hill et al. (1991) and using 3 μg of transforming DNA. Recombinants were verified both by growth assay and PCR-based detection of the kanMX6 cassette. To measure growth, the wild type yeasts and three transformed clones of each *S. cerevisiae* strain were grown in duplicate in YNBG medium supplemented with diff erent concentrations of G418 (0, 200, 400, 800, 1,000, 1,200, 1,400, 1,800, and 2,000 μg/mL) using a Tecan Genios plate-reading spectrophotometer (Tecan, Germany). Specifically, fresh cell cultures in YPD medium (aerobic condition, 30°C, 24 h) were used to inoculate 100 μL of YNBG medium at 0.1 OD 600 nm in a 96-well plate. Cellular growth was monitored at 595 nm every 15 min for a period of 24 h. For the PCR confirmation, DNA was extracted by colony PCR protocol consisting in a treatment of 5 μL of one full size colony dissolved in 200 μL ddH_2O with 20 μL of Zymolase lysis buffer [1 μL of 5 U/μL Zymolase (Zymo Research, CA, USA) + 99 μL phosphate buffer] at 37°C for 2/3 h. After a step at 95°C for 15 min and centrifugation at 2,000 rpm for 7 min (Hettich Zentrifugen, Mikro 200), 3–5 μL of supernatant were used for the amplification with primers GMX6_F and GMX6_R (**Table 1**).

The resulting G418-resistant transformed cells were exposed to a second transformation by electroporation following the method reported by DiCarlo et al. (2013) with few modifications. Briefly, cells were grown in YNBG liquid medium supplemented with 200 μg/mL G418 at 30°C up to stationary phase. Thus, 50 μL of pre-culture were inoculated in 100 mL of the same above medium and grown overnight. Cells have been collected by centrifugation (18,000 g for 10 min) between 0.7 and 1.5 OD_{600nm} and re-suspended in 25 mL of lithium/acetate buffer (0.1 M lithium acetate, 10 mM DDT, 10 mM Tris-HCl pH 7.5, 1 mM EDTA pH 8) at room temperature for 1 h. Yeast cells were then washed twice in 25 mL of cold ddH_2O and once in 10 mL of 1 M cold sorbitol. After that, cells were pelleted 10,000 g for 15 min at 4°C and re-suspended in 100 μL of 1 M cold sorbitol. Each transformation treatment required 40 μL of competent cells and 10 μL of DNA containing 200 ng of vector expressing gRNA corresponding to the *CAN1* gene and the Nat resistance cassette, and 2 μg of donor dsDNA (**Table 1**). The transforming mix was kept on ice for 5 min before electroporation at 2.5 kV, 25 μF, 200Ω in 0.2 cm cuvettes (BioRad Micropulser, BioRad, CA, USA). One mL of 1 M cold sorbitol and YPD medium (1:1 ratio) was added immediately after the current application and the cell suspension was incubated at 30°C for 3–6 h in static condition. Recombinant clones were isolated first on selective plates of YNBG with 200 μg/mL G418 and 50 μg/mL Nat. Subsequently, cells were replicated on YNBG plus 100 μg/mL Can and transformants were verified by: (i) targeting the Nat cassette using the colony PCR protocol with Nat_F/Nat_R couple of primers (**Table 1**); (ii) amplifying (CAN1_F/CAN1_R primers) and sequencing the *CAN1* gene by an external provider (TCAG, Toronto, CA).

The transformation efficiency was calculated as the number of transformants generated per μg of supercoiled plasmid DNA (Hayama et al., 2002). All data are calculated by applying the algebraic average between the calculated transformation efficiencies of each independent treatment. The mutation efficiency was calculated as reported by Jakočiunas et al. (2015) by picking 5 clones resistant to canavanine and submitting them to Sanger sequencing, with the primer pair CAN1_F/CAN1_R, to confirm the mutation in the expected position of the target gene.

TABLE 1 | Materials used in the present study.

Material	Description	References
STRAINS		
E. coli TOP10	F$^-$, *mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(ara-leu)7697 galU galK rpsL (StrR) endA1 nupG*	Invitrogen (Carlsbad, CA, USA)
EC1118	*S. cerevisiae* Lalvin EC1118	Lallemand Inc, France
AWRI 796	*S. cerevisiae* AWRI 796 (Australian Wine Research Institute)	Maurivin, South Africa
ScEC1118*can1*	*S. cerevisiae* EC1118 Gly70stop *CAN1* (-GGC- → -TAG-)	This study
ScAWRI796*can1*	*S. cerevisiae* AWRI796 Gly70stop *CAN1* (-GGC- → -TAG-)	This study
VECTORS		
p414-TEF1p-Cas9-CYC1t	CEN6/ARSH4 origin, *TRP1*, TEF1p promoter, codon optimized Cas9 with C-terminal SV40 tag, Amp^R	(DiCarlo et al., 2013); Addgene, USA
p426-SNR52p-gRNA.CAN1.Y-SUP4t	2μm ori, *URA3,* SNR52 promoter, gRNA CAN1.Y expression cassette, SUP terminator, Amp^R	(DiCarlo et al., 2013); Addgene, USA
p414-G418-TEF1p-Cas9-CYC1t	CEN6/ARSH4 origin, *kan*MX6 cassette, TEF1p promoter, codon optimized Cas9 with C-terminal SV40 tag, Amp^R	This study
p426-Nat-SNR52p-gRNA.*CAN1*.Y-SUP4t	2μm ori, *nat*MX6 cassette, SNR52 promoter, gRNA CAN1.Y expression cassette, SUP terminator, Amp^R	This study
pFA6a	Extraction of the *kan*MX6 espression cassette (promoter and terminator TEF1)	Bahler et al., 1998
P4339	Extraction of the natMX espression cassette (promoter and terminator TEF1)	Tong and Boone, 2006
PRIMERS		
Nat_F	CGGCCGACATGGAGGCCCAGAATA ($T_m = 78.4°C$)	This study
Nat_R	CATATGCAGTATAGCGACCAGCATT ($T_m = 65.7°C$)	This study
GMX6_F	GGTACCCGACATGGAGGCCCAGAAT ($T_m = 75.7°C$)	This study
GMX6_R	TACGTACAGTATAGCGACCAGCATT ($T_m = 59.7°C$)	This study
CAN1_F	GACAAATTCAAAAGAAGACGCCGA($T_m = 66°C$)	This study
CAN1_R	AAATATGATATAAGAGCGCCCACTG ($T_m = 62°C$)	This study
gRN_F	TGTAGTGCCCTCTTGGGCTA	This study
gRNA_R	TCGAGCGTCCCAAAACCTTC	This study
CAN1.can1.Y.90.NCOD	TTCACTTCAGCGTTCTGTACTTCTCCTTCATCTTCATCACCTATCTAATCCTC CATAGAGAACGTATCCTCGCCATTTACTCTCGTCGGG	DiCarlo et al., 2013
CAN1.can1.Y.90.COD	CCCGACGAGAGTAAATGGCGAGGATACGTTCTCTATGGAGGATTAGATAGG TGATGAAGATGAAGGAGAAGTACAGAACGCTGAAGTGAA	DiCarlo et al., 2013

Yeast Fermentations

Fermentation trials were carried out in synthetic and natural grape musts. The composition of synthetic must grape was obtained from the OIV protocol (Directive 22/06/2012, Appendix I) with few modifications: 1.7 g/L YNB without amino acids and ammonium sulfate (Sigma-Aldrich, Germany), 115 g/L Glucose, 115 g/L Fructose, 5 g/L Tartaric acid, 3 g/L Malic acid, 0.2 g/L Citric acid, 2 g/L L-Arginine, pH 3.5. Two grape musts produced in the Franciacorta area (Brescia, Italy) in vintage 2016 were used in this study: a red grape must of Cabernet Sauvignon and a white grape must of Chardonnay. Sugar composition of grape musts was: Cabernet Sauvignon 123.8 g/L Glucose, 123.2 g/L Fructose and for Chardonnay 93 g/L Glucose, 99 g/L Fructose. The APA content of Cabernet Sauvignon was 47.35 mgN/L while Chardonnay contained 250 mg/L. To obtain a final content of APA as 250 mg/L, the Cabernet Sauvignon grape must was corrected with a 10 g/L Supervit solution (Enartis SC, Novara) containing ammonium sulfate, ammonium phosphate and thiamine. Two g/L of arginine were added before the cell inoculation to each grape must (Amerine and Ough, 1980).

Both synthetic and natural grape musts were used to carry out fermentation in flasks. Each strain was separately grown in YPD broth in aerobic condition at 25°C, overnight and then it was inoculated in the synthetic grape must and in the grape musts to obtain an initial cell concentration of about 2×10^6 UFC/mL. Fermentations were performed in triplicate in 250 mL glass flasks containing 200 mL of medium. Fermentations in laboratory conditions were performed in aerobic conditions at 25°C while the ones in oenological conditions were run at 20 ± 2°C. In order to establish a limiting oxygen condition as happens in natural vinifications, flasks with grape must were capped with Muller's valves containing 12% (v/v) sulphuric acid. This enables escape of carbon dioxide and avoids oxygenation of the musts. While in synthetic must, yeast cell growth was monitored by OD at 600 nm. In natural grape musts it was also determined by CO_2 loss through reduction of glass flask weight. At the end of the alcoholic fermentation, when no weight variation is detected for three consecutive days, wines were centrifuged at 18000 g for 5 min and supernatants were maintained at −20°C for further chemical analyses.

Chemical Determinations and Statistical Analysis

The content of L-arginine/urea/ammonia, ethanol and sugars (glucose/fructose) was determined by enzymatic kits (Megazyme, Ireland) following manufacturer instructions. All data are expressed by means of tree replications and standard deviation (±SD). Nitrogen content in natural grape musts was assayed by formol titration (Fracassetti and Tirelli, 2015). Urea yield values were subjected to the one-way ANOVA in order to infer the effect of strains (not-transformed and mutant yeasts); statistically significant differences between means were defined at p-value < 0.001.

RESULTS

The results presented here show the successful editing of the *CAN1* gene of two *S. cerevisiae* wine yeast strains using a CRISPR/Cas9 system that consists of three elements: two expression vectors carrying the Cas9 gene and the gRNA, respectively, and a donor dsDNA fragment. To select the most suitable selectable markers for plasmid construction, tests were conducted to assess the sensitivity of the yeast strains toward drugs generally used in genetic engineering trials. After transformation, mutant strains were tested for their capability of forming urea in synthetic and natural grape musts. Fermentations in synthetic must, containing arginine as sole nitrogen source, led to the quantification of the urea production in wild type vs. mutant strains in absence of the nitrogen catabolite repression. Whether experiments carried out in grape must with several nitrogen sources allowed to validate the actual contribution of the *CAN1* gene in the urea production in oenological conditions.

Drug Sensitivity of *S. cerevisiae* Wine Strains

Saccharomyces cerevisiae EC1118 and AWRI796 strains had not been previously analyzed for their resistance to common agents commonly used for selectable markers. Therefore, assessment of drug sensitivity was required to choose markers for vector maintenance and recombinant yeast strains. Due to a possible interaction between drug and nitrogen source contained in one medium (Cheng et al., 2000), the capability of strains to growth under the presence of G418, Nat, Hyg, and Can was tested in YNBA and YNBG. All spot tests were run in duplicate. All the tested yeast strains grew on both media without drug supplementation in 3 days at 30°C. After 7 days, differences in the ability of forming colonies were observed among the analyzed yeasts. Results showed that *S. cerevisiae* AWRI796 resulted more sensitive than the EC1118 strain to canavanine (**Table 2**). A higher drug sensitivity was detected when L-glutamic acid, rather than ammonium sulfate, was added to the medium (**Table 2**). In particular, this difference was observed in media with geneticin and hygromycin B. Indeed, in presence of these two drugs both strains grew up to a final concentration of 400 μg/mL when ammonium sulfate was added to the medium, while growth was already inhibited at 200 μg/ml in medium containing L-glutamic acid. Based on these data, all transformations and fermentation trials were carried out in YNBG medium (liquid or solid) and G418 and Nat cassettes were chosen as selectable markers.

Construction of Plasmids

All recombinant strains, plasmids and primer pairs used are listed in **Table 1**. The p414-G418-TEF1p-Cas9-CYC1t and p426-Nat-SNR52p-gRNA.CAN1.Y-SUP4t plasmids were obtained from p426-SNR52p-gRNA.CAN1.Y-SUP4t and p414-TEF1p-Cas9-CYC1t vectors, respectively. In vector p414-TEF1p-Cas9-CYC1t the *TRP1* gene was removed and replaced with the *kan*MX6 cassette (1365 bp), conferring resistance to G418. In the vector p426-SNR52p-gRNA.CAN1.Y-SUP4t the *URA3* gene was substituted with the *nat*MX cassette (1,126 bp) for the resistance to Nat. The cassette conferring Nat resistance was amplified from p4339 plasmid (Tong and Boone, 2006). The strategy used to change the selective markers of the original plasmids was similar for both new vectors. As a first step, *TRP1* cassette was excised from p414-TEF1p-Cas9-CYC1t using *Kpn*I/*Sma*BI enzymes and *URA3* was removed from p426-SNR52p-gRNA.CAN1.Y-SUP4t by digestion with *Eag*I/*Nde*I enzymes. Compatible ends at the 5′ and 3′ extremities of the *kan*MX6 and *nat*MX cassettes were generated by PCR amplification using primers GMX6_F/GMX6_R and Nat_F/Nat_R, respectively. Finally, the linearized plasmids and the corresponding resistance cassettes were ligated in order to generate p414-G418-TEF1p-Cas9-CYC1t (9,311 kb) and p426-Nat-SNR52p-gRNA.CAN1.Y-SUP4t (5,718 bp) plasmids (**Figure 1**).

Transformation Trials

During the first round of transformation, the plasmid p414-G418-TEF1p-Cas9-CYC1t (containing the *kan*MX6 marker for geneticin resistance) was transferred into cells using the lithium/acetate protocol applied to about 10^8 cells per transformation reaction. While a transformation efficiency of about 222 ± 16 transformants per μg of DNA was calculated for *S. cerevisiae* AWRI796, a considerably lower value was obtained for *S. cerevisiae* EC1118 strain that showed a recovery of only 90 ± 6 transformants per μg of DNA. However, this difference was not confirmed by subsequent PCR assay for presence of the *kan*MX6 cassette; indeed, unlike what was observed for *S. cerevisiae* EC1118 where all the analyzed clones produced the expected fragment (1,365 bp), *S. cerevisiae* AWRI796 showed that only one to three clones had been correctly transformed. Finally, for each recombinant strain yielding positive PCR assay for the selectable marker, two isolates were further inoculated in liquid medium to assess G418 resistance. We found the *kan*MX6 cassette to confer resistance to the highest amount of antibiotic tested (2 mg/mL) (**Figure 2**). Interestingly, both clones of *S. cerevisiae* EC1118 showed the same growth fitness in YNBG, unlike *S. cerevisiae* AWRI796 which grew more poorly than wild type (data not shown).

For the second transformation by electroporation, approximately 5×10^8 cells containing the plasmid p414-G418-TEF1p-Cas9-CYC1t were co-transformed with the vector p426-Nat-SNR52p-gRNA.CAN1.Y-SUP4t (containing the *nat*MX marker for nourseothricin resistance) and

the donor dsDNA. Selection occurred in YNBG agar medium supplemented with geneticin and nourseothricin. A transformation efficiency of (5.50 ± 3.25) × 10^3 and (1.00 ± 0.17) × 10^4 transformants for *S. cerevisiae* AWRI796 and EC1118 per μg of DNA was calculated, respectively. In this case, the high transformation efficiency could be linked to the time of cell recovery applied to electroporated cells (3 h at 30°C) before plating. Then, the recombinant cells underwent a second canavanine selection on YPD agar medium. Homologous recombination occurred with efficiency of 32 ± 2 and 22 ± 4 transformants per μg of DNA for *S. cerevisiae* AWRI796 and EC1118, respectively. The PCR amplification of the *nat*MX cassette confirmed the presence of the correct band at 1,126 bp from 10 selected clones per strain. Sequence analysis of the *CAN1* gene in the recombinant strains showed the presence of a stop codon at the expected position with a mutation efficiency of 100% of 5 isolates (**Figure 3**).

TABLE 2 | The first symbol on the left corresponds to the ability of growth in YNBA and the symbol on the right refers to the yeast growth in YNBG medium.

Drug concentration (μg/mL)	G418	Nat	Hyg	Can
0	+/+	+/+	+/+	+/+
50	nd/nd	−/−	nd/nd	nd/nd
100	nd/nd	−/−	nd/nd	AWRI796(−), EC1118 (±)/−
200	+/−	−/−	+/−	−/−
300	nd/nd	−/−	nd/nd	−/−
400	±/−	nd/nd	+/−	nd
600	−/−	nd/nd	−/−	nd
800	−/−	nd/nd	−/−	nd

G418, geneticin; Nat, nourseothricin; Hyg, Hygromycin B; Can, Canavanine. Cellular growth is reported as: (+): full growth with no visible isolated colonies; (−): no cell growth; (±): countable isolated colonies; nd: not determined. Spot tests were repeated twice.

Urea Production from Wild Type and *can1* Mutant Strains

In a chemically defined medium with arginine as sole nitrogen source, recombinant strains carrying the mutation in *CAN1* gene showed a statistically significant decrease in urea yield of 18.5–35.5% for *S. cerevisiae* AWRI796 and EC1118, respectively (**Table 3**). Indeed, a small amount of arginine was not consumed by mutant cells [0.1 g/L for AWRI796*can1* and 0.22 for EC1118*can1*, as compared with the AWRI796 and EC1118 wildtype strains that exhausted the available arginine (2 g/L)]. Interestingly, *S. cerevisiae* AWRI796*can1* showed a decrease in the specific growth rate in comparison to its wild type (0.07 vs. 0.09 h^{-1} μ_{max}) (**Figure 4**).

Must fermentations were carried out inoculating *S. cerevisiae* AWRI796, AWRI796*can1*, EC1118 and EC1118*can1* strains in two grape musts obtained from Chardonnay (white) and Cabernet Sauvignon (red) cultivars. The two wild type strains completed the must fermentation in 8 days in both musts while the two mutants ended their growth at the 12th and at the 9th day in red in white musts, respectively (**Figure 5**). The biomass production ranged from 2.23 to 3.02 g/L as an average for all the tested strains, both wild and transformed; starting from the same amount of sugars as for in synthetic must, the presence

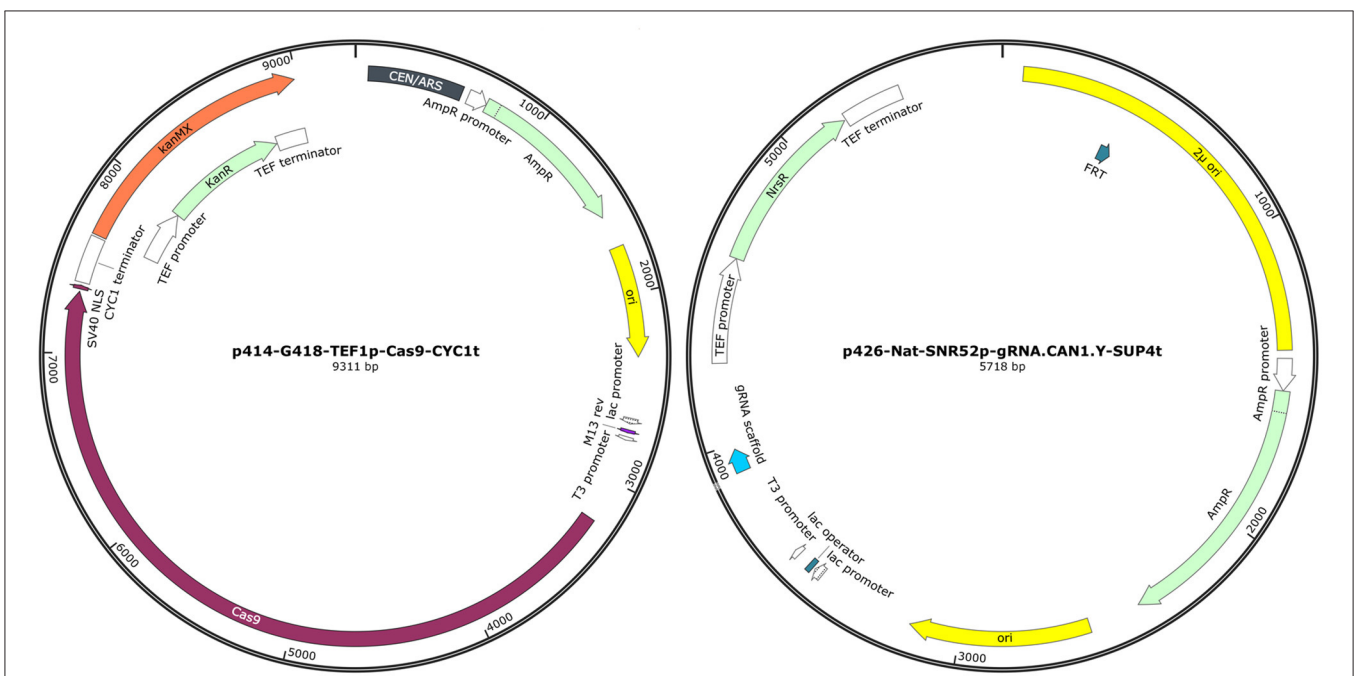

FIGURE 1 | Representation of plasmid p414-G418-TEF1p-Cas9-CYC1t and p426-Nat-SNR52p-gRNA.CAN1.Y-SUP4t (SnapGene® Viewer 3.3.4).

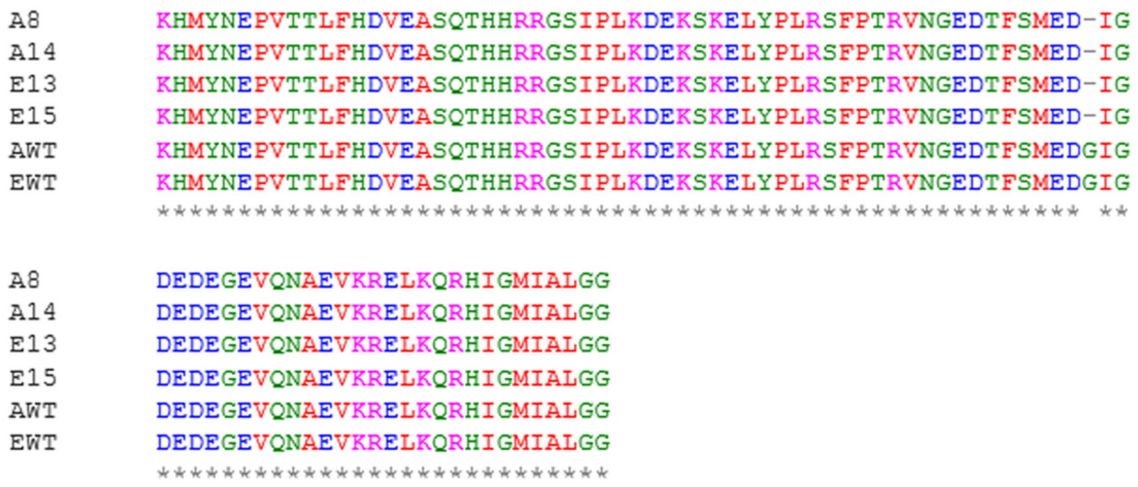

FIGURE 2 | Example of the ClustalΩ multiple sequence alignment. Partial amino acid sequence of *CAN1* genes in wild type and recombinant strains: A8, *S. cerevisiae* AWRI796*can1* clone #8; A14, *S. cerevisiae* AWRI796*can1* clone #14; E13, *S. cerevisiae* EC1118*can1* clone #13; E15: *S. cerevisiae* EC1118*can1* clone #15; AWT, *S. cerevisiae* AWRI796, EWT, *S. cerevisiae* EC1118. A glycine amino amino residue (*G* = ggc) has been replaced by a STOP codon (– = tag) in position 70 from the methionine at the N-terminal of the protein.

of a complete pool of amino acids improved the cellular growth (**Table 3**).

In both musts, although the wild type strain of *S. cerevisiae* AWRI796 and EC1118 showed a better fermentative power in comparison to the mutant strains (total grams of CO_2 produced/volume of fermentation) (**Figure 5**), the ANOVA highlighted that statistically significant differences were not found among strains (**Table 3**). Comparing the two transformed yeasts in terms of oenological traits, *S. cerevisiae* AWRI*can1* and EC1118*can1* were most performant in red rather than white must with a fermentative vigor (g CO_2 produced in 48 h) of about 6.9 and 8.4 g, respectively. Although the mutant strains are able to finish the fermentation in both white and red must, they showed a delay of about 4 days; in terms of sustainability, this behavior should be better investigated if strains are used in a real oenological condition.

Finally, both *can1* mutants failed to produce urea (**Table 3**) and a lower consumption of arginine was detected in fermentations carried out with the *can1* mutant strains in comparison to those with wild types (1.3 vs. 1.7 g/L).

DISCUSSION

Selected yeast starter strains are widely used because they possess very good fermentative and oenological capabilities, contributing to the standardization of fermentation process, wine quality and safety. However, due to their polyploid nature, these strains are still poorly characterized from a genetic point of view. Here we outline a strategy to modify wine yeasts with the CRISPR/Cas9 system, an efficient, cheap and easy-to-use tool for genome editing that allows the simultaneous modification of all the alleles of a target gene. To prove the robustness of the CRISPR/Cas9 system in wine strains of *S. cerevisiae* and to provide a gene-editing pipeline suitable for metabolic pathways relevant in wine production, two commercial strains of *S. cerevisiae* (EC1118, AWRI796) have been genetically engineered in *CAN1* genes to generate strains with a reduced urea production. The *CAN1* gene, which encodes plasma membrane arginine permease, was selected as a model gene for its dual significance: (i) it allows the system validation by a negative selection of the transformed clones using canavanine and (ii) it represents the first enzyme of the arginine degradation pathway that is involved in the production of urea, the main precursor of ethyl carbamate (EC), a toxic compound (Ough et al., 1988).

Urea can be released by wine yeasts as the metabolic intermediate from arginine breakage (Vincenzini et al., 2009). According to this path, arginine is transported into the cell through specific and/or general amino acid permeases (encoded by *CAN1* and *GAP* genes, respectively) and is cleaved by arginase (*CAR1* gene) into ornithine and urea. Urea can then be excreted through Dur4p, a passive urea permease, or transformed by Dur1p/Dur2p, two urea amidolyases, in ammonium and CO_2. Urea can undergo to a spontaneous, non-enzymatic, reaction with ethanol forming EC, which is known to be genotoxic and carcinogenic in a number of mammalian species and which affects several fermented food products. The development of techniques to prevent and/or reduce its content in wine represents an important goal in wine industry. Genetically modified yeasts in the genes of the arginine degradation pathway have been already been obtained for sake and sherry wine production (Coulon et al., 2006; Chiva et al., 2009; Dahabieh et al., 2010; Wu et al., 2014; Zhao et al., 2014). However, no study has investigated the role of *CAN1* gene in the production of urea in any fermentable source yielding human-consumed products.

By exploiting the CRISPR/Cas9 system, in the present work we generated *can1* mutants in *S. cerevisiae* wine strains in order to investigate the urea production in oenological conditions. Prior deciding how to construct useful vectors, yeasts were analyzed

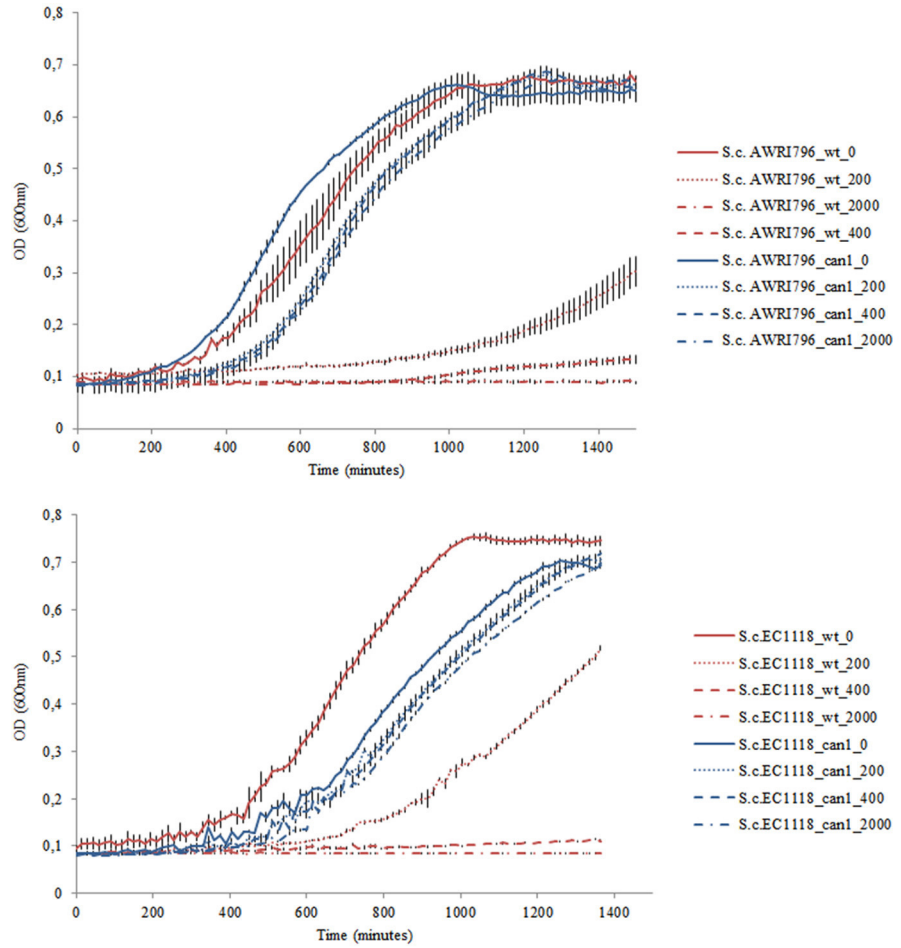

FIGURE 3 | Example of the growth kinetics of *S. cerevisiae* wild type strains (AWRI796 and EC1118) and recombinant clones (clone #23 and clone#8 for AWRI796 and EC1118, respectively) inoculated in YNBG liquid medium in duplicate under different concentrations of G418 (0, 200, 400, and 2,000 μg/mL). Cellular proliferation was monitored for 24 h, 30°C using a Tecan microtitres reader (Tecan, Germany). The optical density was measured at 600 nm every 15 min up to stationary phase. Data are expressed by means of two replicates.

for their sensitivity to drugs commonly used in biotechnological studies. For all the analyzed compounds, we observed different levels of inhibition in the growth due mainly to the nitrogen source present in the media as shown by Cheng et al. (2000) and, only in the case of canavanine in rich medium, on the strain. We also assessed the efficiency of each step in our sequential transformation protocol. Low transformation efficiency was observed for the vector expressing Cas9p. While a value of about 3–5 $\times$ 10^4 transformants/μg of plasmid DNA was expected (Hill et al., 1991), an efficiency of two order of magnitude less was calculated. Two possible hypotheses can be formulated; first, the plasmid size was too large (9,311 bp for p414-G418-TEF1p-Cas9-CYC1t) and reduced DNA uptake and/or Cas9p expression yielded toxicity leading to cell death (Ryan and Cate, 2014). By contrast, the second transformation, mediated by electroporation, yielded an efficiency similar to that reported in literature (Gysler et al., 1990; Pribylova and Sychrova, 2003). Finally, the *CAN1* gene was successfully modified with a 100% mutation frequency for both wine strains as shown by DiCarlo et al. (2013) for a lab strain of *S. cerevisiae*.

The resulting phenotypes of the *can1* mutants were evaluated in a wine-model environment using laboratory and oenological conditions. In a synthetic must, recombinant strains carrying the mutation in *CAN1* gene show a decrease in urea production between 18.5 and 36.5%. This result can be due to presence of the *GAP1* gene, the general acid permease gene that transports arginine into the cell (Chiva et al., 2009). In fact, the *GAP1* deletion could further reduce urea production (Wu et al., 2014) but it might also further reduce specific growth rate due to a reduced intake of arginine and other amino acids into the cells. In this study, possibly because of the metabolic modification of *CAN1* gene, a variation of the specific growth rate was observed in *S. cerevisiae* AWRI796*can1* in comparison to its wild type (0.07 vs. 0.09 h^{-1} μ_{max}). Can1p inactivation may have an effect on the specific growth rate due to a reduced arginine uptake; however, further physiological

TABLE 3 | Urea yield and percentage of urea reduction in wild type and recombinant strains.

Fermentation type	AWRI796	AWRI796*can1*	EC1118	EC1118*can1*
Synthetic must				
Urea (g/L)	0.190 ± 0.008	0.150 ± 0.015	0.120 ± 0.005	0.080 ± 0.004
Biomass (g/L)	2.30 ± 0.20	2.35 ± 0.05	1.92 ± 0.18	2.10 ± 0.25
Urea yield	0.081[a]	0.066[b]	0.062[b]	0.040[c]
Red must				
Fermentative power (gCO2/250 mL)	22.1 ± 0.7	20.6 ± 0.6	22.0 ± 0.9	20.4 ± 0.3
Urea (g/L)	0.05 ± 0.002	n.d.	0.04 ± 0.002	n.d.
Biomass (g/L)	3.02 ± 0.08	2.23 ± 0.13	2.52 ± 0.075	2.38 ± 0.19
Urea yield	0.017[a]	0[b]	0.016[a]	0[b]
White must				
Fermentative power (gCO2/250 mL)	17.1 ± 0.5	16.1 ± 0.5	17.5 ± 0.5	17.0 ± 0.5
Urea (g/L)	0.020 ± 0.001	n.d.	0.01 ± 0.001	n.d.
Biomass (g/L)	2.55 ± 0.07	2.38 ± 0.13	2.50 ± 0.08	2.52 ± 0.02
Urea yield	0.008[a]	0[b]	0.004[a]	0[b]

Yields were calculated at the maximum level of biomass (g/L). The standard error of enzymatic assays is calculated at 3%. The entry n.d = not detected (<0.13 mg/L, according to the detection limit of the enzymatic kit). The ANOVA was applied to urea yields calculated from fermentations performed in synthetic must and natural musts (red and white), respectively. Mean values, on the same line, showing statistically significant differences (p-value<0.001) are superscripted with different letters.

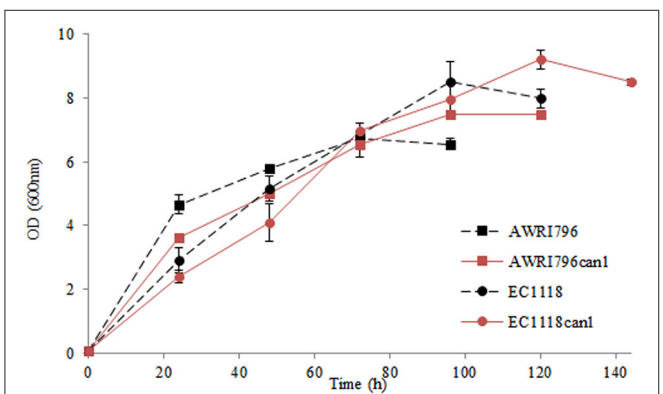

FIGURE 4 | Kinetics of growth of wild types and recombinant strains in synthetic must. Data are expressed by means of three replicates and standard deviation (±SD).

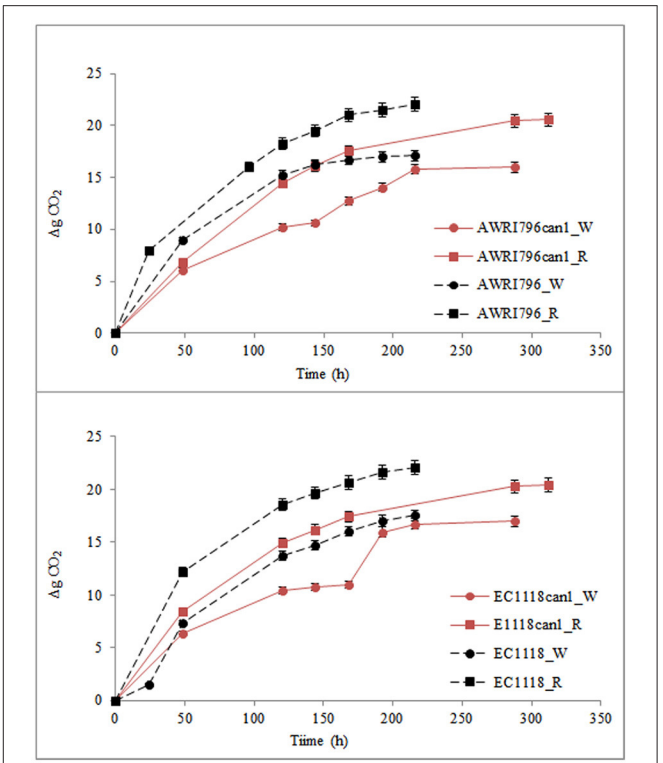

FIGURE 5 | Production of CO_2 by *S. cerevisiae* wild type (AWRI796 and EC1118) and recombinant strains (AWRI796*can1* and EC1118*can1*) in oenological conditions. R, Cabernet Sauvignon must; W, Chardonnay must. Data are expressed by means of three replicates and standard deviation (±SD).

experiments should be carried out to verify this metabolic behavior.

Since arginine is the most common organic nitrogenous compound in grape juice, the growth rate and the biomass production in *can1* mutant strains could be more affected than the wild type. In a wine-model environment, consisting of two grape musts obtained from Chardonnay and Cabernet Sauvignon cultivars, all the analyzed yeasts completed the must fermentation. The most important result is that recombinant strains, carrying only a mutation in the *CAN1* gene, show a complete reduction of urea in both musts. Of course, this achievement has yet to be confirmed in other musts and under actual winemaking conditions. Yeast can sense the numerous available nitrogen sources in a medium and "tune" nitrogen catabolite repression toward optimal utilization of nitrogen. In a grape must, the presence of ammonia, a yeast could down-regulate a pathway necessary to import arginine or other amino acids. Therefore, rather than changing other enzymes of the arginine degradation pathway with possible consequences on the yeast fitness, the sole mutation in *CAN1* could be enough to reduce the urea production.

In conclusion, this study demonstrates that CRISPR/Cas9 system can be successfully established in *S. cerevisiae* wine yeasts, and the editing of the *CAN1* gene thereby yields a reduction of urea production.

AUTHOR CONTRIBUTIONS

IV contributed to the design of the work, to the construction of plasmids and to perform the spot tests for drug sensitivity. IV contributed also contributed to the acquisition, the analysis, and the interpretation of data for the work, to draft the work and revising it and ensured that questions related to the accuracy or integrity of any part of the work were appropriately

investigated and resolved; MG contributed to the design of the work and the implementation of the CRISPR/Cas9 system, to the interpretation of data for the work, to draft the work and revising it; AB contributed to the fermentation growth of yeast strains; RF contributed to the interpretation of data for the work and to draft the work; FR contributed to draft the work and revising it for important intellectual content and to ensure that questions related to the accuracy or integrity of any part of the work were appropriately investigated and resolved.

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The Impact of *Saccharomyces cerevisiae* on a Wine Yeast Consortium in Natural and Inoculated Fermentations

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Natural, also referred to as spontaneous wine fermentations, are carried out by the native microbiota of the grape juice, without inoculation of selected, industrially produced yeast or bacterial strains. Such fermentations are commonly initiated by non-*Saccharomyces* yeast species that numerically dominate the must. Community composition and numerical dominance of species vary significantly between individual musts, but *Saccharomyces cerevisiae* will in most cases dominate the late stages of the fermentation and complete the process. Nevertheless, non-*Saccharomyces* species contribute significantly, positively or negatively, to the character and quality of the final product. The contribution is species and strain dependent and will depend on each species or strain's absolute and relative contribution to total metabolically active biomass, and will therefore, be a function of its relative fitness within the microbial ecosystem. However, the population dynamics of multispecies fermentations are not well understood. Consequently, the oenological potential of the microbiome in any given grape must, can currently not be evaluated or predicted. To better characterize the rules that govern the complex wine microbial ecosystem, a model yeast consortium comprising eight species commonly encountered in South African grape musts and an ARISA based method to monitor their dynamics were developed and validated. The dynamics of these species were evaluated in synthetic must in the presence or absence of *S. cerevisiae* using direct viable counts and ARISA. The data show that *S. cerevisiae* specifically suppresses certain species while appearing to favor the persistence of other species. Growth dynamics in Chenin blanc grape must fermentation was monitored only through viable counts. The interactions observed in the synthetic must, were upheld in the natural must fermentations, suggesting the broad applicability of the observed ecosystem dynamics. Importantly, the presence of indigenous yeast populations did not appear to affect the broad interaction patterns between the consortium species. The data show that the wine ecosystem is characterized by both mutually supportive and inhibitory species. The current study presents a first step in the development of a model to predict the oenological potential of any given wine mycobiome.

Keywords: yeast consortium, population dynamics, yeast interactions, wine fermentation, ARISA

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INTRODUCTION

The alcoholic fermentation of grape must, whether inoculated or not with commercial starter cultures, is initiated by a complex yeast community comprising a high proportion of oxidative and weakly fermentative yeasts (Jolly et al., 2003a; Ghosh et al., 2015; Wang et al., 2015). These species are rapidly outgrown by strongly fermentative yeasts that dominate the middle and end of fermentation (Pretorius et al., 1999; Jolly et al., 2003b; Zott et al., 2008; Bagheri et al., 2015; Ghosh et al., 2015; Setati et al., 2015; Wang et al., 2015; Morgan, 2016; Portillo et al., 2016; Tristezza et al., 2016). The growth and metabolic activity of these yeast species are influenced by physicochemical conditions that prevail during the fermentation process including the rapid depletion of nutrients and oxygen and the accumulation of ethanol (Sainz et al., 2003; Mendoza et al., 2009). However, beyond such environmental or chemical factors, ecological interactions between yeast species will primarily determine the wine fermentation dynamics and the outcome of the fermentation process (Nissen and Arneborg, 2003; Pina et al., 2004; Sadoudi et al., 2012; Renault et al., 2013; Morales et al., 2015; Wang et al., 2015; Shekhawat et al., 2017). For many years, research evaluated interactions between strains of *S. cerevisiae*, the main wine fermenting yeast, with a focus on killer toxin-producing strains (Branco et al., 2014; Williams et al., 2015; Albergaria and Arneborg, 2016; Pérez-Torrado et al., 2017). However, with the growing interest in non-*Saccharomyces* yeast species and the commercialization of a few species for use as co-inoculants in controlled mixed starter fermentations, attention has turned toward evaluating yeast–yeast interactions holistically (Ciani and Comitini, 2015; Albergaria and Arneborg, 2016; Ciani et al., 2016; Wang et al., 2016). Undoubtedly, wine microbial consortia are difficult to scrutinize. Consequently, some studies have employed simplified models in which the interaction between two species mainly *S. cerevisiae* and non-*Saccharomyces* species were investigated (Andorra et al., 2011; Wang et al., 2014; Englezos et al., 2015; Shekhawat et al., 2017). Several aspects, including inoculum ratio, the timing of inoculation of *S. cerevisiae*, cell-cell contact and production of inhibitory metabolites, have been investigated in order to decipher the mechanisms underlying yeast–yeast interactions during wine fermentation (Gobbi et al., 2013; Branco et al., 2014, 2015; Izquierdo Cañas et al., 2014; Kemsawad et al., 2015; Lencioni et al., 2016). Despite these efforts, the overall interactions among wine yeast species in a fermentation modulated by multiple species remain unclear.

Synthetic microbial consortia composed of a subset of culturable strains that simulate the natural community and preserve the indigenous interactions shaped by co-adaptation/evolution, provide a tractable model system with reduced complexity (De Roy et al., 2014; Ponomarova and Patil, 2015), which makes it easier to study interspecific interactions (Jagmann and Philipp, 2014; Jiang et al., 2017). Such a model system also opens opportunities to employ methods inapplicable to complex systems, e.g., species quantitation can easily be done with selective plating, quantitative PCR, fluorescent *in situ* hybridization (FISH), and flow cytometry (Xufre et al., 2006; Grube and Berg, 2009; Zott et al., 2010; Ponomarova and Patil, 2015). These methods have been applied successfully to monitor population dynamics in wine fermentation. However, they are not without limitations. For instance, FISH and qPCR, require species-specific probes and primers whereas, flow cytometry requires prior knowledge of initial microbial population in order to label different species (Deere et al., 1998; Malacrinò et al., 2001; Prakitchaiwattana et al., 2004; Hierro et al., 2006a; Xufre et al., 2006; Andorrà et al., 2010a,b; Zott et al., 2010). In contrast, Automated Ribosomal Intergenic Spacer Analysis (ARISA), which mainly relies on the heterogeneity of the ITS1-5.8S rRNA-ITS2 gene, has been used successfully in several ecological studies (Brežná et al., 2010; Kraková et al., 2012; Ghosh et al., 2015). Like other methods, ARISA may also introduce bias since it is unable to differentiate live and dead cells. However, ARISA is an efficient and rapid tool that can provide a snapshot of the population dynamics (Hierro et al., 2006a; Ramette, 2009; Brežná et al., 2010; Kraková et al., 2012; O'Sullivan et al., 2013; Cangelosi and Meschke, 2014; Ženišová et al., 2014; Ghosh et al., 2015).

The current study aimed to evaluate the application of a multi-species yeast consortium as a tool to investigate population dynamics and yeast–yeast interactions in wine fermentation. The constructed model consortium resembles natural wine yeast consortia in so far as comprising species with different fermentative capacities (i.e., weakly fermentative, medium fermentation capacity and strongly fermentative). Moreover, the consortium was formulated based on species that have been encountered and found in sometimes dominant numbers in grape musts from different South African wine regions and cultivars (Jolly et al., 2003a; Weightman, 2014; Bagheri et al., 2015; Ghosh et al., 2015; Morgan, 2016). The model consortium was evaluated in synthetic must in the presence and absence of *S. cerevisiae*, as well as in a real grape juice that differed significantly from the synthetic must. To allow for a rapid and accurate monitoring of the population dynamics, ARISA was optimized and assessed for its suitability and reliability as a tool to semi-quantitatively monitor yeast dynamics in the model consortium.

The data show that *S. cerevisiae* strongly and specifically suppresses certain non-*Saccharomyces* yeast species, while also favoring the persistence of other species. The findings suggest that the complex modulation of the yeast ecosystem by *S. cerevisiae* will influence the outcome of wine fermentation by selectively changing the contribution of non-*Saccharomyces* species.

MATERIALS AND METHODS

Yeast Strains and Culture Conditions

Sixteen yeast isolates obtained from the culture collection of the Institute for Wine Biotechnology (IWBT) and two commercial yeast species, *S. cerevisiae* Lalvin EC1118 (Lallemand, Canada) and *Torulaspora delbrueckii* BIODIVA (Lallemand, Canada) were used in this study (**Table 1**). The yeast stock cultures were maintained in 20% (v/v) glycerol at −80°C and were streaked out on Wallerstein Laboratory Nutrient agar (WLN) (Sigma–Aldrich,

TABLE 1 | Strains used in this study and their ITS1-5.8S rRNA-ITS2 gene sizes.

Species	Strains number	ITS Size (bp)
Hanseniaspora uvarum (Hu)	Y1104	747
Hanseniaspora vineae (Hv)	Y980	740
Hanseniaspora opuntiae (Ho)	Y866	748
Pichia terricola (Pt)	Y974	419
Issatchenkia orientalis (Io)	Y1130	490
Starmerella bacillaris (Sb)	Y975	458
Candida apicola (Cap)	Y957	457
Candida azyma (Ca)	Y979	436
Candida parapsilosis (Cp)	Y842	522
Candida glabrata (Cg)	Y800	884
Torulaspora delbrueckii (Td)	BIODIVA	797
Rhodotorula glutinis (Rg)	Y824	614
Rhodosporidium diobovatum (Rd)	Y840	618
Kazachstania aerobia (Ka)	Y845	751
Lachancea thermotolerans (Lt)	Y973	675
Saccharomyces cerevisiae (Sc)	EC1118	842
Wickerhamomyces anomalus (Wa)	Y934	618
Metschnikowia pulcherrima (Mp)	Y981	377

Spain) when required. The plates were incubated at 30°C for 3–5 days.

Automated Ribosomal Intergenic Spacer Analysis (ARISA)

Single colonies of each yeast species were inoculated into 5 mL YPD broth (10 g/L yeast extract, 20 g/L peptone and, 20 g/L glucose) and incubated for 16 h at 30°C. Two milliliters of cultures were centrifuged at 5630 × *g* for 10 min to collect the cells. Genomic DNA was extracted using the method described by Sambrook and Russell (2006). DNA concentration was determined spectrophotometrically, using the NanoDrop®ND-1000 (NanoDrop Technologies Inc., Wilmington, DE, United States). The ITS1-5.8S rRNA-ITS2 gene was amplified using the carboxy-fluorescein labeled ITS1 primer (5′-6-FAM- TCC GTA GGT GAA CCT TGC GG-3′) and ITS4 (5′- TCC GTA GGT GAA CCTTGC GG-3′) in a 25 μL reaction, containing 50 ng DNA, 1U Takara Ex Taq, DNA polymerase (TaKaRa Bio Inc., Olsu, Shiga, Japan), 1 × Taq buffer, 0.25 μM of each primer, 400 μM dNTP mix and 1 mM $MgCl_2$. The PCR reaction was performed under the following conditions: an initial denaturation of 3 min at 94°C, followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 54°C for 30 s, extension at 72°C for 45 s and a final extension step of 72°C for 10 min (Slabbert et al., 2010). Three independent PCR reactions were performed. The PCR products were excised from the gel and purified using the Zymoclean™ Gel DNA Recovery Kit Short Protocol (Zymo Research Corporation, Irvine, CA, United States). The ARISA fragments were separated by capillary electrophoresis at the Stellenbosch University Central Analytical Facility on an ABI 3010x Genetic Analyzer (Applied Biosystems) with a ROX 1.1 labeled size standard (75-1121 base pairs). ARISA profiles were analyzed using Genemapper software version 4.1 (Applied Biosystems). Only fragments with peak area larger than 0.5% of the total fluorescence were considered for further analysis. A bin size of 3 bp for species with ITS region below 700 and 5 bp for species with ITS region above 700 bp, was employed to minimize the inaccuracies in the ARISA analysis (Slabbert et al., 2010). The relative abundance of each peak was calculated by dividing individual peak area with the total peak areas for the respective sample.

Micro-Fermentations

Fermentation in Synthetic Grape Must

Eight yeast species *viz. Metschnikowia pulcherrima, Pichia terricola, Starmerella bacillaris, Candida parapsilosis, Wickerhamomyces anomalus, Lachancea thermotolerans, Hanseniaspora vineae,* and *S. cerevisiae* were selected to establish a consortium based on (i) their frequent occurrence in grape juices from SA and other wine producing regions, (ii) easy and consistent resolution in ARISA, and (iii) easy morphological detection on WL agar (Jolly et al., 2003a; Combina et al., 2005; Di Maro et al., 2007; Lopandic et al., 2008; Romancino et al., 2008; Salinas et al., 2009; Sun et al., 2009; Suzzi et al., 2012; Weightman, 2014; Maturano et al., 2015; Morgan, 2016). Fermentations were carried out, by inoculating the selected yeast species, in synthetic grape juice medium (pH 3.5) adapted from Bely et al. (1990) and Henschke and Jiranek (1993). The medium contained 200 g/L sugars (100 g/L glucose and 100 g/L fructose) and 300 mg/L assimilable nitrogen (460 mg/L NH_4Cl and 180 mg/L amino acids). Five hundred milliliters of the juice was dispensed into 500 mL Erlenmeyer flasks, fitted with CO_2 traps. The juice was inoculated with the *NS-Sc* (non-*Saccharomyces-Saccharomyces*) consortium comprising of 7 non-*Saccharomyces* yeast species (*M. pulcherrima*, *P. terricola*, *S. bacillaris*, *C. parapsilosis*, *W. anomalus*, *L. thermotolerans,* and *H. vineae*), each inoculated at 10^6 cells/mL and *S. cerevisiae* at 10^3 cells/mL, and the *NS* (non-*Saccharomyces*) consortium which only consisted of the seven non-*Saccharomyces* yeasts. The fermentations were performed at 25°C with no agitation. Fermentations were monitored by weighing the flasks regularly to measure CO_2 loss. Furthermore, samples were collected regularly to determine sugar concentrations using Fourier Transform Infra-Red Spectroscopy on the Foss Wine scan 2000 (Rhine Ruhr, Denmark). Samples were withdrawn at 2-day intervals and yeast population dynamics was monitored by direct plating on WLN agar and ARISA.

Real Must Fermentation

Fifty liters of clarified Chenin blanc grape juice was obtained from a commercial cellar. The chemical composition of juice was measured, using spectroscopy technique by Foss wine scan 2000 (Rhine Ruhr, Denmark). The yeast community composition of the juice was determined by serial dilution and direct plating on WL-agar, followed by identification through ITS-5.8S rRNA amplification, RFLP, and sequencing as described in Bagheri et al. (2015). Subsequently, 1.5 L Chenin blanc grape juice was dispensed into 2 L fermentation bottles. Three sets of fermentations were performed: (i) spontaneous (ii) *Sc*-inoculated fermentation (at 10^3 cells/mL, *S. cerevisiae* EC1118), and (iii) *NS-Sc* consortium inoculated (7 non-*Saccharomyces* at 10^6 cells/mL

vs. *S. cerevisiae* at 10^3 cells/mL). The fermentations were performed in triplicate, at 25°C, and without SO_2 addition. The fermentations were weighed daily to monitor CO_2 release and samples were withdrawn at 2-day intervals to monitor population dynamics. The residual sugar at the end of fermentation was measured. The fermentations were considered complete when residual sugars in wine were less than 2 g/L and the yeast population dynamics was monitored by direct plating on WLN agar.

Statistical Analysis

The DNA extraction, ARISA analysis, and fermentations were performed in triplicate. The values were presented as means ± SD. The differences between treatments were determined using analysis of variance (ANOVA) with the statistical software Statistica version 13.0 (StatSoft Inc., Tulsa, OK, United States). The differences were considered significant should the *p*-values were equal or less than 0.05. For multivariate data analysis, the Principal Component Analysis was performed, using XLSTAT in Microsoft®Excel (2016).

RESULTS

Selection of Yeast Species for the Consortium

Eighteen yeast species commonly isolated from South African grape musts (Jolly et al., 2003a; Weightman, 2014; Bagheri et al., 2015; Morgan, 2016), were initially evaluated for DNA extractability and resolvability in ARISA analysis. The ARISA profile of the mixed community only revealed 13 peaks (**Figure 1**). An overlap between *Rhodotorula glutinis* (614 bp), *R. diobovatum* (618 bp) and *W. anomalus* (618 bp) was observed. Similarly, *H. uvarum* (747 bp), *H. opuntiae* (748 bp), and

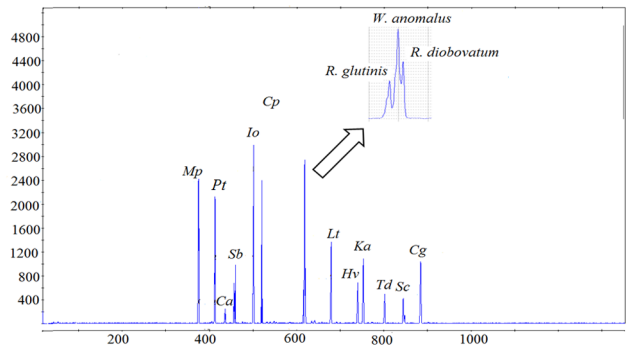

FIGURE 1 | Electropherogram of a mixed culture of 18 yeast species, generated via PCR amplification with ITS1F-ITS4 primers. The *x*-axis represents the fragment size (bp) and the *y*-axis represents the relative fluorescence intensity. The following abbreviations were used for names of yeast species. *Mp*, *Metschnikowia pulcherrima; Pt, Pichia terricola; Ca, Candida azyma; Sb, Starmerella bacillaris; Io, Issatchenkia orientalis; Cp, Candida parapsilosis; Lt, Lachancea thermotolerans; Hv, Hanseniaspora vineae; Ka, Kazachstania aerobia; Td, Torulaspora delbrueckii; Sc, Saccharomyces cerevisiae; Cg, Candida glabrata.*

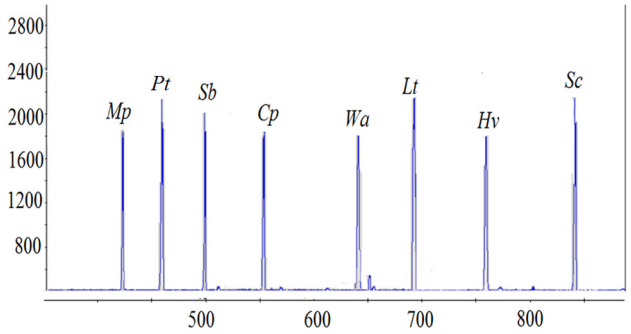

FIGURE 2 | Quantitative validation between the ARISA peaks of eight selected yeast species and CFU/mL. All yeast species were inoculated at 10^5 CFU/mL. The *x*-axis represents the fragment size (bp) and the *y*-axis represents the relative fluorescence intensity.

Kazachstania aerobia (751 bp), as well as *S. bacillaris* (458 bp) and *C. apicola* (458 bp) co-migrated and could not be resolved. Consequently, eight species (*M. pulcherrima, P. terricola, S. bacillaris, C. parapsilosis, W. anomalus, L. thermotolerans, H. vineae,* and *S. cerevisiae*), which could be reliably resolved in ARISA, and could be distinguished based on their colony morphology on WLN agar, were selected to establish a model consortium. The efficiency of DNA extraction method and ARISA on the consortium was evaluated. In addition, standard curves of optical density ($OD_{600\ nm}$) vs. colony forming units (CFU/mL) were established for each species (data not shown). A cell suspension containing approximately each at 10^5 CFU/mL was prepared. Total genomic DNA was extracted from the mixed culture and ARISA was performed. Similar peak heights and peak areas were observed for all species, suggesting that the DNA extraction method and ARISA were efficient for all of them (**Figure 2**).

Validation of ARISA in the Model Consortium

The detection limit of ARISA was investigated in different inoculation scenarios, representing low and high levels of selected yeast species (**Table 2**). The data indicated that when all species were inoculated at the same level, they could be detected even at 10^3 CFU/mL while, in a situation where one species

TABLE 2 | Yeast inoculum combinations used to determine ARISA detection limits.

Yeast species	A	B	C
H. vineae	10^3	10^4	10^3
S. bacillaris	10^3	10^4	10^3
C. parapsilosis	10^3	10^4	10^3
P. terricola	10^3	10^4	10^3
L. thermotolerans	10^3	10^6	10^3
W. anomalus	10^3	10^4	10^3
M. pulcherrima	10^3	10^4	10^3
S. cerevisiae	10^3	10^4	10^6

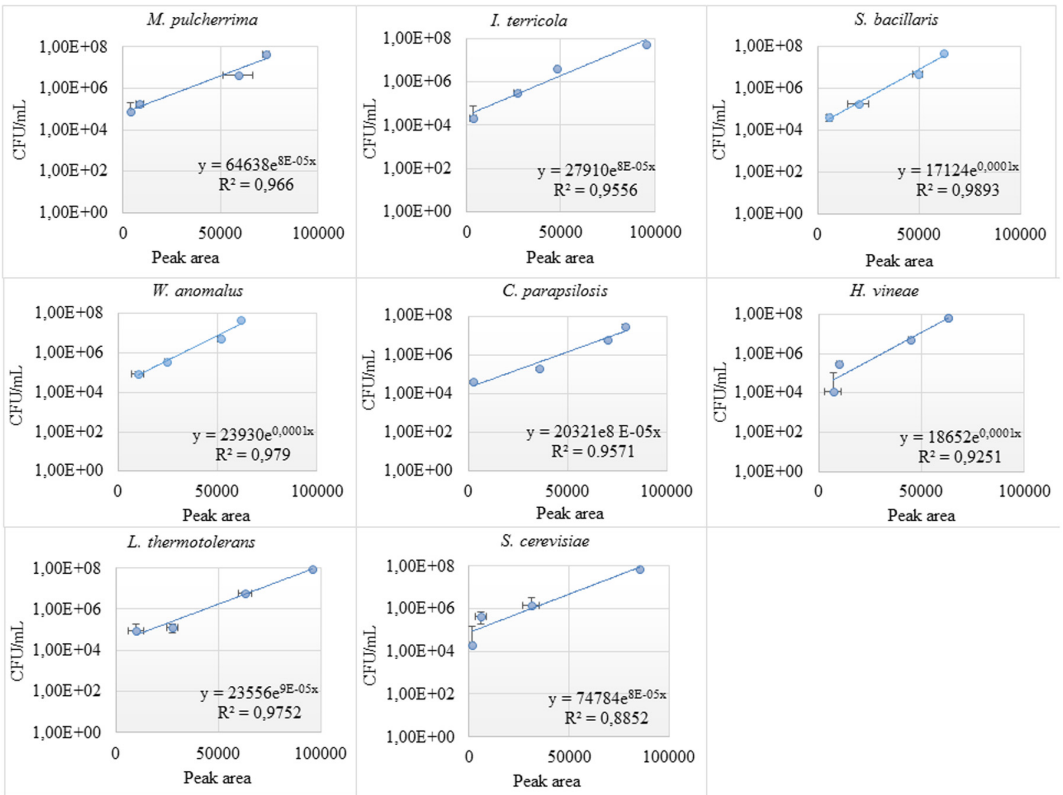

FIGURE 3 | Standard curves of individual yeast species in the consortium. The correlation between the colony forming unit and peak area (bp) was investigated at different dilutions (10^3–10^7 CFU/mL) for individual yeast species in the consortium.

was significantly higher in concentration ($\geq 10^6$ CFU/mL), other species could be detected if present at 10^4 CFU/mL but not at 10^3 CFU/mL (Supplementary Figure S1). Therefore, the detection limit of ARISA was defined as the lowest cell concentration (10^4 CFU/mL) that resulted in a positive signal and fluorescence intensity above 50 relative fluorescence units (RFU).

To test the repeatability and reliability of ARISA for monitoring the yeast dynamics throughout the fermentation, three independent DNA extractions were performed from a sample in which the yeasts were mixed in different concentrations. In each case, similar peak profiles were observed for triplicates with minor variations in peak intensities (Supplementary Figure S2).

For better quantification of the individual yeast species, standard curves correlating colony forming units and peak areas were established. Strong linear correlation between CFU/mL and ARISA peak area, with an R_2 value of ≈0.9 was observed, for individual yeast species (**Figure 3**). However, at lower biomass, the correlation between peak area and viable counts was non-linear.

Fermentation in Synthetic Grape Juice

Fermentation and Growth Kinetics

The applicability of the consortium and ARISA as a model was tested in the synthetic grape juice fermentation, inoculated with *NS-Sc* and *NS* only. The two sets of fermentations displayed distinct kinetics, with the *NS-Sc* fermentation reaching dryness (residual sugar < 2 g/L) within 21 days, while the fermentation with the *NS* consortium was sluggish and still had a total of 88 g/L residual sugar by day 30 (**Figure 4**). The *NS* fermentation got stuck at this level since the residual sugar was found to be the same after 40 days.

Yeast Population Dynamics in Synthetic Grape Juice

Comparison of ARISA and viable counts from the *NS-Sc* fermentation revealed similar trends in the relative abundance of the individual species in the early stage of fermentation (**Figure 5**). However, in the middle and final fermentation stages, ARISA consistently showed higher levels of *S. cerevisiae* and lower levels of *H. vineae* than direct plating (Supplementary Table S1). In addition, *M. pulcherrima* and *P. terricola* were detectable by ARISA until the end of fermentation while, they could not be observed and enumerated on agar plates.

Analysis of the yeast dynamics in the *NS-Sc* fermentation by standard plating on WLN agar revealed an initial increase in the population of non-*Saccharomyces* species until 10% of the sugar was consumed. The individual non-*Saccharomyces* species reached up to 10^7–10^8 CFU/mL and maintained viability at these levels for a brief period, before starting

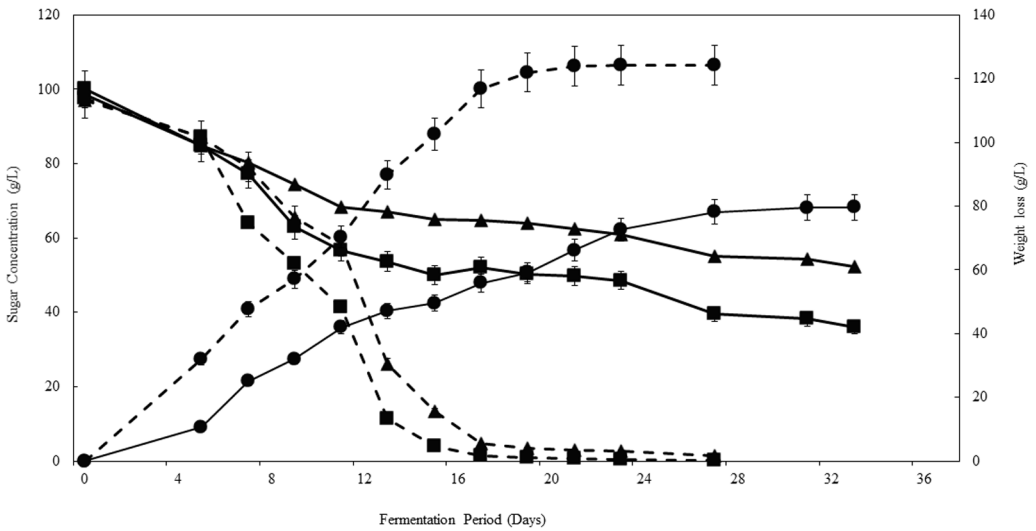

FIGURE 4 | Progress curves showing the kinetics of fermentations performed in the synthetic must. Fermentation performed with *NS-Sc* consortium is indicated with broken lines while fermentation with *NS* consortium is indicated with solid lines. Glucose (■), fructose (▲) and CO_2 release (●) were monitored throughout fermentation.

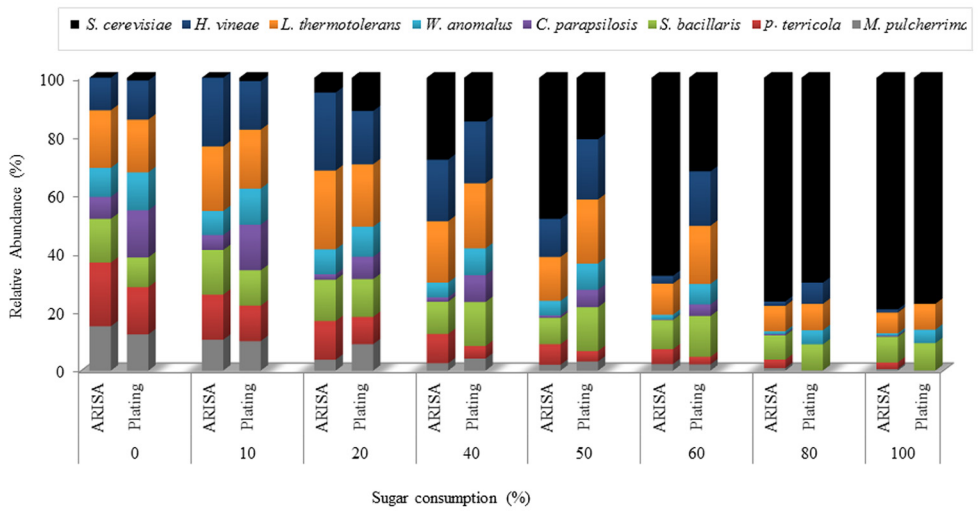

FIGURE 5 | Relative abundance of yeast species throughout the *NS-Sc* fermentation in synthetic grape must. Yeast population dynamics were monitored using ARISA and plating methods.

to decline. *P. terricola* and *C. parapsilosis,* dropped below detection by 50% sugar consumption, whereas *M. pulcherrima* and *H. vineae* were below detection after 70 and 90% sugar consumption, respectively (**Figure 6**). In contrast, the population of *S. cerevisiae* increased steadily from 10^3 CFU/mL to 4.37×10^4 CFU/mL (20% sugar consumption) where the population of all non-*Saccharomyces* species declined to 10^6 CFU/mL. When *S. cerevisiae* reached to 6.47×10^4, a decline in the population of *W. anomalus* (3.70×10^5), *P. terricola* (3.10×10^5) and *M. pulccherrima* (1.90×10^5) was observed whereas, the population of *C. parapsolosis, H. vineae, S. bacillaris,* and *L. thermotolerans* remained at 10^6 CFU/mL. Finally, *S. cerevisiae* dominated the fermentation and reached to 7.19×10^7 CFU/mL. *L. thermotolerans* (8.40×10^4), *S. bacillaris* (8.03×10^4), and *W. anomalus* (1.10×10^4) remained viable until the end of fermentation.

In the *NS* fermentation, the levels of *S. bacillaris, P. terricola,* and *L. thermotolerans* increased moderately and maintained dominance until 40% of the sugar was consumed while, *M. pulcherrima* and *C. parapsilosis* declined steadily from the onset of fermentation. Using the standard curves constructed as described in the previous section, the population of *S. bacillaris,*

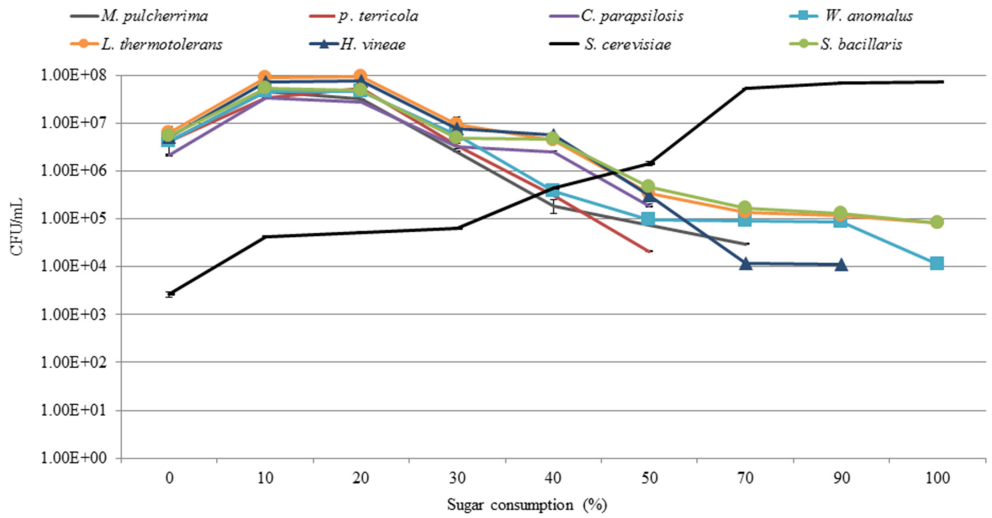

FIGURE 6 | Growth profiles of yeast population throughout *NS-Sc* fermentation in the synthetic must.

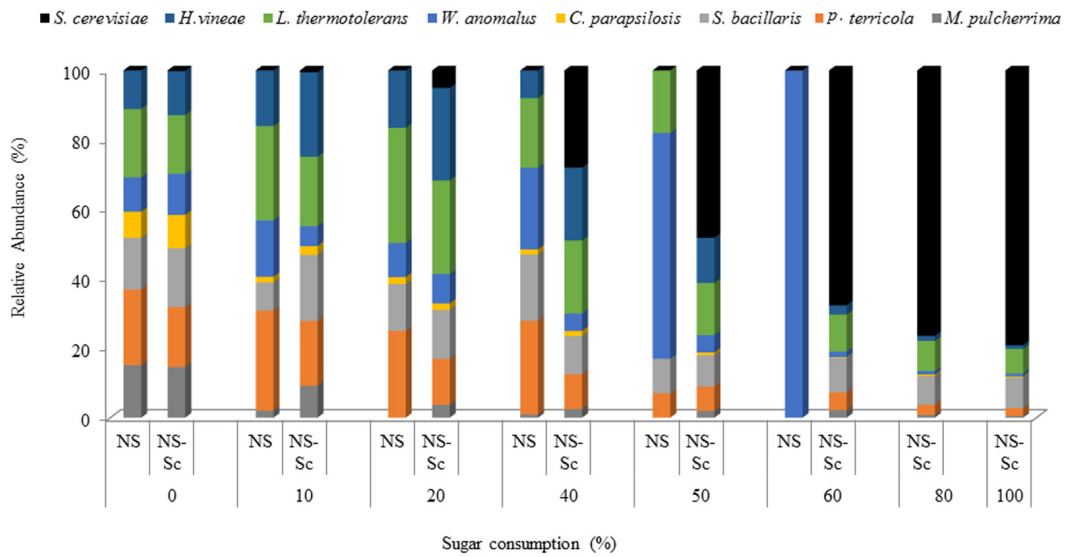

FIGURE 7 | Relative abundance of yeast species during fermentations performed with *NS-Sc* and *NS*. Yeast population dynamics was monitored using ARISA.

P. terricola, and *L. thermotolerans* was estimated to be 1.48×10^5, 5.33×10^5, and 2.82×10^5 CFU/mL, respectively, whereas the population of *M. pulcherrima* and *C. parapsilosis* was 1.22×10^3 and 1.69×10^3 CFU/mL. The population of *H. vineae* at 40% sugar consumption was estimated to be 2.07×10^3 CFU/mL.

After 50% of the sugar was consumed, only four species (*L. thermotolerans*, *S. bacillaris, P. terricola,* and *W. anomalus*) were detected, with *W. anomalus*, accounting for 65% of the population. The population of *L. thermotolerans*, *S. bacillaris, P. terricola,* and *W. anomalus* based on the standard curves were 2.74×10^5, 5.58×10^4, 2.77×10^4, and 7.23×10^6 CFU/mL, respectively. The fermentation got stuck at 60% of sugar consumption and *W. anomalus* was the only detectable yeast at this stage (**Figure 7**). The level of *W. anomalus* based on the standard curve was estimated to be 9.67×10^6 CFU/mL by 60% of sugar consumption in *NS* fermentation while *S. cerevisiae* reached up to 7.19×10^7 CFU/mL by the end of the *NS-Sc* fermentation.

Chemical Parameters and Yeast Diversity in Chenin Blanc Juice

The Chenin blanc juice used in the current study was at 21.7 °Brix with a total acidity of 3.23 g/L, pH 3.37 and a yeast assimilable nitrogen (YAN) of 195 mg/L. Sugar content and YAN concentration were higher in Chenin blanc juice compared to the synthetic must (**Table 3**). One hundred and eighty four yeast isolates obtained from the Chenin blanc juice were identified

TABLE 3 | Chemical parameters of Chenin blanc compared to the synthetic grape juice.

Chemical parameter	Chenin blanc juice	Synthetic grape juice
Sugar (°Brix)	21.7	20
YAN (mg/L)	195	300
pH	3.37	3.5

and revealed that the initial indigenous yeast population comprised *M. pulcherrima* (2.39×10^3 CFU/mL), *H. uvarum* (4.21×10^3 CFU/mL), *L. thermotolerans* (2.70×10^3 CFU/mL), *W. anomalus* (3.34×10^3 CFU/mL) and *S. cerevisiae* (4.85×10^3 CFU/mL).

Chenin Blanc Fermentations

A comparison of the spontaneous fermentation, the *Sc*-inoculated, and the *NS-Sc* inoculated fermentations, revealed that the *Sc* fermentation was the fastest and reached dryness in 24 days, followed by the spontaneous fermentation at 26 days, while, *NS-Sc* fermentation took 28 days to reach dryness (**Figure 8**).

The spontaneous fermentation of the juice was characterized by an initial increase in the yeast population from $\approx 10^3$ CFU/mL to 6.27×10^5 CFU/mL, by 10% sugar consumption. Subsequently, a decline in some non-*Saccharomyces* species was observed; amongst them, *W. anomalus* and *M. pulcherrima* declined rapidly and could not be detected by 30% sugar consumption, while *H. uvarum* persisted until 50% of the sugar was consumed. In contrast, *L. thermotolerans* increased in growth up to 2.3×10^6 CFU/mL at 50% sugar consumption and persisted until the end of fermentation. The indigenous *S. cerevisiae* (*IND-Sc*) increased from $\approx 10^3$ CFU/mL to a maximum of 1.82×10^8 CFU/mL (**Figure 9A**). Similar trends were observed in the *Sc*-inoculated fermentation. However, *W. anomalus* only grew up to 4×10^4 CFU/mL and *H. uvarum* persisted until 40% sugar consumption (**Figure 9B**). In addition, *L. thermotolerans* only reached a maximum of 8×10^5 CFU/mL. Within the *S. cerevisiae* population, *IND-Sc* and EC1118 displayed similar growth patterns. However, *IND-Sc* persisted at a higher level, reaching a maximum of 2.1×10^8 CFU/mL, while EC1118 reached 4.5×10^7 CFU/mL (**Figure 9B**). When the *NS-Sc* consortium was inoculated, *H. uvarum* (the only indigenous non-*Saccharomyces* yeast that was not part of the consortium), grew from 4.4×10^3 to 6.20×10^4 CFU/mL by 10% sugar consumption followed by a steady decline until it could not be detected by 50% sugar consumption (**Figure 9C**). Amongst the remainder of the non-*Saccharomyces* yeasts which were inoculated at $\approx 10^6$ CFU/mL, *P. terricola* and *C. parapsilosis* declined below detection after 10% sugar consumption, followed by *M. pulcherrima* and *W. anomalus* by 28% sugar consumption. In contrast, *H. vineae* declined gradually until 78% sugar consumption; *S. bacillaris* persisted at 10^6 CFU/mL until 78% sugar consumption before dropping to 8×10^4 CFU/mL at the end of fermentation, while, *L. thermotolerans* persisted at 10^6 CFU/mL until the end of fermentation. The *S. cerevisiae* population behaved in a similar way as observed in the *S. cerevisiae* inoculated fermentation, albeit at 10 times less cell concentrations. For instance, *IND-Sc* reached a maximum of 3.2×10^7 CFU/mL, while EC1118 reached 6.9×10^6 CFU/mL.

DISCUSSION

The current study aimed to establish and validate a model system for reliable monitoring and prediction of the temporal trajectories of yeast populations within the wine fermentation ecosystem. To this end, a yeast consortium comprising *S. cerevisiae* and seven non-*Saccharomyces* yeast species of varying fermentative capacities was constructed. These yeast species are all regularly encountered in SA grape juices, and some species have sometimes been detected in significant numbers. Furthermore, all of these non-*Saccharomyces* species have been isolated in countries with several wine producing regions such as Italy, France, Argentina, China, and Brazil (Jolly et al., 2003a; Combina et al., 2005; Di Maro et al., 2007; Lopandic et al., 2008; Romancino et al., 2008; Salinas et al., 2009; Sun et al., 2009; Suzzi et al., 2012; Tofalo et al., 2012; Weightman, 2014; Maturano et al., 2015; Morgan, 2016). These yeast species also differed in their ITS1-5.8S rRNA-ITS2 gene sizes, which made ARISA a suitable method to monitor their dynamics. Our data show that in this semi-complex consortium, the detection limit of ARISA could be as low as 10^3 CFU/mL when all species are present at low levels. However, at lower biomass (10^{3-4} CFU/mL) larger deviations were observed, possibly due to the bias introduced by DNA extraction or preferential amplification in PCR (Giraffa, 2004; Ramette, 2009). Furthermore, in a typical wine fermentation scenario where dominant taxa grow up to 10^{7-8} CFU/mL, minor taxa would not be detected below 10^4 CFU/mL. ARISA is also unable to differentiate between strains of the same species, limiting its ability to monitor strain-specific dynamics. However, species-specific interactions of significantly contributing species can be easily detected and quantified (Ramette, 2009; Ženišová et al., 2014; Ghosh et al., 2015; Setati et al., 2015). The limits are similar to those obtained for FISH (Xufre et al., 2006) and PCR-DGGE (Prakitchaiwattana et al., 2004) and they are less sensitive than qPCR (10^2 CFU/mL) and flow cytometry (10^3 CFU/mL) methods (Malacrinò et al., 2001; Hierro et al., 2006a,b; Zott et al., 2010). However, ARISA does not require species-specific primers and is less technically demanding than qPCR and flow cytometry. Overall, ARISA generated similar growth patterns for individual yeast species in the consortium as observed with viable counts. However, some discrepancies were observed in the middle and final stage of fermentation. These discrepancies might reflect biases and limitation in both methods. For instance, plating method might show bias against cells in a VBNC state and injured population (Divol and Lonvaud-Funel, 2005; Renouf et al., 2007) while ARISA is unable to differentiate between live and dead cells (Xie et al., 2007; O'Sullivan et al., 2013). Consequently, an overestimation of most of the species (e.g., *M. pulcherrima, P. terricola, H. vineae, L. thermotolerans, S. bacillaris, and S. cerevisiae*) by one order of magnitude was evident with ARISA compared to the plating method. The data in the current study suggest that up to 3% of dead cells could possibly be detected

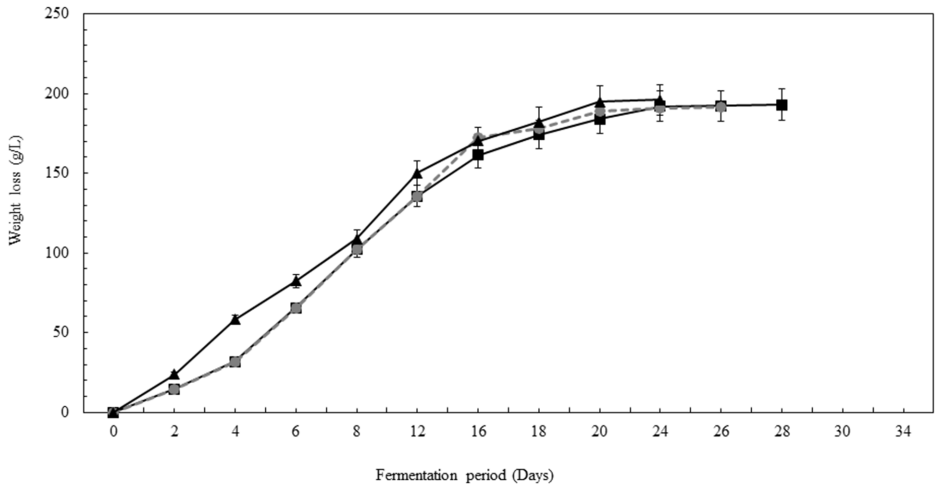

FIGURE 8 | Progress curves displaying the kinetics of spontaneous fermentation (●), fermentation inoculated with *Sc* (▲), and fermentation inoculated with *NS-Sc* consortium (■).

by ARISA. Similarly, Salinas et al., (2009) indicated that qPCR overestimate the number of live cells in average one order higher compared to microscopy analysis, which according to Hierro et al. (2006a) could represent up to 1% of the dead cells.

Our study showed that the yeast species constituting the consortium responded differently to the wine fermentation ecosystem, and the behavior of the non-*Saccharomyces* species was differentially influenced by the presence of *S. cerevisiae*. The data showed that in the absence of *S. cerevisiae*, some non-*Saccharomyces* species such as *M. pulcherrima* and *C. parapsilosis* experienced a decline from the onset of fermentation whereas, species such as *S. bacillaris, P. terricola,* and *L. thermotolerans* experienced a moderate increase followed by a steady decline in the absolute numbers by the middle of fermentation. On the contrary, *W. anomalus* suppressed the rest of non-*Saccharomyces* species and increased in cell concentration back to the initial inoculum level. This suggests that *W. anomalus* can withstand the chemical milieu created in the early stages of the fermentation better than the other yeast species and may utilize the nitrogen released by dead cells. In contrast, in the presence of *S. cerevisiae*, specifically, this yeast declines early in fermentation, suggesting that *S. cerevisiae* creates an unconducive environment, which suppresses *W. anomalus*. Indeed, an antagonistic interaction between *S. cerevisiae* and *W. anomalus*, has been proposed in other fermentation ecosystems (Ye et al., 2014). *S. cerevisiae* may inhibit other organisms through a variety of mechanisms including the production of short chain fatty acids and glycoproteins (killer toxin), and the specific antagonism exerted by *S. cerevisiae* modulates the ecosystem (Vannette and Fukami, 2014; Boynton and Greig, 2016). Conversely, other yeast species such as *M. pulcherrima*, *P. terricola,* and *C. parapsilosis* consistently declined in the early stages of the fermentation, both in the presence and in the absence of *S. cerevisiae*, suggesting that the decline could be due to another factor such as oxygen limitation. Several studies have shown that the growth and survival rate of *M. pulcherrima* and *C. parapsilosis* was markedly enhanced in aerated fermentations (Oh et al., 1998; Rossignol et al., 2009; Morales et al., 2015; Shekhawat et al., 2017). Furthermore, in the presence of *S. cerevisiae*, *L. thermotolerans,* and *S. bacillaris* could survive until late fermentation. The survival of *L. thermotolerans* until end of the fermentation has been shown previously (Gobbi et al., 2013). In addition, *S. bacillaris* strains are typically fructophilic and therefore preferentially utilize fructose, which is less preferred by *S. cerevisiae*. Interestingly, our study revealed that *H. vineae* survives better in the presence *S. cerevisiae* suggesting a positive interaction between the two yeasts. Such an interaction is perhaps not coincidental since other studies have shown that in nutrient-rich conditions, co-fermentations using strains of these two species often reflect a significant contribution of *H. vineae* to wine aroma and flavor (Viana et al., 2011; Medina et al., 2013).

Based on our current findings, we can infer that the mutualism (*S. cerevisiae* and *H. vineae*) and antagonism (*S. cerevisiae* and *W. anomalus*) observed in the wine ecosystem, could be a species-specific interaction that occurs as a result of the presence of *S. cerevisiae*. However, the strength of the mutualism or antagonism in the wine consortium may vary between different strains of one species requires further investigation. Indeed, species-specific patterns throughout the wine fermentation process are probable and comprehensible. For instance, it is well established that some species decline rapidly by early or mid-fermentation (*Cryptococcus carnescens, Aureobasidium pullulans, P. terricola,* and *M. pulcherrima)*, others repeatedly persist until late fermentation (*S. bacillaris, L. thermotolerans, T. delbrueckii*) regardless of the strain variability (Jemec et al., 2001; Sun et al., 2009; Cordero-Bueso et al., 2011; Bezerra-Bussoli et al., 2013; Gobbi et al., 2013; Milanović et al., 2013; Bagheri et al., 2015).

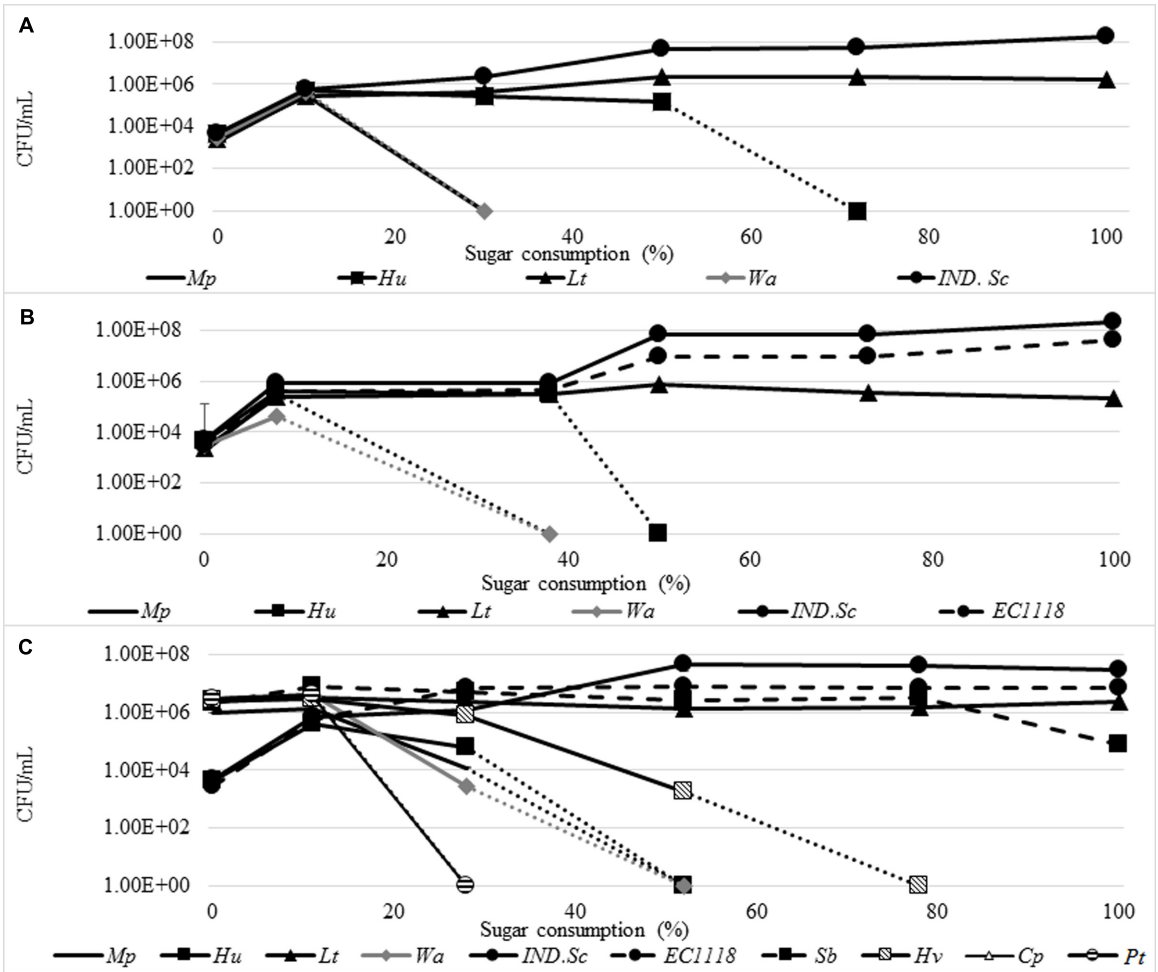

FIGURE 9 | Yeast population dynamics in Chenin blanc spontaneous fermentation **(A)**, *S. cerevisiae* inoculated fermentation **(B)** and *NS-Sc* consortium fermentation **(C)**. The following abbreviations were used for names of yeast species. *Mp*, *M. pulcherrima; It, P. terricola; Sb, S. bacillaris; Cp, C. parapsilosis; Lt, L. thermotolerans; Hv, H. vineae; Hu, H. uvarum; IND.Sc,* Indigenous *S. cerevisiae.*

One of the goals of the current study was to establish a consortium that would serve as a representative model to predict yeast dynamics in wine fermentation. In order to validate the suitability of this consortium, it was used as an inoculum in Chenin blanc must and the dynamics was monitored throughout the fermentation. Interestingly, four of the yeast species (*M. pulcherrima*, *L. thermotolerans, W. anomalus*, and *S. cerevisiae*) which form part of the consortium were also present in the natural yeast community of the Chenin blanc must, confirming once more the representative nature of our consortium. Our study shows that all the species in the consortium could compete with the native yeast species in a non-sterilized must. While we were unable to differentiate between the indigenous strains and inoculated strains (e.g., *W. anomalus*), the population dynamics observed were similar to those described for the synthetic grape juice, suggesting species, and not strain specific drivers of interactions. This is further supported by the fact that the dynamics were preserved although the environmental conditions, including nitrogen and sugar levels, differed consoderably between the two matrices (Supplementary Table S2). We also observed that the indigenous *S. cerevisiae* population displayed better growth than the EC1118 inoculated strain although they were at similar levels at the beginning of the fermentation, further indicating that the selective drivers were species and not strain-dependent. Our data show that the consortium constructed in the current study serves as a viable and robust model to assess yeast population dynamics during wine fermentation since the matrix did not have a considerable influence on the dynamics as such. We suggest that the yeast dynamics observed in the current study is mainly due to species-specific interactions and the selective pressure applied by *S. cerevisiae* to other species. Our data suggest that inoculation with *S. cerevisiae* favors the persistence of some non-*Saccharomyces* species in wine fermentation whereas; it clearly suppresses the growth and contribution of other non-*Saccharomyces* species.

The dynamics of the wine ecosystem is driven by a multitude of positive and negative yeast–yeast interactions. The main

challenge in microbial ecology is to link microbial composition to function. Here, we demonstrate that a model consortium approach can be used as a tool to predict the microbial behavior in a complex natural environment. Such a model consortium can be easily perturbed under well-controlled conditions in order to gain a deep understanding of the effect of environmental parameters on yeast–yeast interactions. In-depth insight on yeast–yeast interactions may allow us to manipulate the microbial community and enhance the population of the beneficial microbes or suppress the population of undesirable yeast species. The study presents a first step in the development of a model to predict the oenological potential of any given wine mycobiome.

AUTHOR CONTRIBUTIONS

FB and MS conceptualized the study. BB, FB, and MS designed the experimental layout. BB performed the experiments, analyzed the data and wrote the first draft of the manuscript. BB, FB, and MS edited subsequent drafts, read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2017.01988/full#supplementary-material

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Metatranscriptomics Reveals the Functions and Enzyme Profiles of the Microbial Community in Chinese Nong-Flavor Liquor Starter

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Chinese liquor is one of the world's best-known distilled spirits and is the largest spirit category by sales. The unique and traditional solid-state fermentation technology used to produce Chinese liquor has been in continuous use for several thousand years. The diverse and dynamic microbial community in a liquor starter is the main contributor to liquor brewing. However, little is known about the ecological distribution and functional importance of these community members. In this study, metatranscriptomics was used to comprehensively explore the active microbial community members and key transcripts with significant functions in the liquor starter production process. Fungi were found to be the most abundant and active community members. A total of 932 carbohydrate-active enzymes, including highly expressed auxiliary activity family 9 and 10 proteins, were identified at 62°C under aerobic conditions. Some potential thermostable enzymes were identified at 50, 62, and 25°C (mature stage). Increased content and overexpressed key enzymes involved in glycolysis and starch, pyruvate and ethanol metabolism were detected at 50 and 62°C. The key enzymes of the citrate cycle were up-regulated at 62°C, and their abundant derivatives are crucial for flavor generation. Here, the metabolism and functional enzymes of the active microbial communities in NF liquor starter were studied, which could pave the way to initiate improvements in liquor quality and to discover microbes that produce novel enzymes or high-value added products.

Keywords: ethanol fermentation, flavor generation, Chinese liquor starter, metatranscriptome, Chinese Nong-flavor liquor, saccharification

INTRODUCTION

Chinese liquor is one of the world's four best-known distilled spirits. It accounts for more than one-third of all spirits consumed (Sweeney, 2013) and is the largest spirit category by sales in the world (Molon, 2013). The unique and traditional Chinese solid-state simultaneous saccharification and fermentation (SSF) and liquor brewing technologies have been in continuous use for several thousand years (Xiao et al., 2011; Yao et al., 2015; Xu et al., 2017). Nong-flavor (NF) liquor accounts

for more than 70% of the Chinese liquor market. The main fermentation process includes two stages: liquor starter production and alcohol fermentation. The production process of NF liquor starter, which is aerobically produced, usually includes approximately 1 month of spontaneous incubation in a fermentation room and 3 months of drying in a storage room to mature (Zheng et al., 2011; Chen et al., 2014). NF liquor starters use wheat as feedstock, and the wetted wheat is shaped into bricks, each weighing approximately 1.5–4.5 kg (Zheng et al., 2011; Zheng and Han, 2016). As NF liquor starter is characterized by a moderately high temperature (62°C), the liquor starter fermenting bricks must be maintained at a moderately high temperature for 8 days in the fermentation room. For the alcohol fermentation process, first, raw sorghum, wheat, corn, rice, and sticky rice are crushed, steamed, cooled, and mixed evenly with liquor starter. Then, the mixture undergoes a solid-state SSF process for approximately 40–45 days in a pit. Finally, the fermented mixture is distilled to produce the liquor (Tao et al., 2014; Yan et al., 2015). The fermentation processes that occur during SSF are mainly attributed to the metabolism and interactions of the microorganisms from the liquor starter, Zaopei and pit mud (Chen et al., 2014). Liquor starter is the most important and essential component for liquor fermentation. During the production of liquor starter, no microorganisms are intentionally inoculated; thus, most of the microbes are enriched from naturally occurring ecosystems, such as feedstock, water, air and the working environment, with high balance and stability. These Chinese NF liquor starter microbial communities have evolved for more than several thousand years and have greatly influenced liquor properties, such as their distinctive flavor and taste.

As the NF liquor starter production process is subjected to extremely severe conditions (50–62°C), the special microbial community enriched in the liquor starter may produce efficient and diverse thermophilic carbohydrate-degrading enzymes. Recently, great efforts have been made to discover novel thermophilic lignocellulases with excellent performance, including high activity and marked stability (McClendon et al., 2012; Balasubramanian and Simões, 2014). The carbohydrate-degrading enzymes from the liquor starter under aerobic and thermophilic conditions are different from those in termite and other herbivore-associated gut communities, which are dominated by anaerobic bacteria. Thus, enzymes from liquor starter may have great potential for industrial applications because lignocellulose decomposition has mainly been demonstrated under aerobic conditions (Robinson et al., 2001). Therefore, global and comprehensive technologies are needed to retrieve multiple thermophilic and synergistic carbohydrate-degrading enzymes from the NF liquor starter system. Elucidating the saccharification capability of liquor starters and identifying other attractive enzymes for industrial applications would also be of great value.

The microbial community of liquor starters has been studied using culture-dependent and denaturing gradient gel electrophoresis methods (Zheng et al., 2012; Yan et al., 2013; Chen et al., 2014; Zhang L. et al., 2014; Wang and Xu, 2015) as well as pyrosequencing techniques (Li et al., 2013; Zhang X. et al., 2014; Wang et al., 2017). Moreover, Huang et al. (unpublished data) compared the dominant microbial community of Jiang-flavor (JF) and NF liquor starters and provided a more complete picture of the microbial composition in liquor starters. These studies have increased the understanding of the microbial community structure of liquor starters. However, not all of these methods are ideal for assessing community functions, and little is known concerning the active microbial community compositions and their metabolic functions in liquor starter. Metatranscriptomics, the direct analysis of mRNA from environmental samples, offers a powerful tool to study the active microbial community composition as well as their active genes and changes in transcriptional regulation when microbes respond to temporal variation (Mitra et al., 2011). Although, several studies have demonstrated the great advantage of metatranscriptomic technology (Bashir et al., 2013; Sanders et al., 2013; Wu et al., 2013), high-quality RNA from complex and difficult environmental samples severely challenges metatranscriptomics projects. The high content of starch and other polysaccharides, the complex fermentation products and the strong colored biomass during fermentation make RNA extraction of the microbial community in liquor starter difficult.

In the present study, we first successfully extracted total RNA from complex liquor starter samples and then applied high-throughput metatranscriptomic technology to globally, comprehensively and functionally analyze the actual microbial composition and metabolic characteristics of the most widely consumed NF liquor starter during the production process. The efforts of this study provide the first step in understanding the metabolism and function of the active microbial communities in liquor starters and pave the way toward the optimization of liquor production, improvement of liquor quality and discovery of microbes that produce valuable and novel enzymes with great potential for industrial applications.

MATERIALS AND METHODS

Sample Collection

NF liquor starter was sampled from a fermentation workshop of the Yibin Hongloumeng Distillery Group Co., Ltd. in Yibing, Sichuan, China, in July 2013. The liquor starter was sampled at different time points. The samples were harvested from three locations in the same liquor starter fermentation room at each time point. N1 was collected at the beginning of liquor starter fermentation (30°C); N2 was collected after 3 days of liquor starter fermentation (50°C); N3 was collected after 9 days of liquor starter fermentation (62°C); and N4 was collected from the mature liquor starter after fermentation for 20 days (25°C). The samples were frozen in liquid nitrogen when they were harvested in the fermentation workshop and were then immediately transferred to 50-ml RNase free Corning CentriStar™ centrifuge tubes (430828, Corning, NY, USA) and kept on dry ice. Finally, all the samples were transferred to the Chengdu Biology Institute at the Chinese Academy of Sciences on the day of sampling and stored in a −80°C freezer until analyses. The liquor starter samples for enzyme analysis were prepared as follows: 5 g of liquor starter was suspended in 20 ml of 0.1% (v/v) Tween

80 solution and transferred to the Chengdu Biology Institute, Chinese Academy of Sciences at room temperature.

Enzyme Profile of the NF Liquor Starter

After the liquor starter in the Tween 80 solution was transferred to the laboratory, the samples were incubated at 25°C with shaking at 100 rpm overnight. The enzyme profile of the supernatant was investigated using insoluble chromogenic AZurine Cross-Linked (AZCL) polysaccharides according to the manufacturer's protocol (Megazyme, Ireland). After incubation at 35, 45, or 55°C for 22 h, the diameter of the blue haloes were measured and recorded in millimeters.

RNA Extraction

Total RNA was extracted from liquor starter according to a previously reported method (Kumar et al., 2011) with some modifications. Briefly, 1 g of liquor starter was homogenized into fine powder in a precooled mortar with liquid nitrogen. Next, 4 ml of a hot (80°C) borate buffer [200 mM sodium borate (pH 9.0), 30 mM ethyleneglycotetraacetic acid (EGTA), 1% (w/v) sodium dodecyl sulfate (SDS), 2% (w/v) polyvinylpyrrolidone (PVP), and 0.5% (v/v) Nonidet-40 (NP-40) combined in 0.1% diethyl pyrocarbonate (DEPC)-treated water and then autoclaved; after cooling and just before use, 10 mM β-mercaptoethanol and 0.03% (v/v) RNase inhibitor were added] and 280 μl of proteinase K (20 mg/ml) were added, and the mixture was incubated at 80°C for 2 min. The lysate was centrifuged for 10 min at 5,000 × g. The supernatant was mixed thoroughly with an equal volume of 70% ethanol by shaking vigorously. The sample was applied to an RNeasy midi column and centrifuged for 5 min at 5,000 × g; this step was repeated for the residual sample. The sample was cleaned following the RNeasy Midi Kit protocol (Qiagen, 75142) and treated with DNase I (Fermentas, USA) according to the manufacturer's protocol. The purity, concentration and RNA integrity number (RIN) were measured using an Agilent 2100 Bioanalyzer. Qualified total RNA was submitted to the Beijing Genomics Institute (BGI)-Shenzhen, China, for RNA sequencing.

cDNA Illumina Library Construction, RNA Sequencing and *De novo* Assembly

More than 20 μg of qualified total RNA from each sample (N1, N2, N3, and N4) was used for RNA sequencing using the HiSeq^{TM} 2000 platform. For eukaryotes, poly (A) mRNA was purified using poly-T oligo-attached magnetic beads. For prokaryotes, rRNA was removed before subsequent library construction steps. The mRNA was mixed with fragmentation buffer and then fragmented. Fragmented mRNAs were synthesized into first-strand cDNA using reverse transcriptase and random primers. This step was followed by second-strand cDNA synthesis. Short fragments were purified and resolved with EB buffer for end reparation and poly (A)-tailing. Thereafter, the short fragments were connected with sequencing adapters, and then 200-bp cDNA fragments were purified for further template enrichment by PCR. The validated 200-bp fragment cDNA libraries were submitted for paired-end (PE) RNA sequencing using the HiSeq^{TM} 2000 platform. Known bacterial, fungal and archaeal sequences were extracted from the National Center for Biotechnology Information (NCBI) Nucleotide (NT) database using in-house scripts, and the filtered reads were mapped to these sequences using the SOAP aligner (version 2.21) (Li et al., 2009). Next, the transcriptome data were assembled *de novo* using Trinity (http://trinityrnaseq.sourceforge.net/; Grabherr et al., 2011). The raw and assembled sequencing data have been deposited in the DDBJ/EMBL/GenBank database under the accession numbers SRR5384077 and GFMA00000000, respectively.

Functional Annotation and Cluster Analysis

The software TransDecoder (http://sourceforge.net/projects/transdecoder/) was used to predict open reading frames (ORFs) based on the assembly results. The predicted amino acid sequences were aligned to diverse databases through BLAST (version 2.2.23), and related information was extracted and summarized using custom scripts. Gene Ontology (GO) classification (Ashburner et al., 2000) was achieved using WEGO (http://wego.genomics.org.cn/cgi-bin/wego/index.pl) (Ye et al., 2006). Enzyme codes were extracted, and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways were retrieved from the KEGG web server (Kanehisa, 1997; Kanehisa et al., 2004, 2006). Carbohydrate-active enzymes were annotated according to the Carbohydrate-Active enZymes database (CAZy) (version: 2011-9-20) (Cantarel et al., 2009). The evolutionary genealogy of genes was extracted from Non-supervised Orthologous Groups (eggNOG) (version 3.0) (Powell et al., 2012). KEGG, GO CAZy and eggNOG function cluster analyses were conducted using custom scripts. A heat map was constructed with the R package (Ihaka and Gentleman, 1996) using custom scripts.

Expression Profiling and Differential Expression Identification

To investigate the expression level of each unigene in the different samples, all the predicted ORFs were removed for redundancy using cd-hit (Version 4.6.1, http://weizhong-lab.ucsd.edu/cdhit_suite/cgi-bin/index.cgi). Unigene expression was calculated according to the fragments per kilobase of transcripts per mapped million fragments method (FPKM) (Ali et al., 2008). The *P*-values and log2-fold-changes ($\log_2$FCs) were calculated, and then the significantly differentially expressed transcripts (DETs) between the two samples were identified using $p \leq 0.05$ and $\log_2 FC \geq 1$. Because thousands of hypothesis tests were performed using the transcriptome data, a suitable *p*-value for an individual test is not sufficient to guarantee a low rate of false discovery. Thus, multiple testing corrections for each individual hypothesis were performed to guarantee an overall low false discovery rate. The false discovery rate (FDR) control is a statistical method used in multiple hypothesis testing to correct for the *p*-value as described previously (Benjamini and Yekutieli, 2001). When the FDR was obtained, the FPKM ratio of the two samples was used at the same time. In this analysis, the values were identified as follows: FDR ≤ 0.001 and FPKM ratio ≥ 2.

TABLE 1 | Species information for the liquor starter samples after blasting the cDNA sequencing reads against the bacterial and fungal databases.

Sample ID	Database	Rate (%)
N1	Bacteria	1.10
N1	Fungi	15.43
N2	Bacteria	7.68
N2	Fungi	48.35
N3	Bacteria	15.30
N3	Fungi	45.05
N4	Bacteria	12.33
N4	Fungi	66.22

RESULTS

Metatranscriptome Sequencing and *De novo* Assembly of the NF Liquor Starter Samples

After sequencing, we obtained 21.789 Gbp of data in total (Table S1). The raw reads were cleaned, pooled and assembled *de novo*. The assembly metrics can be found in Table S2. The maximum contig lengths were 13,340, 17,819, 12,496, and 12,933 bp, and the N_{50} lengths were 404, 538, 1,084, and 1,105 bp for N1, N2, N3, and N4, respectively. The coding DNA and protein sequences were predicted and translated based on the assembled transcripts. Scanning the ORFs of all the contigs identified 25387, 58884, 56927, and 28618 ORFs with lengths longer than 600 bp for N1, N2, N3, and N4, respectively (Table S3).

Functional Profiling and Characterization of the NF Liquor Starter Metatranscriptome

The high-quality sequences were aligned to the bacterial and fungal sequences in the NT database. The composition of active species is presented in **Table 1**. The identified active fungal community was more prevalent than the bacterial community during the entire liquor starter production process as more active mRNA can be detected. The fungal component even increased up to 66.22% for sample N4. However, the bacterial component increased to the highest value of 15.30% for sample N3 and dropped to 12.33% for sample N4.

GO, CAZy, eggNOG, and KEGG annotation combined with BLASTX was performed to profile the active genes and related pathways in the NF liquor starter. A total of 29418 unigenes from all the NF samples were annotated with the GO database, accounting for 17.32% of all the unigenes. The annotation was mainly clustered into three general sections: biological processes, cellular components and molecular functions (Figure S1). Primary metabolic processes and catalytic activities were the most enriched GO terms in the biological processes and molecular functions sections, respectively. This result was further confirmed by KEGG analysis. For the NF liquor starter samples N1, N2, N3, and N4, 5047, 16419, 17034 and 8662 unigenes were found in 254, 264, 260, and 250 reference pathways, accounting for 19.88%, 27.88, 29.92, and 30.27% of the total unigenes, respectively.

The 20 most abundant KEGG pathways of the four samples are shown in **Figure 1**. Notably, oxidative phosphorylation was ranked as the first most abundant pathway for sample N2. Among the 20 pathways, glycolysis, pyruvate metabolism, the citrate cycle, fructose and mannose metabolism, starch and sucrose metabolism and the pentose phosphate pathway were highly represented, particularly in samples N2 and N3. Additionally, butanoate metabolism and propanoate metabolism were found among the 20 pathways. Metabolism of 10 amino acids, i.e., lysine, alanine, aspartate, glutamate, valine, leucine, isoleucine, cysteine, methionine and glutathione, were also ranked in the top 20 and were dominant in samples N2 and N3.

The specific pathways related to saccharification, ethanol fermentation and flavor generation in the NF liquor starter metatranscriptome are schematically presented in **Figure 2**. First, polymers such as cellulose, hemicellulose, starch and protein are converted to monomers by diverse carbohydrate-active enzymes and proteases with high activity. Next, the monomers are taken up and further utilized by the microbial community. The products and intermediates of primary metabolism, such as glycolysis and the citrate cycle, as well as by-products of metabolism, mainly contribute to ethanol production and flavor development.

Identification of Carbohydrate-Active Enzymes

In the present study, we analyzed the enzyme profiles of the NF liquor starter samples at different reaction temperatures (35, 45, and 55°C). Low enzyme activity was detected in the initial sample (N1) (**Table 2**). However, a broad spectrum of carbohydrate-active enzymes was detected in sample N2. More importantly, endo-β-1,3-1,4-glucanase, endo-1,3-β-D-glucanase, α-amylase, endo-1,5-α-L-arabinanase and endo-protease thermophilic activity was obviously present with higher activity at higher temperatures. Furthermore, endo-β-1,3-1,4-glucanase, rhamnogalacturonanase and endo-protease thermostable activity was clearly present in the N3 sample. The mature liquor starter sample N4 also had a broad spectrum of enzymatic activity.

The metatranscriptome data were annotated to further identify carbohydrate-active enzymes at the molecular level. The profiles of the carbohydrate-active enzymes varied among the four samples (**Figure 3**). The N3 sample had the highest number of carbohydrate-active enzymes, including 478 glycoside hydrolases, 397 glycosyl transferases, 57 carbohydrate esterases and 64 carbohydrate-binding modules, followed by the N2 sample (**Figure 3** and Table S4). The most highly expressed glycoside hydrolases in the NF liquor starter were mainly classified as cellulases (GH5, GH7, GH9 and GH45), endo-hemicellulases (GH10, GH11, GH12 and GH28), cell wall extension enzymes (GH16, GH17 and GH81), cell wall debranching enzymes (GH51 and GH67) and oligosaccharide-degrading enzymes (GH1, GH2, GH3, GH29, GH35, GH38, and GH43).

In this study, the most abundant and diverse auxiliary activity 9 (AA9) genes (up to 12 unique encoding genes) were found

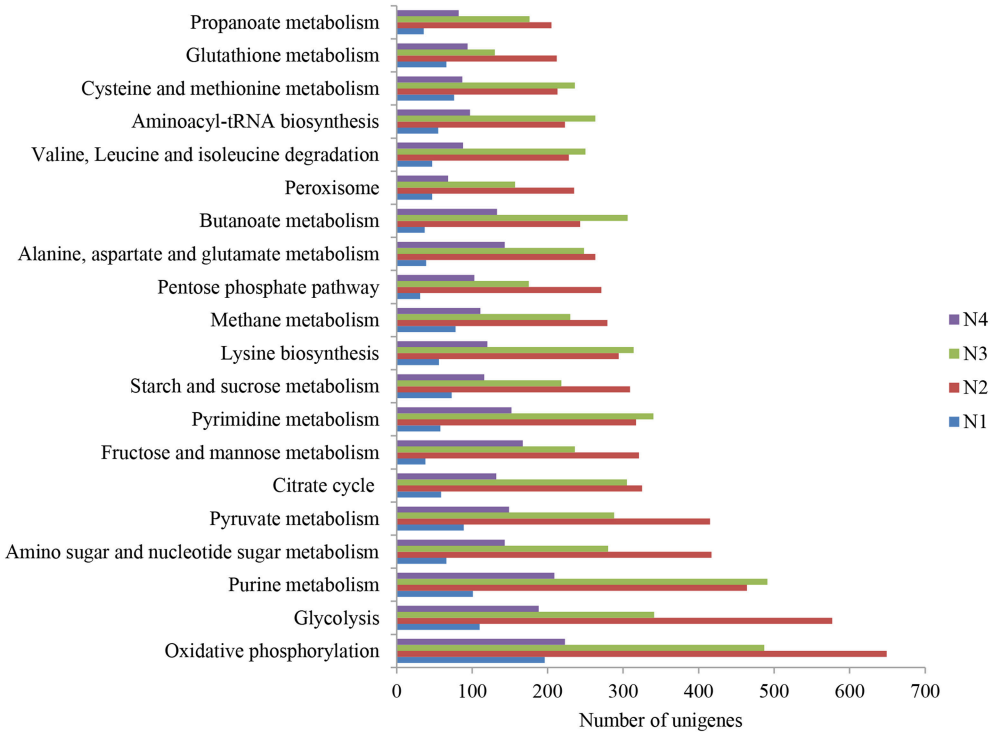

FIGURE 1 | The 20 most abundant KEGG pathways in the metatranscriptome of Nong-flavor liquor starter samples (N1, N2, N3, and N4). N1 was sampled at the beginning of liquor starter production, N2 was sampled after 3 days of liquor starter fermentation, N3 was sampled after 9 days of liquor starter fermentation, and N4 was the mature liquor starter. The temperatures of N1, N2, N3, and N4 were 30, 50, 62 and 25°C, respectively.

in the N3 sample (**Table 3**). The AA9 genes were mainly from *Thermoascus aurantiacus*, *Trichocomaceae* and *Emericella nidulans*. Only one AA10 protein from *Bacillales* was found in the N3 sample. All of these AA9 and AA10 proteins secreted by thermophilic fungi and bacteria were first identified in the NF liquor starter with low identity (41–75%) to the reported protein sequences. Additionally, the phylogenetic tree (Figure S2) showed that the AA9 proteins are diverse in sequence.

Starch Metabolism, Glycolysis, Ethanol Metabolism, and Pyruvate Metabolism

Enzymes related to starch metabolism were analyzed among the four samples (**Figure 4A**). Most of the enzymes were more abundant in the N2 and N3 samples, especially the important enzymes related to the conversion of starch to glucose, such as α-amylase, starch phosphorylase, maltose phosphorylase, β-glucosidase, glucoamylase, glucan 1,3-β-glucosidase and endoglucanase (colored in red in **Figure 4A**). Moreover, α-amylase had the highest expression level, with an RPKM value of up to 1398.5 in the N2 sample.

We then comparatively analyzed the glycolysis metabolism of the NF liquor starter samples. The results indicated that there were various expression levels for the 9 enzyme-catalyzed reactions that convert hexose to pyruvate in all the samples (Figure S3). The N2 sample had the highest total numbers and expression levels of the glycolysis genes (**Figure 1** and **Figure 4B**). Among these genes, glyceraldehyde 3-phosphate dehydrogenase had the highest expression level with an RPKM value of 6004.1. Many of the enzyme genes also exhibited comparable expression levels in sample N3 (**Figure 4B**). Furthermore, three key enzymes in glycolysis, hexokinase, 6-phosphofructokinase and pyruvate kinase (colored in red in **Figure 4B**), were significantly up-regulated, with $\log_2$Ratio values (N2/N1) ranging from 3.6 to 16.6 (Figure S3 and Table S5). In total, 8 hexokinases, 10 6-phosphofructokinases and 16 pyruvate kinases were up-regulated, and the up-regulated genes were mainly from *Saccharomycetales* and *Mucorales*.

Pyruvate can be converted to ethanol in conditions of insufficient oxygen, which possibly occurs inside the liquor starter brick. As shown in Figure S3, alcohol dehydrogenase, aldehyde dehydrogenase (NAD+) and aldehyde dehydrogenase (NAD(P)+) are three key enzymes in ethanol metabolism. The highest expression levels of alcohol dehydrogenase and aldehyde dehydrogenase (NAD(P)+) were in N3, and the highest expression level of aldehyde dehydrogenase (NAD+) (RPKM: 1878.7) was in N2, with a comparable level (RPKM: 1300.4) in N3 (colored in blue in **Figure 4B**). Furthermore, compared with the N1 sample, the alcohol dehydrogenase levels in the N2, N3, and N4 samples from different microbial community members were up-regulated. In particular, in the N2 sample, 10 types of alcohol dehydrogenases were up-regulated from diverse microorganisms, such as *Leuconostocaceae*, *Millerozyma farinose*, *Weissella thailandensis*, *Saccharomycetales*,

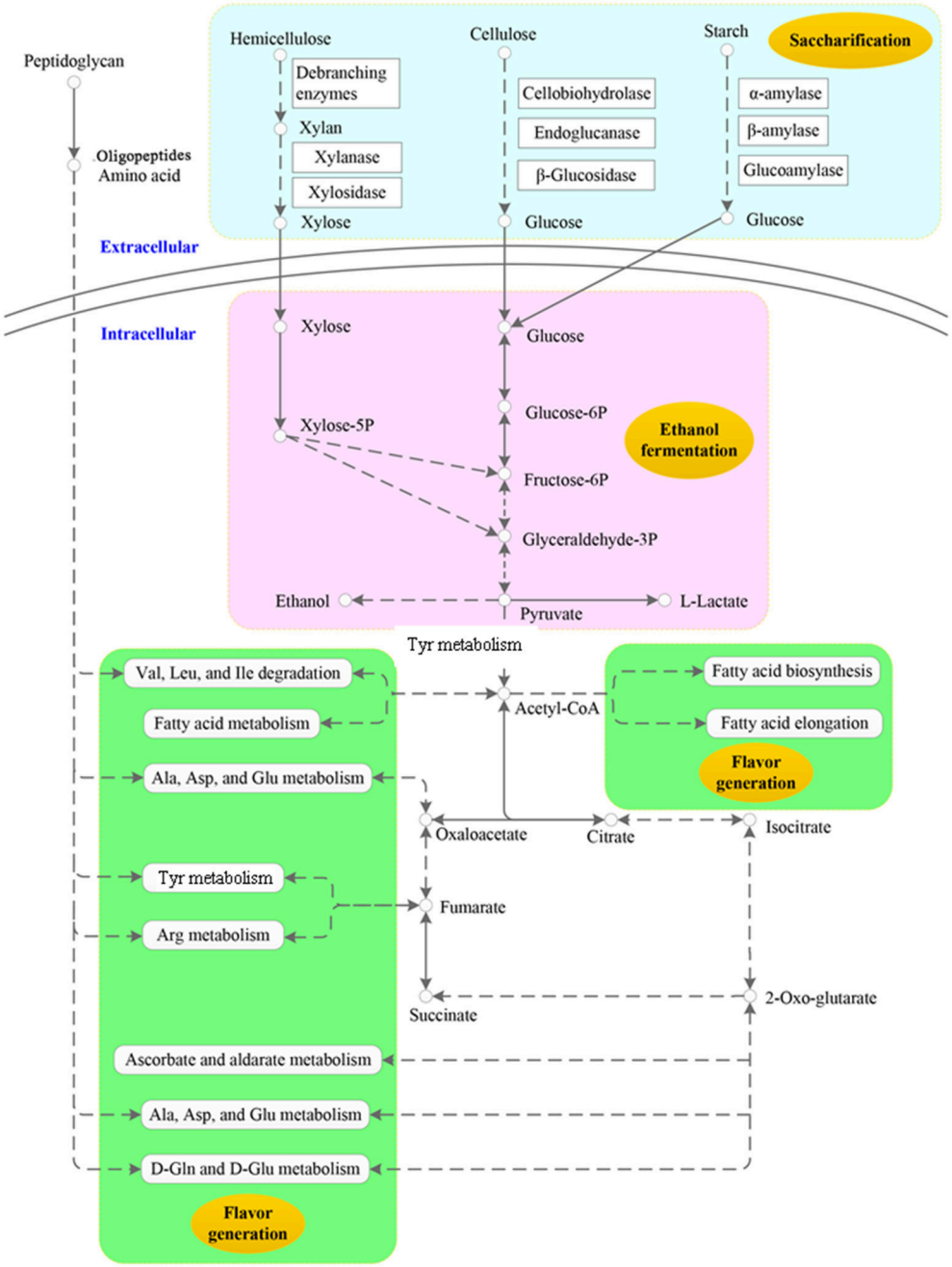

FIGURE 2 | Key metabolic profiles of scarification, ethanol fermentation and flavor generation in the Nong-flavor liquor starter. In this schematic summary, extracellular and intracellular reactions are separated by the cell membrane, but these reactions are not restricted to one cell. The light-blue color indicates the scarification profile. The pink color indicates the ethanol fermentation profile, and the green color indicates that the derivatives may be related to flavor generation.

Dikarya, *Rhizopus oryzae*, and *Lactobacillales* (**Table 4**). The $\log_2$Ratio (N2/N1) values of the alcohol dehydrogenases varied from 9.5 to 14.2. When the temperature was increased to 62°C, some aldehyde dehydrogenases (NAD+) and aldehyde dehydrogenases (NAD(P)+) were also up-regulated in the N3 sample. The RPKM value of one aldehyde dehydrogenase (NAD(P)+) produced by *Trichocomaceae* increased from 9.7 to 237.6, and the $\log_2$Ratio (N3/N2) value of aldehyde dehydrogenase (NAD+) produced by bacteria was the highest value of 15.6 (**Table 5**).

Another important intermediate of pyruvate metabolism is acetyl-CoA, which is produced by pyruvate dehydrogenase E1 and pyruvate dehydrogenase E2 (Figures S3, S4). Pyruvate dehydrogenases E1 and E2 (colored in green in **Figure 4B**) both

TABLE 2 | Carbohydrate-active enzyme analysis for Nong-flavor liquor starter using insoluble chromogenic AZurine Cross-Linked (AZCL) polysaccharides.

Substrate	Enzyme	Diameter (mm)			
		N1	N2	N3	N4
		35/45/55°C	35/45/55°C	35/45/55°C	35/45/55°C
AZCL-beta-glucan	Cellulase (endo-β-1,3-1,4-glucanase)	3/3/3	17.5/20/23	7/8/13	13/16/22
AZCL-galactan	endo-1,4- β -D-galactanase		0.5/0.5/0	6/9/2	12/12/7
AZCL-curdlan	endo-1,3- β -D-glucanase	4/4/4	10/15/18		4/9.5/7
AZCL-amylose	α-amylase	2/6/6	9.5/17/17	3/4/4	14.5/15/16
AZCL-collagen	endo-proteases				
AZCL-debranched arabinan	endo-1,5-α-L-arabinanase	0.5/0.5/0.5	0/7.5/8	7/7/6	10/8/10
AZCL-galactomannan	endo-1,4- β -D-mannanase	5.5/6/5	8/14/13	11/3/0.5	6.5/8/8
AZCL-xyloglucan	endo-β-1,4-xyloglucanase		5.5/5/7		
AZCL-xylan	endo-1,4- β -D-xylanase				13/16/18
AZCL-he-cellulose	cellulase (endo-β-1,4-glucanase)				
AZCL-pullan	microbial pullulanase				
AZCL-chitosan	Chitosanase				
AZCL-dextran	endo-1,6-α-D-glucanase				
AZCL-rhmnogalacturonan I	Rhamnogalacturonanase		14/3/4	7/8/11	3/15/17
AZCL-casein	endo-proteases		7/14/14	0/6/6	6/12/12.5
AZCL-arabinoxylan	endo-1,4- β -D-xylanase			4/3/0	11/21.5/22

showed their highest expression levels in the N2 sample, which was followed by N3. Meanwhile, other enzymes involved in the complicated metabolism of pyruvate were also compared among the four samples. Most of them showed relatively high expression levels in the N2 and N3 samples (**Figure 4C** and Table S6). For the 19 most highly expressed pyruvate metabolism enzymes, 8 enzymes exhibited their highest RPKM values in the N2 sample, and 7 enzymes exhibited their highest RPKM values in N3. Malate dehydrogenase, D-lactate dehydrogenase/D-lactate dehydrogenase (cytochrome), L-lactate dehydrogenase and pyruvate oxidase (colored in red in **Figure 4C**) are responsible for reversibly converting pyruvate to L-malate, D-lactate, L-lactate and acetyl phosphate, respectively. Meanwhile, phosphate acetyltransferase, malate synthase and acetyl-CoA hydrolase (colored in blue in **Figure 4C**) are responsible for irreversibly converting acetyl-CoA to acetyl phosphate, L-malate and acetate, respectively. More importantly, acetyl-CoA carboxylase (ACAC/accA), acetyl-CoA C-acetyltransferase and homocitrate synthase (colored in green in **Figure 4C**) are responsible for producing intermediates from acetyl-CoA for fatty acid biosynthesis, butanoate metabolism and leucine biosynthesis, respectively (Figure S4). In all, the N2 and N3 samples exhibited large capacities for converting pyruvate to pivotal intermediates for carbohydrates, fatty acids and amino acids, which further contribute to specific flavor.

Pyruvate can also be converted to lactic acid in conditions of insufficient oxygen, which likely occurs inside the liquor starter brick. In the NF liquor starter, low numbers of L-lactate dehydrogenase (cytochrome), D-lactate dehydrogenase, D-lactate dehydrogenase (cytochrome) and L-lactate dehydrogenase were detected in the N1 (total RPKM: 80.5) and N4 (165.0) samples, but higher numbers were found in N2 (RPKM: 611.2) and N3 (RPKM: 600.2) (**Figures 4B,C** and Table S7).

The Citrate Cycle and Flavor Generation

A high total number of citrate cycle genes were found in the N2 sample (**Figure 1**). However, the number of key enzymes increased in N3 (Figure S5). Most of the top 24 most highly expressed citrate cycle enzymes had definitively higher expression levels in N2 and N3, with 13 enzyme genes having their highest expression levels in N3 and 6 genes in N2 (**Figure 4D** and Table S8). Meanwhile, isocitrate dehydrogenase (NAD+) (IDH3) and isocitrate dehydrogenase (IDH1) showed comparably high expression levels in N2 and N3. Furthermore, a comparison between the N2 and N3 samples was deeply analyzed, and most of the key enzyme genes were up-regulated in N3. The RPKM and $\log_2$Ratio (N3/N2) values of the key enzymes, citrate synthase, isocitrate dehydrogenase, 2-oxoglutarate dehydrogenase, dihydrolipoamide succinyl transferase and dihydrolipoamide dehydrogenase, are shown in **Table 6**. These key enzymes were mainly produced by bacterial and fungal community members such as *Eurotiomycetidae*, *Bacillales*, *Trichocomaceae*, *Firmicutes,* and *E. nidulans*.

DISCUSSION

This study globally and comprehensively explored the active microbial community and its function in the highly consumed NF liquor starter. A promising number of thermophilic enzymes were also discovered. The results showed that the fungal communities were much more diverse and the enzymes produced by them were more abundant than that of bacteria communities during the process of making the liquor starter, especially during the high temperature period. This interesting

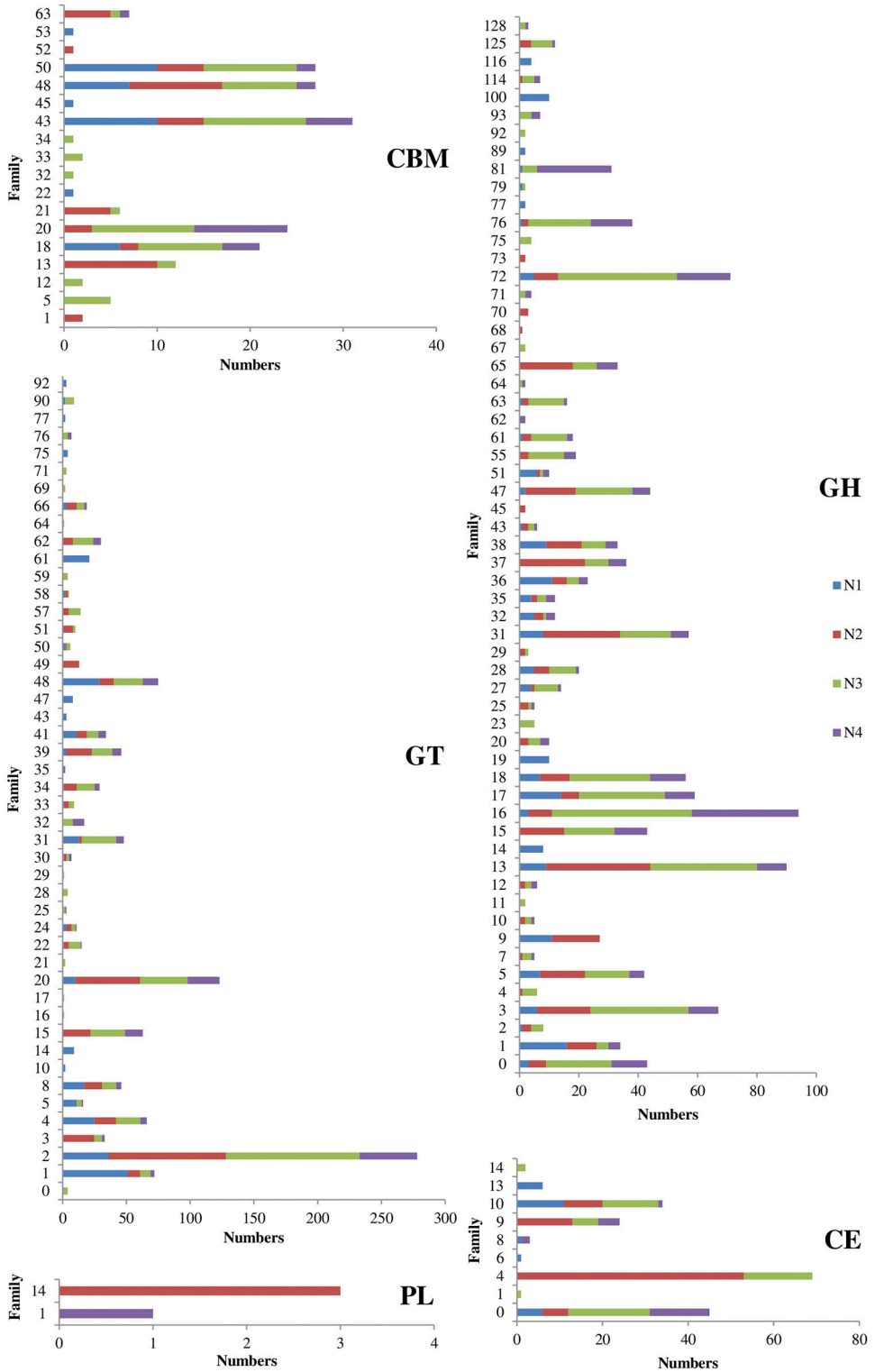

FIGURE 3 | Matched numbers of carbohydrate-active enzymes from the Nong-flavor liquor starter samples. CBM, Carbohydrate-Binding Module; GT, Glycosyl Transferase; PL, Polysaccharide Lyase; GH, Glycoside Hydrolase; and CE, Carbohydrate Esterase. N1 was sampled at the beginning of liquor starter production, N2 was sampled after 3 days of liquor starter fermentation, N3 was sampled after 9 days of liquor starter fermentation, and N4 was the mature liquor starter. The temperatures of N1, N2, N3, and N4 were 30, 50, 62, and 25°C, respectively.

TABLE 3 | Inventory of putative AA9 and AA10 proteins in Nong-flavor liquor starter at different time periods.

Sample	Gene_id	Identity (%)	Family	Species information
N1	N1_25028	59.56	AA9	*Pyrenophora*
N2	N2_38569	71.26	AA9	*Thermoascus aurantiacus*
	N2_47469	72.87	AA9	*Trichocomaceae*
	N2_47471	75.18	AA9	*Eurotiomycetidae*
N3	N3_22651	71.26	AA9	*Thermoascus aurantiacus*
	N3_25796	72.87	AA9	*Trichocomaceae*
	N3_25797	73.39	AA9	*Trichocomaceae*
	N3_27422	65.71	AA9	Unknown
	N3_48067	75.81	AA9	*Thermoascus aurantiacus*
	N3_49513	40.82	AA9	*Trichocomaceae*
	N3_53678	42.57	AA9	*Trichocomaceae*
	N3_31319	–	AA9	*Paracoccidioides brasiliensis*
	N3_6630	58.86	AA9	*Emericella nidulans*
	N3_7220	41.74	AA9	*Trichocomaceae*
	N3_8741	72.87	AA9	*Trichocomaceae*
	N3_14765	43.21	AA9	*Emericella nidulans*
	N3_43070	–	AA10	Bacteria
	N3_51026	73.55	AA10	*Bacillales*
N4	N4_12386	61.65	AA9	*Trichocomaceae*
	N4_26828	66.52	AA9	*Emericella nidulans*

finding is complementary to the results of 16S rRNA and ITS sequencing study (Huang et al., unpublished data), which indicated that the diversity and richness of the total bacterial community was much higher than that of the total fungal community. Thus, metatranscriptomics offered an important and excellent platform to actually understand the dynamics of microbial metabolism at the transcript level in liquor starter.

In this study, we discovered diverse and abundant carbohydrate-active enzymes from the NF liquor starter, especially in the N2 and N3 samples, which were characterized by high temperature and an aerobic environment. As mentioned above (**Table 2**), some thermostable carbohydrate-active enzymes were only detected in special stages, such as endo-1,3-β-D-glucanase and endo-1,5-α-L-arabinanase in N2, rhamnogalacturonanase in N3 and N4, endo-proteases and endo-β-1,3-1,4-glucanase in N2, N3, and N4, and endo-1,4-β-D-xylanase in N4. Thus, this study highlights the benefits of specifically mining for thermostable enzymes from one special stage (N2, N3, or N4) and not just from the mature starter (N4). The liquor starter production system is markedly different from other types of environmental systems, such as the microbes in cow rumens (Hess et al., 2011), wood-feeding termite hindguts (Warnecke et al., 2007), leaf-cutter ant fungal gardens (Aylward et al., 2012), and panda guts (Zhu et al., 2011), which have been well studied using metagenomic and metaproteomic strategies. Relatively high numbers of carbohydrate-active enzymes have been found in these systems by metagenomic technologies (Table S9). However, these metagenomic analyses could not reflect the genes that are actively expressed at any given time and in response to external environmental conditions. Additionally, the microbial communities associated with these various gut systems were dominated by anaerobic bacterial taxa. Notably, industrial-scale lignocellulose degradation has mostly been demonstrated under aerobic conditions (Robinson et al., 2001). By contrast, liquor starter is made in an aerobic environment, and both bacteria and fungi were enriched, with the microbial composition dynamically changing during the production process. The highest number of carbohydrate-active enzymes was found at 62°C and thus potentially offers thermophilic enzymes for lignocellulosic biomass degradation. The enzymatic conversion of polysaccharides in agricultural waste is a promising technology. However, it is still limited by the heterogeneity of the plant cell wall and recalcitrant biomass (Himmel et al., 2007). Previous studies have found that AA9 and AA10 can act synergistically with cellulose, hemicellulose, starch and chitin (Harris et al., 2010; Horn et al., 2012; Lo Leggio et al., 2015; Paspaliari et al., 2015; Kojima et al., 2016) because they have flat substrate-binding surfaces and have an oxidative mechanism to cleave polysaccharide chains in the crystalline context (Vaaje-Kolstad et al., 2010). The AA9 genes in the N3 sample were produced by *T. aurantiacus*, *Trichocomaceae,* and *Emericella nidulans*. *Thermoascus aurantiacus* is a promising thermophilic fungus for enzyme production and biomass degradation (McClendon et al., 2012). One of the *T. aurantiacus* AA9 enzymes can reduce commercial enzyme loads and is part of a well-understood synergistic system (Rosgaard et al., 2006; Harris et al., 2010). The *Trichocomaceae* family has been reported to have diverse physiological properties and can grow under extreme conditions. Some members of this family have been exploited in biotechnology for the production of enzymes (Houbraken, 2013). The thermotolerant *E. nidulans* (also called *Aspergillus nidulans*) can also utilize a broad spectrum of biomass to produce enzymes with high specific activities (Kango et al., 2003). The present study was the first to identify an AA10 protein from *Bacillales* in liquor starter, which might boost cellulose degradation. These highly expressed AA9 and AA10 members might contribute to the robust degradation capabilities of NF liquor starter, and made themselves potential candidates for industrial application. Based on these results, approximately 60 complete carbohydrate-active enzyme genes, including several AA9 proteins sequences, have been amplified from the cDNA of sample N3. They will be further cloned, expressed and characterized in future work.

Functional profiling and comparative analysis of the 4 NF liquor starter samples showed that oxidative phosphorylation was the most abundant pathway in sample N2, indicating that the microbial community quickly metabolized and released ample energy to drive energy-requiring reactions, as well as producing considerable heat that increased the room temperature to 50°C in 3 days. Among the 20 abundant pathways, most of them were closely related to energy and sugar metabolism, indicating that the microbial community has a great capability to degrade sugars and convert them to important products, such as ethanol. Additionally, butanoate metabolism and propanoate metabolism were also active in

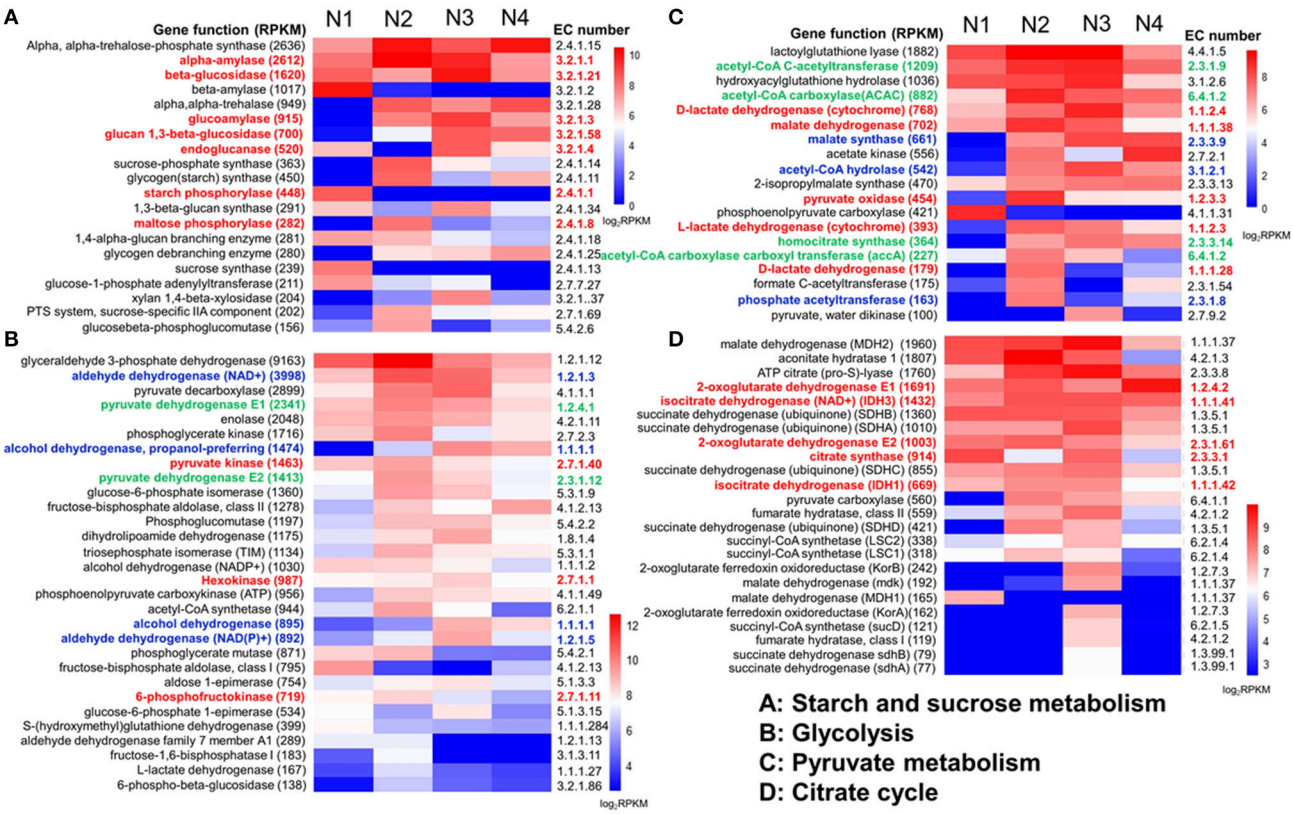

FIGURE 4 | Genes related to carbohydrate and energy metabolism were relatively highly expressed in the liquor starter samples (N1, N2, N3, and N4). Four abundant carbohydrate and energy metabolisms were analyzed here, i.e., starch and sucrose metabolism **(A)**, glycolysis **(B)**, pyruvate metabolism **(C)**, and the citrate cycle **(D)**. For each metabolism, relatively high gene expression levels were presented by function, EC number and total RPKM. Relative expression (log_2RPKM)) is shown between the high (red) and low (blue) expression levels. The key enzymes are highlighted with color.

TABLE 4 | Changes in alcohol dehydrogenase (EC1.1.1.1), a key enzyme in the ethanol metabolism pathway, between liquor starter samples N2 and N1.

GeneID	Ko_ name	Ko_defi	Ko_EC	N1-RPKM	N2-RPKM	log_2 Ratio (N2/N1)	Up-Down-Regulation (N2/N1)	*P*-value	FDR	Species information
N2_19311	adh	Alcohol dehydrogenase	1.1.1.1	0.0	6.4	12.7	Up	0.1625	0.1940	*Leuconostocaceae*
N2_23390	adhP	Alcohol dehydrogenase. Propanol-preferring	1.1.1.1	0.0	6.9	12.8	Up	0.0983	0.1224	*Millerozyma farinosa*
N2_24864	adhP	Alcohol dehydrogenase. Propanol-preferring	1.1.1.1	0.0	16.0	14.0	Up	0.0021	0.0033	*Leuconostocaceae*
N2_25713	adh	Alcohol dehydrogenase	1.1.1.1	0.0	3.3	11.7	Up	0.5243	0.5758	*Leuconostocaceae*
N2_29242	adh	Alcohol dehydrogenase	1.1.1.1	0.0	18.3	14.2	Up	0.0000	0.0001	*Weissella thailandensis*
N2_29640	adhP	Alcohol dehydrogenase. Propanol-preferring	1.1.1.1	0.0	12.9	13.7	Up	0.0029	0.0046	*Saccharomycetales*
N2_700	adhP	Alcohol dehydrogenase. Propanol-preferring	1.1.1.1	0.0	0.7	9.5	Up	0.8662	0.8807	*Dikarya*
N2_38029	adh	Alcohol dehydrogenase	1.1.1.1	0.0	11.9	13.5	Up	0.0001	0.0002	*Rhizopus oryzae*
N2_38923	adhP	Alcohol dehydrogenase. Propanol-preferring	1.1.1.1	0.0	11.6	13.5	Up	0.0029	0.0046	*Saccharomycetales*
N2_15497	adhP	Alcohol dehydrogenase. Propanol-preferring	1.1.1.1	0.0	19.2	14.2	Up	0.0001	0.0001	*Lactobacillales*

the liquor starter samples. Butanoate and propanoate are the most important substrates for ethyl caproate biosynthesis. Ethyl caproate is a key component that affects the flavor and quality of NF liquor (Tao et al., 2014). More interestingly, amino acid metabolism was robust in the NF liquor starter. The metabolisms of 10 amino acids were found to be dominant

TABLE 5 | Changes in aldehyde dehydrogenase (NAD+) (EC1.2.1.3) and aldehyde dehydrogenase (NAD(P)+) (EC1.2.1.5), key enzymes in the ethanol metabolism pathway, in liquor starter samples N3 and N2.

GeneID	Ko_defi	Ko_EC	N2-RPKM	N3-RPKM	log_2 Ratio (N3/N2)	Up-Down-Regulation (N3/N2)	*P*-value	FDR	Species information
N3_18183	Aldehyde dehydrogenase (NAD+)	1.2.1.3	0.0	32.5	15.0	Up	4.44E-16	1.89E-15	Bacteria
N3_20191	Aldehyde dehydrogenase (NAD+)	1.2.1.3	0.0	50.5	15.6	Up	4.44E-16	1.65E-15	Bacteria
N3_20791	Aldehyde dehydrogenase (NAD+)	1.2.1.3	0.0	23.3	14.5	Up	4.44E-16	1.40E-15	*Bacillales*
N3_2443	Aldehyde dehydrogenase (NAD+)	1.2.1.3	0.0	27.0	14.7	Up	4.44E-16	1.87E-15	Bacteria
N3_19461	Aldehyde dehydrogenase (NAD(P)+)	1.2.1.5	0.0	16.7	14.0	Up	4.44E-16	1.32E-15	*Trichocomaceae*
N3_20602	Aldehyde dehydrogenase (NAD(P)+)	1.2.1.5	0.0	15.3	13.9	Up	4.44E-16	1.48E-15	*Trichocomaceae*
N3_20661	Aldehyde dehydrogenase (NAD(P)+)	1.2.1.5	0.0	41.9	15.4	Up	4.44E-16	1.40E-15	*leotiomyceta*
N3_21915	Aldehyde dehydrogenase (NAD(P)+)	1.2.1.5	9.7	237.6	4.6	Up	4.84E-14	1.29E-13	*Trichocomaceae*
N3_39954	Aldehyde dehydrogenase (NAD(P)+)	1.2.1.5	12.0	159.3	3.7	Up	0	0	*Eurotiomycetidae*
N3_16852	Aldehyde dehydrogenase (NAD(P)+)	1.2.1.5	2.2	61.0	4.8	Up	3.92E-08	7.09E-08	*Trichocomaceae*

TABLE 6 | Changes in citrate synthase (EC2.3.3.1), isocitrate dehydrogenase (EC1.1.1.42 and EC1.1.1.41), 2-oxoglutarate dehydrogenase (EC1.2.4.2), dihydrolipoamide succinyl transferase (EC2.3.1.61), and dihydrolipoamide dehydrogenase (EC1.8.1.4), key enzymes in the citrate cycle, in liquor starter samples N3 and N2.

GeneID	Ko_defi	Ko_EC	N2-RPKM	N3-RPKM	log_2 Ratio (N3/N2)	Up-Down-Regulation (N3/N2)	*P*-value	FDR	Species information
N3_22492	Citrate synthase	2.3.3.1	0.8	5.5	2.9	Up	1.15E-05	1.71E-05	*Eurotiomycetidae*
N3_26467	Citrate synthase	2.3.3.1	0.0	55.4	15.8	Up	4.44E-16	1.48E-15	*Bacillales*
N3_30325	Citrate synthase	2.3.3.1	0.0	165.1	17.3	Up	4.44E-16	1.92E-15	*Bacillales*
N3_41412	Citrate synthase	2.3.3.1	0.0	130.5	17.0	Up	4.44E-16	1.63E-15	*Trichocomaceae*
N3_20330	Isocitrate dehydrogenase (IDH1)	1.1.1.42	30.4	202.7	2.7	Up	0	0	*Eurotiomycetidae*
N3_26466	Isocitrate dehydrogenase (IDH1)	1.1.1.42	0.0	54.0	15.7	Up	4.44E-16	1.34E-15	*Firmicutes*
N3_30327	isocitrate dehydrogenase (IDH1)	1.1.1.42	0.0	122.5	16.9	Up	4.44E-16	1.46E-15	*Firmicutes*
N3_723	Isocitrate dehydrogenase (IDH1)	1.1.1.42	0.0	52.5	15.7	Up	4.44E-16	1.45E-15	*Trichocomaceae*
N3_6975	Isocitrate dehydrogenase (IDH1)	1.1.1.42	0.0	116.1	16.8	Up	4.44E-16	1.69E-15	*Eurotiomycetidae*
N3_21675	Isocitrate dehydrogenase (NAD+) (IDH3)	1.1.1.41	26.6	127.7	2.3	Up	1.60E-13	4.10E-13	*Trichocomaceae*
N3_36039	Isocitrate dehydrogenase (NAD+) (IDH3)	1.1.1.41	0.0	62.9	15.9	Up	4.44E-16	1.51E-15	*Trichocomaceae*
N3_25689	2-oxoglutarate dehydrogenase E1	1.2.4.2	0.0	131.4	17.0	Up	4.44E-16	1.85E-15	*Eurotiomycetidae*
N3_25690	2-oxoglutarate dehydrogenase E1	1.2.4.2	11.5	89.7	3.0	Up	0	0	*Eurotiomycetidae*
N3_32950	2-oxoglutarate dehydrogenase E1	1.2.4.2	0.3	15.5	5.8	Up	0.000137615	0.000182959	Bacteria
N3_17371	2-oxoglutarate dehydrogenase E2	2.3.1.61	0.0	27.6	14.8	Up	4.44E-16	1.75E-15	*Emericella nidulans*
N3_21757	2-oxoglutarate dehydrogenase E2	2.3.1.61	24.8	156.1	2.7	Up	4.11E-13	1.03E-12	*Proteobacteria*
N3_9942	2-oxoglutarate dehydrogenase E2	2.3.1.61	0.0	53.8	15.7	Up	4.44E-16	1.43E-15	*Emericella nidulans*
N3_32516	Dihydrolipoamide dehydrogenase	1.8.1.4	0.0	35.0	15.1	Up	4.44E-16	1.35E-15	*Bacillales*
N3_33478	Dihydrolipoamide dehydrogenase	1.8.1.4	0.0	64.4	16.0	Up	4.44E-16	1.62E-15	*Bacillales*
N3_12553	Dihydrolipoamide dehydrogenase	1.8.1.4	30.0	307.4	3.4	Up	6.75E-13	1.66E-12	*Trichocomaceae*

in the N2 and N3 samples. Aliphatic and branched-chain amino acids are the main pre-substrates for liquor flavor generation (Zhuang, 2007). Thus, these highly expressed genes involved in butanoate, propanoate and amino acid metabolism indicate that the liquor starter has great potential for flavor development.

We further analyzed starch metabolism, glycolysis and ethanol and pyruvate metabolism because they are important for ethanol production and are related to flavor generation. The results showed that the microbial community had a high capability for degrading starch with different functional enzymes throughout the liquor starter production process, especially during the high

temperature period. The key enzymes of glycolysis metabolism in the N2 and N3 samples were highly expressed. All the up-regulated genes in the glycolysis pathways were mainly from *Saccharomycetales* and *Mucorales*. *Saccharomycetales* are multifunctional microorganisms that saccharify sugar polymers, improve esterification, contribute to aroma precursors, utilize feedstocks efficiently and affect flavor. *Mucorales* belongs to the Zygomycetes family of filamentous fungi. They are robust fungi that show great promise for ethanol fermentation (Abedinifar et al., 2009) and the production of efficient and diverse carbohydrate-active enzymes (Battaglia et al., 2011). Therefore, the extremely highly expressed key glycolytic enzymes from *Saccharomycetales* and *Mucorales* were important for saccharification and ethanol fermentation. Additionally, more diverse microbial community members, including both fungi and bacteria, mainly contributed to pyruvate and ethanol metabolism at 50 and 62°C. Analysis of the important enzymes of pyruvate metabolism further showed that the NF liquor starter exhibited large capacities for converting pyruvate to intermediates, i.e., acetate and acetyl-CoA, which further contribute to other metabolic functions and specific flavor. The enzymes involved in the conversion of pyruvate to lactic acid were higher in the N2 and N3 samples and reduced in N4 sample. To some extent, this result was consistent with finding in the study by Huang et al. (unpublished data); *Lactobacillus* increased quickly when the liquor starter had incubated for 3 days (N2) and decreased markedly when the temperature reached its maximum of 62°C (N3), and fewer was found in N4. A markedly low level of lactic acid in the mature NF liquor starter is associated with high quality, and such starter can be further used for ethanol fermentation (Li, 2000; Lai, 2007).

The intermediates of the citrate cycle also have important functions in specific flavor generation. Thus, the citrate cycle is essential for many biochemical pathways in the liquor starter microbial community and it is necessary to understand the multiple functions of this cycle in the liquor starter process. The key enzymes of the citrate cycle were produced by both bacterial and fungal community members in the N2 and N3 samples. *Bacillales* species were the dominant bacterial community members when the room temperature increased to 62°C (Huang et al., unpublished data). The other relevant microbial community members were fungi. The high expression levels of the fungal community members also confirmed the high abundance of the active fungal community at the highest temperature point. All of these fungi have been reported to have high capabilities for degrading carbohydrates, fats and amino acids. Therefore, the N2 and N3 samples were more active and would have large capacities for generating energy and producing intermediates for liquor flavor generation. However, liquor flavor development is determined by a much more complex metabolic process; it is determined not only by the microbial community from the liquor starter but also by the Zaopei and pit mud. Thus, the mechanism of liquor flavor generation requires further comprehensive analysis of the microbial communities from the liquor starter, Zaopei and pit mud.

CONCLUSIONS

Chinese liquor starter is produced in a thermophilic and aerobic system. The present study used metatranscriptomics to globally and comprehensively explore the active microbial communities and their functional transcripts in NF liquor starter. The results demonstrated that fungi were the most abundant active community members during the liquor starter production process. The identified abundant pathways, diverse thermophilic carbohydrate-active enzymes, and up-regulated key enzyme genes that are involved in glycolysis, ethanol metabolism, pyruvate metabolism and the citrate cycle at 50 and 62°C implied that the liquor starter is capable of robust saccharification, fermentation and production of flavor-generating agents. A breakthrough occurred during this study regarding the understanding of microbial metabolism and the function of Chinese liquor starter, paving the way for the optimization of liquor production and for the discovery of special and scarce microbial resources and thermophilic enzymes. To obtain encompassing insights into Chinese liquor, which has been produced for several thousand years and involves a complex and dynamic ecosystem, further temporal and spatial studies are needed concerning the microbial communities involved throughout the entire liquor brewing process.

AUTHOR CONTRIBUTIONS

HZ, YH, KH, DL, HL, YJ, and YF designed the experiment; YH and ZY wrote the main manuscript, performed the experiment and analyzed data; YH, DZ, and HH collected samples and communicated with the liquor factory; YH, ZY, YF, and HZ revised the manuscript. All authors revised and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.01747/full#supplementary-material

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A Response Surface Methodology Approach to Investigate the Effect of Sulfur Dioxide, pH, and Ethanol on *DbCD* and *DbVPR* Gene Expression and on the Volatile Phenol Production in *Dekkera/Brettanomyces bruxellensis CBS2499*

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Dekkera/Brettanomyces bruxellensis, the main spoilage yeast in barrel-aged wine, metabolize hydroxycinnamic acids into off-flavors, namely ethylphenols. Recently, both the enzymes involved in this transformation, the cinnamate decarboxylase (*DbCD*) and the vinylphenol reductase (*DbVPR*), have been identified. To counteract microbial proliferation in wine, sulfur dioxide (SO_2) is used commonly to stabilize the final product, but limiting its use is advised to preserve human health and boost sustainability in winemaking. In the present study, the influence of SO_2 was investigated in relation with pH and ethanol factors on the expression of *DbCD* and *DbVPR* genes and volatile phenol production in *D. bruxellensis* CBS2499 strain under different model wines throughout a response surface methodology (RSM). In order to ensure an exact quantification of *DbCD* and *DbVPR* expression, an appropriate housekeeping gene was sought among *DbPDC*, *DbALD*, *DbEF*, *DbACT,* and *DbTUB* genes by GeNorm and Normfinder algorithms. The latter gene showed the highest expression stability and it was chosen as the reference housekeeping gene in qPCR assays. Even though SO_2 could not be commented as main factor because of its statistical irrelevance on the response of *DbCD* gene, linear interactions with pH and ethanol concurred to define a significant effect ($p < 0.05$) on its expression. The *DbCD* gene was generally downregulated respect to a permissive growth condition (0 mg/L mol. SO_2, pH 4.5 and 5% v/v ethanol); the combination of the factor levels that maximizes its expression (0.83-fold change) was calculated at 0.25 mg/L mol. SO_2, pH 4.5 and 12.5% (v/v) ethanol. On the contrary, *DbVPR* expression was not influenced by main factors or by their interactions; however, its expression is maximized (1.80-fold change) at the same conditions calculated for *DbCD* gene. While no linear interaction between factors

influenced the off-flavor synthesis, ethanol and pH produced a significant effect as individual factors. The obtained results can be useful to improve the SO_2 management at the grape harvesting and during winemaking in order to minimize the *D./B. bruxellensis* spoilage.

Keywords: *D./B. bruxellensis*, volatile phenols, cinnamate decarboxylase gene, vinylphenol reductase gene, gene expression, response surface methodology

INTRODUCTION

During the aging of red wines, mainly if they are stored in barrels, undesirable metabolites (off-flavors) can appear due to the growth of contaminating yeasts, such as *Dekkera/Brettanomyces bruxellensis* species (Silva et al., 2004). This sensory modification resulting in wine defect is termed "Brett character" and it is described by "leather," "horse sweat," "medicinal," "barnyard," and "bacon" descriptors (Chatonnet et al., 1995). In general, the spoilage by *Dekkera/Brettanomyces* yeasts can causes huge economic loss in wine industry and several methods for its rapid detection has been proposed (Tofalo et al., 2012; Vigentini et al., 2012; Uusitalo et al., 2017).

The origin of volatile phenols (VPs) involves the sequential action of enzymes acting on hydroxycinnamic acids, substrates that can be obtained through the activity of cinnamoyl-esterase enzyme on their respective cinnamic acids or released by fungal enzymes or by grape juice heating (Gerbaux et al., 2002). Being toxic for many microorganisms, hydroxycinnamic acids are decarboxylated by the action of cinnamate decarboxylase (CD), thus allowing a detoxification of the environment (Edlin et al., 1998).

It has been reported that the activity of CD releases vinyl derivatives (4-vinylphenol, 4-vinylguaiacol, and 4-vinylcatechol) (Dias et al., 2003a; Edlin et al., 1995). In particular, in *B. bruxellensis* LAMAP2480 a CD was identified as phenylacrylic acid decarboxylase (PAD1p), which is responsible for the production of 4-vinylphenol from *p*-coumaric acid, and encoded by the corresponding *DbPAD* gene (Godoy et al., 2014). Vinyl phenols are reduced into their corresponding ethyl derivatives (4-ethylphenol, 4-ethylguaiacol, and 4-ethylcatechol) in a step catalyzed by a vinylphenol reductase (VPR) that represents the key enzyme designating *D./B. bruxellensis* species as the spoilage yeast able to produce ethyl phenols. VPR enzyme was identified in *D. bruxellensis* CBS4481 as a Zn/Cu superoxide dismutase (SOD1) belonging a NAD(P)H-dependent oxidoreductases of the Short-chain Dehydrogenases/Reductases (SDRs) family (Granato et al., 2014). The cloning of *DbVPR* gene in *Saccharomyces cerevisiae*, a species not producing ethyl phenols, has recently confirmed its role in the off-flavor production (Romano et al., 2017).

The concentration of some wine components (i.e., ethanol, sugars, and VPs) and some chemical factors (i.e., pH and sulfur dioxide) have been demonstrated affecting the occurrence of off-flavors by *D./B. bruxellensis* (Dias et al., 2003b; Godoy et al., 2008; Sturm et al., 2014). This evidence has posed the need to investigate the interaction among multiple aspects on the production of VPs (Ganga et al., 2011; Chandra et al., 2014). For example, the influence of interactions due to the presence of *p*-coumaric acid, ferulic acid, and ethanol on CD activity and the expression of its putative gene has been studied (Ganga et al., 2011). Results outlined that although oenological concentrations of *p*-coumaric and ferulic acids alone did not produced any significant effect on the enzyme activity, this was influenced by interactions between ethanol and cinnamic acid or temperature. Recently, Chandra et al. (2014) analyzed the effect of glucose, ethanol and SO_2 on the growth and VP production by *B. bruxellensis* ISA 2211. A negative linear and quadratic effect triggered by SO_2 occurred on growth and 4-ethylphenol production; in particular, a SO_2 concentrations higher than 20 mg/L, at pH 3.50, induced immediate loss of cell culturability even under growth permissive levels of ethanol.

"Bret" character is often associated to the capability of *Brettanomyces* yeasts to grow under low level of molecular SO_2 concentration (Barata et al., 2008; Curtin C. et al., 2012; Vigentini et al., 2013). Thus, using high concentrations of SO_2 could ensure failure of *Brettanomyces* spoilage. However, reducing sulfite in wine represents a valuable task in view of a sustainable implementation in winemaking and a better acceptability for the consumers' health. The present study has investigated the expression of *DbCD* and *DbVPR* genes, being recently identified with certainty (Godoy et al., 2014; Romano et al., 2017), and the production of VPs in relation with wine's factors as SO_2, pH, and ethanol throughout a response surface methodology (RSM). The choice of the factors ensued taking into consideration that molecular SO_2 concentration depends on pH, ethanol concentration, and temperature (Usseglio-Tomasset and Bosia, 1984; Ribéreau-Gayon et al., 2006) and that, the latter is possibly the only manageable factor in aging process. Moreover, in order to ensure an exact quantification of mRNA transcription profile of *DbCD* and *DbVPR*, in the condition under study, an appropriate housekeeping gene (HKG) was identified.

MATERIALS AND METHODS

Yeast Strain and Maintenance

Dekkera bruxellensis CBS2499 was used in this study. Its whole genome sequence is available at http://genome.jgi.doe.gov/Dekbr2/Dekbr2.home.html (Piškur et al., 2012). Cells were stored in YPD medium (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose, 5.5 pH) supplemented with 20% (v/v) glycerol at −80°C. Cell revitalization was performed inoculating the glycerol stock at 1% (v/v) in YPD broth. Cultures were placed into an incubator (Heidolph, Schwabach, Germany) at 30°C for 3 days.

Growth Media and Culture Conditions

Experiments were run to collect yeast biomass for RNA extraction, retrotranscription and the analysis of gene expression by real-time quantitative PCR (qPCR). All fermentations were carried out in simil-wine Medium (SWM) [2.50 g/L glucose, 2.50 g/L fructose, 5 g/L glycerol, 5 g/L tartaric acid, 0.50 g/L malic acid, 0.20 g/L citric acid, 4 g/L L-lactic acid, 1.70 g/L yeast nitrogen base w/o AA and ammonium sulfate (Difco, Sparks, MD, United States), 0.005 g/L oleic acid, 0.50 mL tween 80, 0.015 g/L ergosterol, 0.020 g/L uracil, 0.010 g/L *p*-coumaric, 0.010 g/L ferulic acid, and 1.50 g/L ammonium sulfate]. Variants of SWM were prepared at different molecular SO_2 (below: SO_2) and ethanol concentration and pH value, adjusted with NaOH, depending on the conditions set by the chosen RSM (**Table 1**). Media was sterilized with 0.20 μm cellulose-nitrate filters. Cultural media were stored at 22°C prior the cell inoculation. SO_2 was added immediately before the inoculum from a 4 g/L sodium metabisulphite in mQ water. The theoretical content of molecular SO_2 was calculated according to Usseglio-Tomasset and Bosia (1984), Ribéreau-Gayon et al. (2006), and Duckitt (2012). Cellular growth was monitored by OD at 600 nm. Fresh cells in YPD broth were centrifuged at 3500 rpm for 15 min (Hettich, ROTINA 380R, Tuttlingen, Germany); then, cells were washed in 0.9% (w/v) NaCl and inoculated at 0.1 $OD_{600\ nm}$ in flask in SWM adjusted at 5% (v/v) ethanol, pH 4.5 and maintaining an air/medium ratio of at least 40% in order to ensure aerobic condition. Cellular pre-cultures were grown at 25°C for 3 days, in aerobic condition. An aliquot of the fresh cultures was analyzed by plate count to calculate the exact number of viable cells transferred into each variant of the SWM for the RSM (**Table 1**). The inoculum was carried out at 0.25 $OD_{600\ nm}$ in SWM modified as required by the RSM scheme (**Table 1**). The inoculated media were divided into 10 mL aliquots in sterile and hermetically closed tubes with no headspace volume, and cultivated at 22°C in static condition. Each aliquot sample was used once for analyses. Cellular growth was monitored daily by total plate count and OD_{600nm} measurement. At 1.00 ± 0.2 $OD_{600\ nm}$ cells two aliquots were pelleted by centrifugation (11000 rpm, 1 min, 4°C) (Hettich, ROTINA 380R, Tuttlingen, Germany), collecting a total cell amount of 20 $OD_{600\ nm}$, immediately frozen with liquid nitrogen and stored at −80°C until use. For the RSM scheme, the cultures were arranged according to the chosen experimental design.

Extraction of Total RNA and cDNA Synthesis

The extraction of total RNA from pellets was carried out using Presto Mini RNA Yeast Kit (Geneaid, New Taipei City, Taiwan) with few modifications. Briefly, cell lysis through mechanic disruption was performed in 500 μL Buffer RB, 5 μL β-mercaptoethanol, and an iso-volume of glass beads (425–600 μm, 154 Sigma–Aldrich, Saint Louis, MO, United States). Three breaking cycles with TissueLyser (Qiagen, Hilden, Germany) for 2 min at the maximum oscillation frequency, interchanged with 1 min on ice, were applied. The supernatant was centrifuged at 16000 × *g* for 3 min (Hettich, Tuttlingen,

TABLE 1 | Runs of Box–Behnken experimental design, normalized relative expression values of *DbCD* and *DbVPR* genes, expressed as fold-change, and quantification of vinyl phenol, vinyl guaiacol, ethyl phenol, and ethyl guaiacol, expressed as ratios between μmoles of product (volatile phenols) on μmoles of relative consumed precursor (coumaric and ferulic acids) for the different trials.

Run	mol. SO_2 (mg/L)	pH	Ethanol (v/v)	*DbCD*	*DbVPR*	Conc. (mg/L)						Yield (μM product/μM consumed acid)					
						p-cumaric acid	Ferulic acid	Vinyl phenol	Vinyl guaiacol	Ethyl phenol	Ethyl guaiacol	Vinyl phenol	Vinyl guaiacol	Ethyl phenol	Ethyl guaiacol
1	0	3.5	8.75	0.49	1.22	2.35	1.64	1.09	0.036	2.45	2.29	0.25	0.01	0.55	0.58
2	0.25	3.5	8.75	0.45	1.33	2.39	1.92	0.71	0.029	4.15	3.46	0.16	0.01	0.94	0.93
3	0	4.5	8.75	0.52	1.47	3.81	1.81	1.13	0	2.67	1.92	0.35	0.00	0.80	0.51
4	0.25	4.5	8.75	0.66	1.80	4.11	2.82	0.35	0	2.54	1.57	0.12	0.00	0.83	0.53
5	0	4	5	0.30	1.47	0.86	0.42	0.40	0	5.73	4.29	0.07	0.00	1.02	0.87
6	0.25	4	5	0.14	0.80	3.86	2.66	0.10	0	2.53	1.96	0.03	0.00	0.77	0.63
7	0	4	12.5	0.24	0.67	3.43	2.43	1.87	0.075	2.16	2.00	0.53	0.02	0.60	0.60
8	0.25	4	12.5	0.38	0.91	3.45	2.78	2.24	0.324	2.52	2.28	0.64	0.11	0.70	0.75
9	0.125	3.5	5	0.33	0.99	0.39	0.37	0.14	0	6.45	5.18	0.02	0.00	1.08	1.04
10	0.125	4.5	5	0.39	1.11	1.26	0.66	0.26	0	5.65	3.90	0.05	0.00	1.07	0.82
11	0.125	3.5	12.5	0.40	0.94	2.34	2.33	1.75	0.092	2.41	2.70	0.40	0.03	0.54	0.80
12	0.125	4.5	12.5	0.65	0.92	4.08	2.07	1.55	0.021	2.06	1.37	0.51	0.01	0.66	0.38
13	0.125	4	8.75	0.21	0.85	3.16	2.13	0.59	0	3.56	2.62	0.16	0.00	0.93	0.74
14	0.125	4	8.75	0.15	0.89	2.29	1.61	0.51	0.024	2.60	2.00	0.11	0.01	0.58	0.50
15	0.125	4	8.75	0.18	0.72	3.17	2.06	0.76	0	3.37	2.61	0.20	0.00	0.88	0.72

Germany). The genomic DNA residue was degraded using 100 μL of 2 KU/mL DNase (Sigma–Aldrich, St. Louis, MO, United States) for 15 min at room temperature. Following steps were carried out according to the manufacturing's instructions. RNA concentration was determined by measuring the absorbance at 260 nm (BioTek, Winooski, VT, United States). The integrity of RNA sample (0.3 μg RNA, 2 μL RNA loading Buffer 5X, H_2O DEPC up to 10 μL) was assessed, after 5 min treatment at 65°C, by electrophoresis on 1.2% agarose gel [90 mL DEPC water, 10 mL 10X formaldehyde gel buffer (200 mM MOPS, 50 mM sodium acetate, and 10 mM EDTA)] adjusted at 7 pH with NaOH prepared in DEPC water 37% (v/v) formaldehyde added. The electrophoretic run was carried out at 100 V for 1 h and bands were UV visualized (Bio-Rad, Berkeley, CA, United States). RNAs were stored at −80°C until cDNA synthesis. The RNA retrotranscription was obtained with the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany). cDNAs were stored at −20°C until used for the qPCR assays.

Primer Design

Five genes, pyruvate decarboxylase (PDC) (*DbPDC*), aldehyde dehydrogenase (*DbALD*), actin (*DbACT*), eukaryotic translational elongation factor (EF) (*DbEF*), and tubulin (*DbTUB*), were analyzed to identify a HKG suitable in the normalization process of the gene expression of CD (*DbCD*) and VPR (*DbVPR*) (**Table 2**). Gene sequences of *DbPDC* and *DbALD* were identified using *S. cerevisiae* S288C (Schifferdecker et al., 2014), *Komagataella phaffii* CBS7435 and GS115, *D. bruxellensis* CBS2499 (Piškur et al., 2012), and *B. bruxellensis* AWRI1499 (Curtin C.D. et al., 2012) genomes. SGD[1], NCBI[2], and ENA[3] databases were used as sequence sources. All alignments were performed through BLAST and ClustalIX2. Primer pairs were obtained at NCBI website[4] and validated for no forming neither self nor cross-dimers[5] (**Table 2**). The *DbCD* gene sequence for primer design was deduced by Godoy et al. (2014).

PCR Assays

Two sets of gene expression analysis were set up under different oenological conditions: (i) to identify a suitable HKG for gene expression normalization; (ii) to analyze the relative expression of *DbCD* and *DbVPR*, by using the gene identified in (i). As far the primers couples designed in this study for *DbCD*, *DbALD,* and *DbPDC* genes, they were also validated by a standard PCR amplification in a 25 μL reaction composed by: 1 U Taq, 200 μM dNTPs (Biotech rabbit, Dusseldorf, Germany), 1X Taq Buffer (Genscript, Piscataway, NJ, United States), 1 mM $MgCl_2$ (5Prime, Hilden, Germany), 0.1 μM primer forward and 0.1 μM primer reverse (Eurofins Genomics, Ebersberg, Germany), and 80–100 ng DNA. The amplification cycle was: 95°C for 6 min, 95°C for 45 s/54°C for 30 s/72°C for 1 min (repeated 34 times), and 72°C for 10 min. Results were visualized on a 2% agarose gel prepared in TAE 1X buffer (20 mL TAE 50X, 980 mL demineralized water) and 0.5 μg/mL ethidium bromide. Electrophoresis was set at 80 V for 1.30 h. PCR products were sequenced by an external provider (Eurofins genomics, Ebersberg, Germany).

As far qPCRs, they were performed in a Realplex Mastercycler EP Gradient Thermocycler (Eppendorf, Hamburg, Germany) using a 15 μL reaction mix composed as follow: 2X SYBR Green Master-Mix (Biotech rabbit, Dusseldorf, Germany), 200 nM-100 nM-50 nM primer forward and primer reverse (Eurofins genomics, Ebersberg, Germany), and 10-fold dilution cDNA. The qPCR amplification cycle was set at 95°C for 30 s, 54°C for 30 s, and 65°C for 30 s; repeated for 40 times. At the end of the reaction (95°C for 15 s), a melt-curve was generated by increasing the temperature from 60 to 95°C, with a step at 0.5°C. All cDNAs were run as technical duplicates in a 96-well plate (Eppendorf, Hamburg, Germany). For each gene, three decimal serial dilutions at least were prepared into DNA LoBind

[1]http://www.yeastgenome.org

[2]https://www.ncbi.nlm.nih.gov

[3]http://www.ebi.ac.uk/ena

[4]https://www.ncbi.nlm.nih.gov/tools/primer-blast

[5]https://www.thermofisher.com

TABLE 2 | Primer pairs used for quantitative PCR (qPCRs).

Oligo name	Sequence (5′ → 3′)	Tm (°C)	Reference
*DbALD*_F	CTATCAAGGTCGGAAACCCA	57.3	This study
*DbALD*_R	TCTCTCACCACCAGTAAGGA	57.3	This study
*DbACT*_F	TTATTGATAACGGTTCTGGTATGT	55.9	Nardi et al., 2010
*DbACT*_R	ACCCATACCGACCATGATAC	57.3	Nardi et al., 2010
*DbEF*_F	CTCCAGTTGTTGACTGCCA	56.7	Nardi et al., 2010
*DbEF*_R	CATCTTAACCATAGCAGCATCAC	58.9	Nardi et al., 2010
*DbPDC*_F	GTGGTTTGCTTTCCGACTAC	57.3	This study
*DbPDC*_R	AAACAGCGGACTTGACCTTAC	57.9	This study
*DbTUB*_F	GTATCTGCTACCAGAAACCAACC	60.6	Rozpędowska et al., 2011
*DbTUB*_R	CCCTCACTAACATACCAGTGGAC	62.4	Rozpędowska et al., 2011
*DbCD*_F	CACAGACTCGAACGGAAAAC	57.3	Godoy et al., 2014
*DbCD*_R	CCAGGGCGTACACATTGATA	57.3	Godoy et al., 2014
*DbVPR*_F	CTAAGGGCACTATCAAGGACA	57.9	Romano et al., 2017
*DbVPR*_R	CTGCAAAGAACCAGCATCA	54.5	Romano et al., 2017

tubes (Eppendorf, Hamburg, Germany) and stored at −20°C. The amplification curves were analyzed with Realplex software (Eppendorf, Hamburg, Germany).

The $2^{-\Delta\Delta C_T}$ method was applied on the basis of Livak and Schmittgen (2001) to calculate the relative expression of *DbCD* and *DbVPR* respect the chosen HKG expression. Results were expressed as fold-changes whereas the expression value of the target gene (normalized against *DbTUB* expression) was expressed as increase or decrease respect to its expression in the calibrator (for equivalent amount of samples) corresponding to the growth condition "LS" [0 mg/L mol. SO_2, pH 4.5 and 5% (v/v) ethanol] described in the paragraph "Gene Expression Stability."

Gene Expression Stability

The expression of *DbPDC*, *DbALD*, *DbACT*, *DbEF*, and *DbTUB* genes was evaluated setting up a qPCR multiplex assay under two different oenological conditions of the SWM called "low-" and "high-" stringent (LS and HS, respectively) growth conditions. In particular, the LS condition was characterize by 0 mg/L mol. SO_2, pH 4.5 and 5% (v/v) ethanol while the HS condition by 0.25 mg/L mol. SO_2, pH 3.5 and 12.5% (v/v) ethanol. Yeast cultures were prepared in duplicate; three RNA extractions and the following cDNA synthesis were performed from each independent culture.

GeNorm analysis (Vandesompele et al., 2002) (Genex software version 4.3.6, MultiD analyses, Gothenburg, Sweden) was used to determine the stability of gene expression (termed *M*-value), by analyzing each reference gene against the others in a pairwise variation that serially excludes the least stable gene (highest *M*-value) from the analysis. At the end, genes are ranked with an accepted cut-off value of 0.50 according to their expression stability. Normfinder algorithm (Genex software version 4.3.6, MultiD analyses, Gothenburg, Sweden) separates the variation into an intra-group and an inter-group contribution. The analysis is repeated without considering the groups and this, estimates a robust standard deviation (SD) for each gene. The accumulated standard deviation (Acc. SD) is a reliable indicator of the number of reference genes to be used. All the genes were analyzed in the same assay to reduce any further experimental variability.

Experimental Design and Response Surface Methodology

In order to investigate the expression of *DbCD* and *DbVPR* genes and the production of VPs in oenological conditions a Box–Behnken experimental design and RSM were applied. SWM samples were formulated with different level % ethanol (v/v) (5 – 8.75 – 12.5), pH values (3.5 – 4.0 – 4.5), and molecular SO_2 (mg/L) (0 – 0.125 – 0.25) (**Table 1**). The 15 trials provided by Box–Behnken experimental design were analyzed using Statgraphics Plus 5.1 software. The expression values of investigated genes were normalized with the HKG expression.

The fit of the model was evaluated by the linearity coefficient (R-squared). The regression approach was used to determine the effects produced by SO_2, pH, and ethanol variables. The main effects (A, B, and C) and both the linear (AB, AC, and BC) and quadratic effects (AA, BB, and CC) were statistically validated by analysis of variance. To identify the most important factors, a standardized Pareto chart is drawn. In particular, each effect is converted to a t-statistic by dividing it by its standard error (data not shown). These standardized effects are then plotted in decreasing order of absolute magnitude. Statistically relevant effects with a *p*-value less than 0.05 (95% confidence level) were reported in a response surface graph where the three-dimensional surface is described by a second-order polynomial equation.

Determination of VPs

The content of hydroxycinnamic acids, namely *p*-coumaric and ferulic acids, vinyl phenol, vinyl guaiacol, ethyl phenol, and ethyl guaiacol in the cultures of the 15 runs of Box–Behnken experimental design was assessed in the obtained samples by an Acquity HClass UPLC (Waters, Milford, MA, United States) system equipped with a photo diode array detector 2996 (Waters). Chromatographic separations were performed with a Kinetex C18 150 mm × 3 mm, 2.6 μm particle size, 100 Å pore size (Phenomenex, Torrance, CA, United States). Eluting solvents were (A) trifluoroacetic acid 0.05% (v/v) and (B) methanol. The gradient program was 0.1 min, 20% B; 0.1–2 min, 35% B; 2–14 min, 58.5% B. The separation run was followed by 7 min of column rinsing and conditioning. The flow rate was 0.5 mL/min and the column temperature was 28°C. The samples were filtered with PVDF 0.22 μm filter prior the injection. Calibration curves were obtained for *p*-coumaric and ferulic acids, vinyl phenol, vinyl guaiacol, ethyl phenol, and ethyl guaiacol concentrations in the range from 0.1 to 20 mg/L. Quantification was performed according to the external standard method. Data acquisition and processing were carried out by Empower 2 software (Waters) at 320, 280, and 260 nm for hydroxycinnamic acids, ethyl phenols, and vinyl phenols, respectively. Yield values of VPs were calculated as the molar ratio between each product (vinyl phenol, vinyl guaiacol, ethyl phenol, and ethyl guaiacol) and the corresponding hydroxycinnamic acid potentially used as substrate. Data were analyzed with Statgraphics Plus 5.1 using the RSM approach.

RESULTS

The aim of the study was to investigate the expression of *DbCD* and *DbVPR* genes and the production of VPs in a range of oenological conditions. To do that, we defined the experimental conditions at the realistic concentrations of some factors found in wines along with the requirement to have conditions compatible with cell growth. Different runs (**Table 1**) were performed to obtain gene expression values workable through a RSM approach under the tested conditions: SO_2 levels ranged from 0 to 0.25 mg/L, pH varied between 3.5 and 4.5 units and ethanol concentrations between 5 and 12.5% (v/v).

Identification of *DbPDC* and *DbALD* Genes in *D. bruxellensis*

DbPDC gene was identified in the scaffold 1 at 1700 bps (e_gw1.1.1485.1) of *D. bruxellensis* CBS2499 genome; in particular, the nucleotide sequence showed about 55% identity

with the *S. cerevisiae* genes encoding for *PDC1*, *PDC5,* and *PDC6* (55.1, 55.8, and 55.5%, respectively). Due to the similar level of identity found among the three isoforms, *PDC1* sequence was chosen for a further investigation in the genome of *B. bruxellensis* AWRI1499. The nucleotide sequence with accession number "EIF49850.1" was identified as a possible homologous of *S. cerevisiae PDC* gene with an identity of 55% (identity of 96.9% with e_gw1.1.1485.1). In *K. phaffii* genome, the gene codifying for *KpPDC* showed two potential isoforms differently located in *K. phaffii* CBS7435 (chromosomes 3 and 4). Only the sequence on the chromosome 3 identified the homologous gene (identity of 100%) on the genome of the strain *K. phaffii* GS115, with accession number XM_002492352.1. Thus, this gene was aligned against *D. bruxellensis* CBS2499 and the sequence in the scaffold 1 (e_gw1.1.1485.1) was confirmed as the potential homologous gene of *KpPDC* (55.5%identity). In conclusion, the open reading frames represented by the accessions e_gw1.1.1485.1 and EIF49850.1 of *D. bruxellensis* CBS2499 and *B. bruxellensis* AWRI1499, respectively, were identified as the homologous genes of *ScPDC1* and *KpPDC*.

As regards *DbALD*, among the three genes (*ScALD3, ScALD2,* and *ScALD6*) encoding for the sequence of *ScALD6* of *S. cerevisiae* S288c genome led to the identification of a possible homologous gene in *D. bruxellensis* CBS2499 genome in the scaffold 4 at 1523 bps (e_gw1.4.403.1) with an identity of 55.6%. *ScALD6* sequence was also aligned against the genome of *B. bruxellensis* AWRI1499 and the resulting amino acid sequence with the accession number "EIF46557.1" showed an identity of 56% (99.4% identity with e_gw1.4.403.1). In *K. phaffii* genome, the gene encoding for *KpALD* was identified on different chromosomes; the nucleotide sequence in the scaffold 20 at 1496 bps (e_gw1.20.29.1) of the chromosome 3 of the strain CBS7435 showed the highest identity (67.8%) with both e_gw1.4.403.1 and EIF46557.1 open reading frames of *D. bruxellensis* CBS2499 and *B. bruxellensis* AWRI1499 genome, respectively. Thus, these last genes were used for primer design being considered the homologous genes of *ScALD6* and *KpALD*.

Primer Validation in Standard PCRs and Optimization of qPCR Experiments

The primer pairs designed on *DbALD*, *DbPDC,* and *DbCD* were evaluated for their ability to produce a specific fragment through a standard PCR and further sequencing of the amplified products. A unique amplification product of 140 bps for all the three genes investigated was obtained (data not shown). This value corresponds to the expected product length on the base of the size (**Table 2**) of *in vitro* primers design. No aspecific products were detected and no amplification was observed with *S. cerevisiae* S288C and *K. phaffii* GS115 used as negative controls. Primer specificity was confirmed by sequencing with a 100% identity with the target sequences.

All primers designed for the amplification of the potential HKGs (*DbALD, DbPDC, DbEF, DbTUB,* and *DbACT*) and the target genes (*DbCD* and *DbVPR*) were validated to assess whether the qPCR reactions were really optimized. Five dilutions of cDNA samples obtained from cell culture of *D. bruxellensis* CBS2499 grown in SWM at LS condition [0 mg/L SO_2, pH 4.5, 5% (v/v) ethanol] were tested to evaluate the ones containing from 10^3 to 10^6 copies of template that were able to give amplification curves between 30 and 20 C_T values, respectively. The obtained C_Ts values were relatively low and similar; the lowest one (about 13) was given by *DbEF* gene, while the highest (about 20) was obtained for *DbCD* gene, thus revealing similar expression levels among the amplified genes. Then, a standard curve was created to assess primer efficiency of both the target genes and potential HKGs, as well as to be used as "standard" within the normalization plate used for HKG identification by qPCR. The R^2 values obtained for all primer pairs ranged from 0.980 to 0.999.

Analysis of the Gene Expression Stability of Potential HKGs

Five genes were evaluated for this purpose (**Table 2**): two genes encoding for metabolic enzymes, PDC and acetaldehyde dehydrogenase (ALD), were chosen based on their important role on fermentative metabolism and on NAD(P)H supply. The three others, encoding for EF, tubulin (TUB), and actin (ACT), have been already used as HKG in other studies (Nardi et al., 2010; Rozpędowska et al., 2011; Moktaduzzaman et al., 2016).

DbALD, DbPDC, DbEF, DbTUB, and *DbACT* were analyzed by a qPCR multiplex assay to identify the reference gene with a constant expression level across the experimental conditions under study. Expression stability of potential HKG genes were assessed at the two extreme growth conditions of the used experimental design, LS [0 mg/L SO_2, pH 4.5, 5% (v/v) ethanol] and HS [0.25 mg/L SO_2, pH 3.5, 12.5% (v/v) ethanol]. The cultures showed a negligible lag phase reaching a similar final biomass (1.4–1.7 $OD_{600\ nm}$) in 8 days. The absolute quantification approach was employed to obtain the qPCR results from the assayed normalization plate. Thus, a direct comparison between C_Ts of each sample and C_Ts of the standards (corresponding to the transcript copy number of each serial dilution of the HKG candidates) was accomplished. Overall, genes presented C_Ts spanning from 11 to 20, with *DbEF* and *DbPDC* having the lower values (**Table 3**). C_T data were submitted to GeNorm (Vandesompele et al., 2002) and Normfinder analysis. Because of the elimination process, GeNorm algorithm cannot identify an optimum reference gene and ended up by suggesting a pair of genes having the best same *M*-value of 0.186, *DbACT* and *DbTUB* (**Table 3**). For a single gene discrimination, Normfinder was employed along with GeNorm algorithm. Since samples came from two different treatment groups, Normfinder algorithm separated the variation into an intra-group and an inter-group contribution. The analysis was then repeated without considering the groups and this allowed to estimate a robust SD; the lowest SD (0.0929) was assigned to *DbTUB* (**Table 3**). A minimal value of the accumulated standard deviation was a great indicator of the optimal number of reference genes to be used for normalization. The highest expression stability revealed by *DbTUB*, attributed by both the lowest *M*-value and the SD, identifying this gene as the HKG for this study.

Effect of SO_2, pH, and Ethanol on *DbCD* and *DbVPR* Gene Expression

Real-time qPCR assays were carried out to test all conditions of the experimental design in order to study the role of SO_2, pH, and ethanol on *DbCD* and *DbVPR* genes expression. All the assays produced amplification curves in the range of the best sensitivity of the qPCR (20–30 C_T values) and a high reproducibility within a single test and among tests was obtained; indeed, an overlapping of the amplification curves of the replicates of both each run and the calibrator was observed. This was particularly evident in the case of *DbTUB* amplification that showed a constant gene expression (C_T value of 23) among the 15 conditions evaluated, confirming once again its reliable role as HKG.

Although the experimental design has to be considered functional to only apply the RSM approach and data cannot be individually interpreted as not obtained from biological replicates (except for runs 13, 14, and 15), it was possible to observe that *DbCD* gene was downregulated in all the tested conditions with fold-change values ranging between 0.14 and 0.66 (**Table 1**). The application of the Box–Behnken results to the RSM approach allowed to analyze how the *DbCD* gene expression was influenced by SO_2, pH, and ethanol by predicting further expression values inside the environment of the tested variables. Indeed, as regards the *DbCD* gene expression, a high R-squared values indicated a good fit of the model to the experimental data explaining the 98.3% (R-squared) of the *DbCD* gene variability (**Table 4**). Main and interaction effects (linear and quadratic) of the factors on the gene expressions are reported in **Table 5** and shown in the standardized Pareto chart (**Figure 1**). While pH and ethanol factors produced a significant effect (P-value < 0.01) on the *DbCD* gene expression, SO_2 did not affect it. On the contrary, linear interactions between SO_2 and pH and SO_2 and ethanol revealed a substantial influence (P-value < 0.05) (**Table 5** and **Figure 1**) thus concurring to define the response represented as three-dimensional surface (**Figures 2A,B**).

The shape of the surface obtained for SO_2 and pH interaction (**Figure 2A**) on the response reflected the predominant inhibition by pH, since the expression of the gene decrease rapidly up to pH 4. In particular, the change in *DbCD* expression occurring from the lowest to the highest level of pH (**Figure 3A**) was the same for both 0.125 and 0.250 mg/L levels of SO_2; the parallel trend of lines indicated that the effect of the pH on the response is probably not dependent from these SO_2 values. Even when pH was in the range 3.5–4 and SO_2 at 0 mg/L, the observed lines were almost parallel with respect to the other lines (with an overlapping between 0 and 0.250 mg/L of SO_2). On the contrary, when pH was set between 4 and 4.5 a moderate interaction of this factor with SO_2 occurred (lines are not parallel) (**Figure 3A**).

TABLE 3 | Candidate genes for their potential as housekeeping genes (HKGs).

Gene	C_T values								*M*-Value	Acc. SD
	LSA	LSA	LSB	LSB	HSA	HSA	HSB	HSB		
DbALD	19.51	19.77	20.10	20.12	19.05	18.62	18.6	18.5	0.373	0.2398
DbPDC	14.2	13.98	14.04	13.94	14.46	14.65	15.06	15.13	0.564	0.1523
DbEF	11.77	11.65	12.22	12.19	12.66	12.64	13.42	13.19	0.741	0.2762
DbTUB	16.71	16.83	16.86	16.49	16.89	17.04	17.28	17.34	0.186	0.0929
DbACT	17.58	17.23	17.27	17.53	18.17	18.13	18.01	17.75	0.186	0.1443

The second row indicates the two tested conditions: LS, low stringent growth condition and HS, high stringent growth condition, performed in two independent replicates (A and B). From each replicate three mRNAs were extracted and analyzed in qPCR assays. M-value is calculated by the GeNorm analysis while Normfinder algorithm and GenEx software calculate the accumulated standard deviation (Acc. SD) that is the expected SD if multiple reference genes are used for normalization.

TABLE 4 | Regression equations which fitted to the data of the Box–Behnken experimental design.

Variable (*y*)	Regression model equation	R^2 (%)
DbCD gene	$y = 17.797 - 5.704*A - 8.5935*B - 0.0976963*C + 5.47467*AA + 0.756*AB + 0.159467*AC + 1.05217*BB + 0.0249333*BC - 0.000245926*CC$	98.3
DbVPR gene	$y = 21.2275 - 12.6293*A - 10.3565*B + 0.186785*C + 19.4453*AA + 0.88*AB + 0.4864*AC + 1.32733*BB - 0.0189333*BC - 0.011603*CC$	87.3
Vinyl phenol yield	$y = -0.10787 + 0.203333*A + 0.173333*B - 0.10463*C + 4.34667*AA - 0.56*AB + 0.08*AC - 0.0183333*BB + 0.0106667*BC + 0.00660741*CC$	94.5
Vinyl guaiacol yield	$y = -0.619907 - 0.523333*A + 0.346667*B - 0.0109259*C + 0.773333*AA + 0.0*AB + 0.048*AC - 0.0416667*BB - 0.00266667*BC + 0.00121481*CC$	81.2
Ethyl phenol yield	$y = 2.62032 + 5.05*A - 0.6825*B - 0.161407*C - 2.61333*AA - 1.44*AB + 0.186667*AC + 0.0966667*BB + 0.0173333*BC + 0.00118519*CC$	71.8
Ethyl guaiacol yield	$y = 2.08079 + 4.24667*A - 0.385833*B - 0.060037*C - 2.02667*AA - 1.32*AB + 0.208*AC + 0.0633333*BB - 0.0266667*BC + 0.00645926*CC$	78.2

Factors are mol SO_2 (A), pH (B), and Ethanol (C). The second-order equations show main (A, B, and C), linear (AB, AC, and BC), and quadratic effects (AA, BB and CC). Coefficients are the regression coefficients for the considered variable. R-squared statistic indicates that the model as fitted explains a certain % of the variability in the considered variable.

TABLE 5 | Statistical analysis (value are expressed as *P*) of main effect of three variables and their interaction for *DbCD* and *DbVPR* expression levels and volatile phenol productions.

			Yield (μM product/μM consumed acid)			
Factor	***DbCD* gene**	***DbVPR* gene**	**Vinyl phenol**	**Vinyl guaiacol**	**Ethyl phenol**	**Ethyl guaiacol**
Mol SO_2 (A)	0.456	0.989	0.3090	0.1805	0.5915	0.5164
pH (B)	**0.003**	0.190	0.4067	0.5201	0.6185	**0.0395**
Ethanol (C)	**0.004**	0.146	**0.0003**	**0.0323**	**0.0283**	0.0934
AA	**0.006**	**0.029**	0.1556	0.3078	0.6576	0.6859
BB	**0.000**	**0.021**	0.9146	0.3727	0.7917	0.8387
CC	0.8635	0.164	0.0710	0.1694	0.8552	0.2733
AB	**0.050**	0.593	0.4111	1.0000	0.3294	0.2973
AC	**0.010**	0.064	0.3810	0.0791	0.3417	0.2277
BC	0.052	0.727	0.6303	0.6456	0.7126	0.5124

Bold values are those considered statistically significant (P < 0.005).

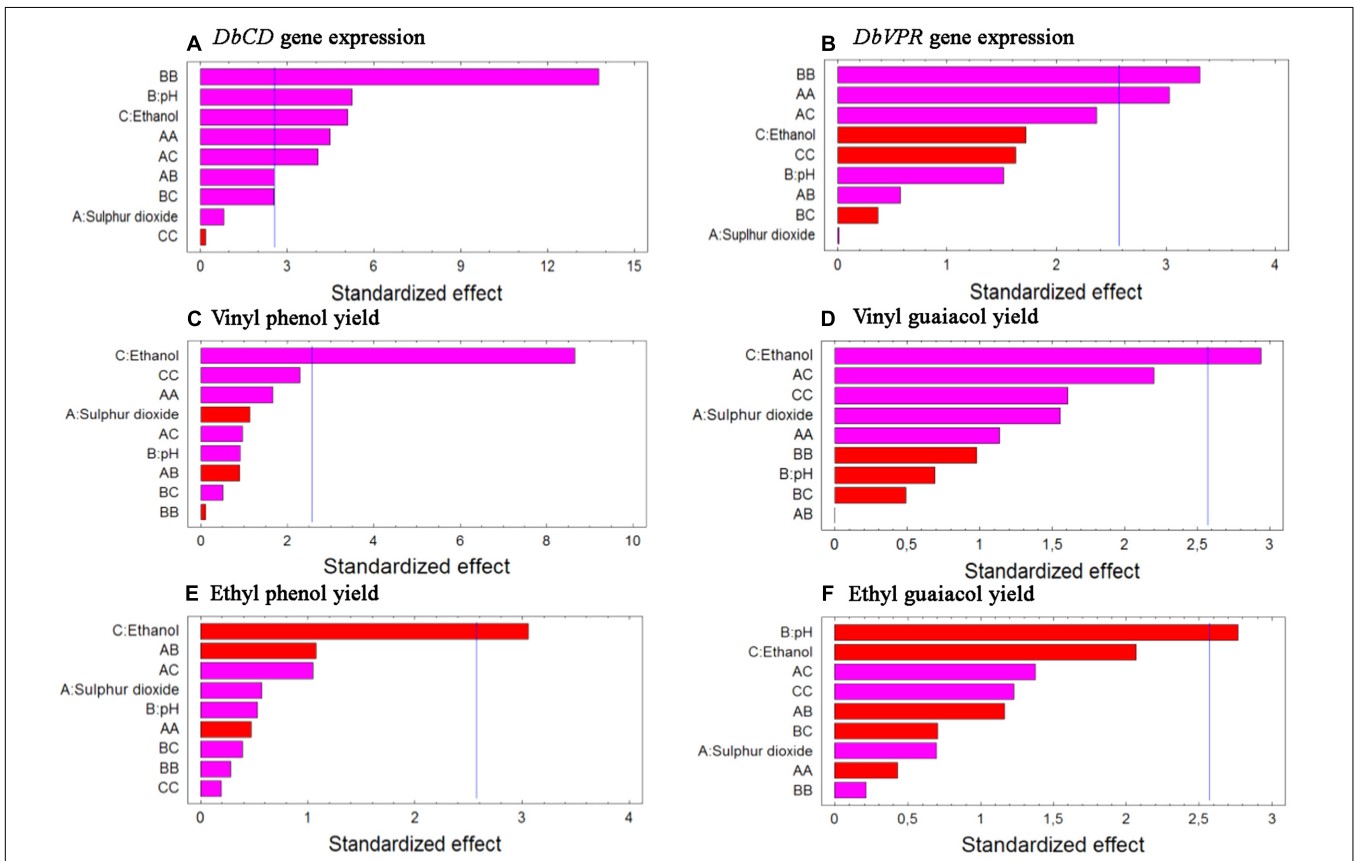

FIGURE 1 | Standardized Pareto charts for each analyzed variable **(A)** *DbCD* and **(B)** *DbVPR* gene expression, **(C)** vinyl phenol, **(D)** vinyl guaiacol, **(E)** ethyl phenol, and **(F)** ethyl guaiacol yields. The color of the bars shows whether an effect is positive (pink) or negative (red). A line is drawn on the chart beyond which an effect is statistically significant at the specified significance level of 5%.

On the other hand, the interaction between SO_2 and ethanol produced a response that changed faster as function of ethanol (**Figure 2B**). In detail, considering ethanol from 5 to 12.5% (v/v) and SO_2 at the concentration of 0 mg/L, 0.125 mg/L or 0.250 mg/L, the observed lines were not parallel indicating that an interaction between ethanol and SO_2 exists (**Figure 3B**). If ethanol at 5% (v/v) interacted with 0.25 mg/L SO_2, the *DbCD* expression was lower than the one revealed by the condition at 0 mg/L SO_2. This is probably due to the effect of ethanol along with SO_2 in determining more stress to the cell. Moreover, the expression at 0 mg/L SO_2 and 8.75% (v/v) ethanol was slightly lower than the one revealed at 0 mg/L SO_2 and 5% (v/v) ethanol.

The comparison between the two interaction plots (**Figures 3A,B**) allowed identifying the SO_2-ethanol as the

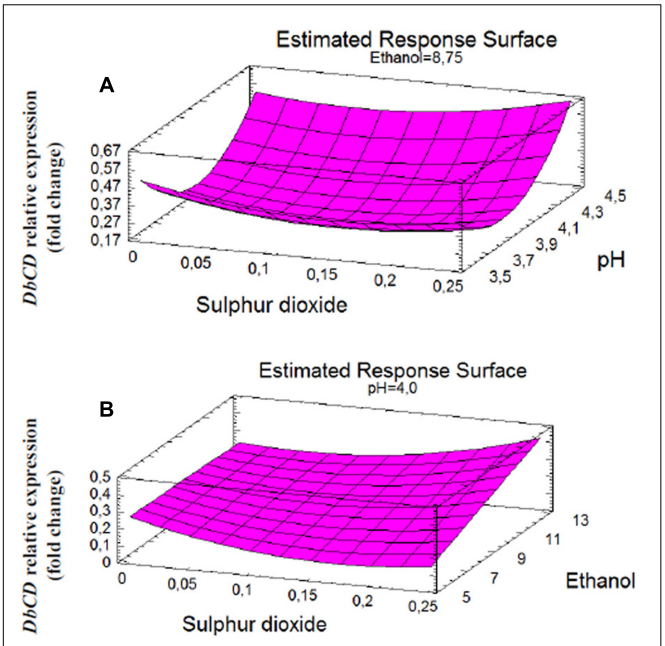

FIGURE 2 | Response surface fitted to experimental data points corresponding to: **(A)** *DbCD* expression as function of SO_2 and pH interaction (AB); **(B)** *DbCD* expression as function of SO_2 and ethanol interaction (AC).

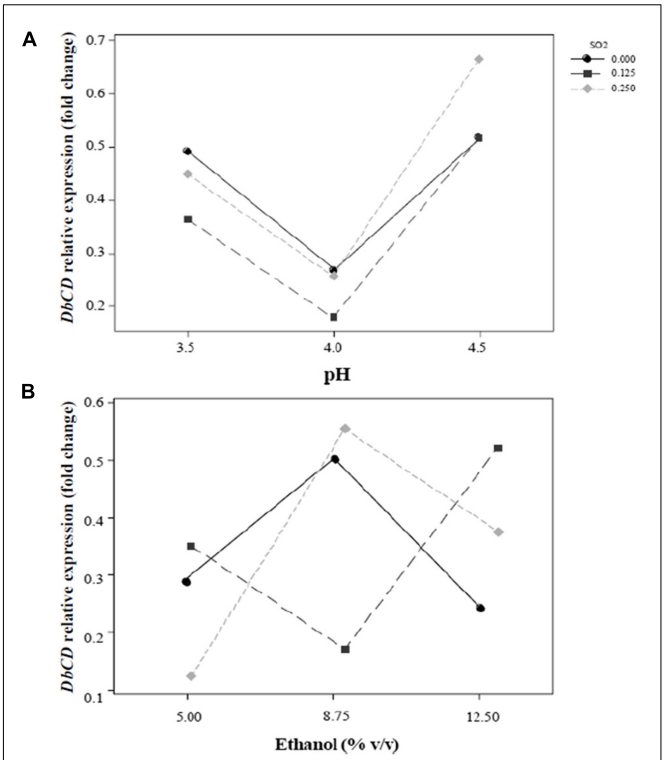

FIGURE 3 | Interaction plots for the expression of *DbCD* gene. **(A)** SO_2 and pH (AB); **(B)** SO_2 and ethanol concentration (AC). Lines represent the predicted responses at further experimental combinations among the analyzed factors. Continuous line (●), 0 mg/L mol. SO_2; long-dashed line (■), 0.125 mg/L mol. SO_2; short-dashed line (♦), 0.25 mg/L mol. SO_2.

stronger interaction to define the expression of *DbCD*, as also showed by the *p*-value of this linear interaction (AC, **Table 5** and **Figure 1**).

Finally, based on the response surfaces for *DbCD* gene expression and the model equation it is also possible to predict further responses in addition to those obtained in this study; according to this prediction approach, the combination of the factor levels that maximizes the *DbCD* expression (0.834-fold change) is at 0.25 mg/L, 4.5 and 12.5% (v/v), respectively, for SO_2, pH, and ethanol.

As far the *DbVPR* gene expression, it showed a different trend in regulation in comparison to *DbCD* gene. Even if data of the experimental design cannot be singularly interpreted, *DbVPR* seemed to be upregulated in runs 1, 2, 3, 4, 5, and 10 with fold-change value ranging between 1.11 and 1.80, whereas in the other cases it was slight downregulated, being values lower than 1. Interestingly, following the results in **Table 1**, although *DbCD* and *DbVPR* genes were expressed at their maximum level under the same growth condition corresponding to SO_2 0.25 mg/L, pH 4.5, ethanol 8.75% (v/v) (run 4). The statistical processing of expression data provided a regression equation of the proposed model with a goodness of fit of 87.3% (R-squared) (**Table 4**). In this case only a positive quadratic effect of SO_2 and pH resulted statistically significant on the *DbVPR* gene expression being all the other factors, main and interactions, characterized by *p*-values higher than 0.05 (**Table 5**). In agreement with the RSM approach, *DbVPR* expression was maximizes (1.80-fold change) at 0.25 mg/L SO_2, pH 4.5, and 12.5% (v/v) ethanol, as observed for the *DbCD*.

Effect of SO_2, pH, and Ethanol on VP Production

The release of VPs was determined in the experimental conditions adopted in the Box–Behnken experimental design. Although 10 mg/L of each hydroxycinnamic acid were added to the SWM, the initial concentrations of *p*-coumaric acid and ferulic acid were estimated at 8.40 ± 0.07 mg/L and 6.71 ± 0.25 mg/L, respectively. As expected, these compounds proportionally decreased as the VPs increased (data not shown). The highest concentration of VPs was reached under a condition that is more permissive the yeast growth [SO_2 0.125 mg/L, pH 3.5 and ethanol 5% (v/v)] in comparison to the expression of *DbCD* and *DbVPR* genes [SO_2 0.25 mg/L, pH 4.5, and ethanol 12.5% (v/v)]. Indeed, VPs are released at a final concentration of 6.45 and 5.18 mg/L of ethyl phenol and ethyl guaiacol, respectively, in run 9 whereas *DbCD* and *DbVPR* genes were approximatively half of the expression values detected in run 4.

In general, some considerations arose from the calculated yields of VPs (**Table 1**). First, the lowest conversion of acids in the corresponding vinyl compounds was detected for the vinyl guaiacol that was mostly produced at trace level in all the analyzed runs (**Table 1**). We could speculate that this behavior could be linked to a higher activity of *DbCDp* toward the coumaric acid rather than the ferulic

acid. On the contrary, ethyl phenol and ethyl guaiacol yields were found relatively balanced each other suggesting a similar capability of the *DbVPR* enzyme to transform its two substrates, the vinyl derivates. However, for this observation studies are required to analyze the activity of *DbCDp* and *DbVPRp* in the metabolic pathway of VPs under enological conditions.

Data processing by the RSM approach released four second-order equations with R-squared values indicating that the model as fitted explained 94.5, 81.2, 71.8, and 78.2% of the variability in vinyl phenol, vinyl guaiacol, ethyl phenol, and ethyl guaiacol molar ratios, respectively (calculated against the corresponding substrates of hydroxycinnamic acids). Main and interaction effects (linear and quadratic) of the factors on the VP production are reported in **Table 5** and shown in the standardized Pareto charts (**Figure 1**). Considering the influence of individual factors, ethanol, and pH produced a significant effect (P-value < 0.05) on the production of such aromatic compounds whereas SO_2 did not result involved in. In particular, ethanol influenced the release of vinyl phenol, ethyl phenol, and vinyl guaiacol while pH was important in determining the variability of ethyl guaiacol. No linear interaction between factors resulted statistically significant for the synthesis of VPs.

DISCUSSION

Wine spoilage by *D./B. bruxellensis* has increased in frequency because of the use of less-severe processing conditions, the great variety of diverse vinification techniques and the tendency to reduce the use of preservatives, such as sulfur dioxide. In particular, the sustainable perspective that to limit SO_2 in bottled wines can reduce undesirable allergenic effects on humans drives the latter action.

The capability of *D./B. bruxellensis* to survive and to grow in wine can be partially ascribed to its high resistance to SO_2; one of the main research question that can be addressed regarding the prevention of this spoilage yeast species is: "how the SO_2 addition can be managed in order to counteract the yeast occurrence during winemaking and in the final product?" Unfortunately, since the active form of SO_2 against microbial proliferation depends on pH, ethanol concentration, and temperature (Usseglio-Tomasset and Bosia, 1984; Ribéreau-Gayon et al., 2006), the answer has to take into consideration that wine is an extremely heterogeneous environment.

Although some wine factors/constituents are reported to play a key role on the off-flavor synthesis by *D./B. bruxellensis*, most of the works carried out to date have independently studied the factors without considering their interactions (Dias et al., 2003b; Godoy et al., 2008; Sturm et al., 2014). With the RSM approach used in this study, the simultaneous effects produced by SO_2, pH, and ethanol on *DbCD* and *DbVPR* gene expression and VPs production have been investigated. Two specific aims are issued in this investigation: (i) the identification of a suitable HKG to assess the relative expression of *DbCD* and *DbVPR* genes and (ii) the setup of an experimental design in order to predict factors and/or possible factor interactions affecting the pathway of VP production.

Regarding the first goal, since real-time qPCR represents the protocol for highly sensitive and reproducible gene expression analysis, accurate and reliable expression results cannot exclude the normalization of real-time qPCR data against a "confident" reference gene in the condition under study. In this work, five genes were evaluated for this purpose and the GeNorm and Normfinder algorithm were used to assay the RNA transcription level of each candidate gene. Despite to the large literature reporting real-time qPCR expression data of several *D./B. bruxellensis* genes, only one manuscript has searched for adequate HKGs to be involved in the data normalization of gene expression assays under oenological conditions (Nardi et al., 2010). In particular, Nardi et al. (2010) choose actin (*ACT1*) and translational elongation factor EF-1α (*TEF1*) genes as housekeeping references. The finding that tubulin (*DbTUB*) was the best reference gene in the present study proves the need of include, as a specific objective of the work, preliminary transcriptional assays to validate the "housekeeping" status of a candidate reference gene under particular experimental conditions.

As concern the second goal, different considerations can be done on the analysis of possible factors that influence the expression of *DbCD* and *DbVPR* genes and the production of VPs.

In general, the main outcome of this study reveals that the highest variability of the response, as a function of the studied factors, was obtained with the expression of *DbCD* that resulted repressed in all the conditions tested by the experimental design in comparison with the condition used as "calibrator." Indeed, being the first enzyme of the metabolic pathway of VPs, the *DbCD* gene is probably more influenced by change of the environmental/oenological conditions in comparison to the *DbVPR* gene.

The expression of *DbCD* is strongly affected by pH and the linear interactions between pH and SO_2, SO_2 and ethanol. Regarding the effect exerted by pH on *DbCD* expression, is important to consider that pH plays an important role on the enzyme substrates, determining the dissociation/undissociation of hydroxycinnamic acids. At wine pH both *p*-coumaric and ferulic acids are mainly under undissociated form (pKa = 4.5), that, due to their lipophilic properties, easily cross the periplasmic membrane and decrease cytoplasmic pH by dissociation into cytosol (Agnolucci et al., 2010). This means that in our study, *DbCD* expression would be expected to increase in the entire range of pH 3.5–4.5, and not only from pH 4 to 4.5, in order to convert acids into the corresponding vinyls. Interestingly, the maximal downregulation can be observed under conditions of pH 4. A hypothesis of this behavior of *DbCD* expression could be related to different mechanisms of the hydroxycinnamic acids uptake in *D. bruxellensis* CBS2499, by passive as well as by active transport, which would deserve more detailed analysis. However, we cannot also exclude the possibility of a strong downregulation resulting from the presence of higher level of SO_2 at low pH.

Although it has been suggested that the entry of the hydroxycinnamic acids into cells is facilitated by the localization

of ethanol close to the dehydrated membrane (Sousa et al., 1996), a high ethanol concentration can generate a cessation of the *DbCD* enzyme activity reducing the conversion of the hydroxycinnamic acids into vinyl phenols (Benito et al., 2009). Moreover, ethanol can also determine a post-transcriptional regulation of the CD affecting the protein activity (Clausen et al., 1994; Cavin et al., 1998). Thus, the same effect that ethanol produces on the membrane permeability is possibly the same exerted on enzyme's conformation since this last depends mainly on the hydrophobic interactions among the amino acid residues of the protein (post-transcriptional regulation). We could speculate that the relative lower level of downregulation of *DbCD* gene observed in cells growing in presence of higher concentration of ethanol [0.25 mg/L, 4.5 and 12.5% (v/v)] could allow the cells compensating, by a transcriptional regulation of *DbCD* gene, a decreased enzyme activity.

Neither a main nor an interaction effect seem to influence *DbVPR* gene expression in the growth conditions under our study. However, the quadratic effect of pH and SO_2 show a significant role in its expression. Indeed, under oenological conditions, SO_2 causes undoubtedly oxidative stress, and we cannot forget that VPR enzyme has been identified in *D. bruxellensis* CBS4481 as a Zn/Cu superoxide dismutase (SOD1) (Granato et al., 2014).

The present study shows that the observed production of VPs, in the tested conditions, depends mainly on ethanol, as single factor, although pH is important in modulating the ethyl guaiacol yield. Moreover, a higher gene expression (run 4, **Table 1**) did not lead to a higher release of VPs (run 9, **Table 1**). This finding suggests that the transformation yield could be affected by factors other than *DbCD* and *DbVPR* regulation.

Ethanol plays a positive linear effect in the transformation of hydroxycinnamic acids to vinyl derivates. This result can support the finding that a lower downregulation of the *DbCD* gene occurs at a high ethanol concentration when cells have to counteract a possible lost in enzyme conformation. Contrarily to what has been observed by Chandra et al. (2014), the SO_2 factor seems to have no effect on the effective production of ethyl phenols, and in general on the off-flavor yields. Nevertheless, different wines and winemaking procedures can affect the content of this chemical and, usually, a higher level is reached during aging, due to a mismanaging use of SO_2 by oenologists. Further experiments are so required to investigate the pathway of VPs by *D./B. bruxellensis* in real wines or under more severe conditions. Finally, due to a diverse capability to counteract the SO_2 stress, different *D./B. bruxellensis* strains could behave differently (Curtin C. et al., 2012; Vigentini et al., 2013); however, this work suggests that the uncontrolled use of sulfur dioxide, besides not representing a sustainable choice, may not be an adequate strategy to protect wine from spoilage.

AUTHOR CONTRIBUTIONS

FV contributed to the design of the work, to the selection of candidate genes for the normalization of gene expression, to perform the qPCR assays and to the interpretation of data for the work, to draft the work and revising it. DF contributed to analysis of volatile phenols, to the interpretation of data for the work and to draft the work. AC contributed to the preparation of cell cultures for the RMS approach, to the extraction of RNAs and the preparation of cDNA, to perform the qPCR assays and to draft the work. CaC contributed to the setup of qPCR assays. RF and CoC contributed to the interpretation of data for the work and to draft the work. IV contributed to the design of the work, to the acquisition, the analysis, and the interpretation of data for the work, to draft the work and revising it for important intellectual content, and ensured that that questions related to the accuracy or integrity of any part of the work were appropriately investigated and resolved.

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frontiers in Microbiology

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Yeast Starter as a Biotechnological Tool for Reducing Copper Content in Wine

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Copper is widely used in agriculture as a traditional fungicide in organic farming to control downy mildew on grapes, consequently it is possible to find this metal during all stages of the vinification process. Low amounts of copper play a key role on the function of key cell enzymes, whereas excess quantities can exert amount-dependent cytotoxicity, resulting in general cellular damage. Nowadays the excessive copper ions in wines is removed by addition of adsorbents, but these additives can influence the sensory characteristics of wine, as well as detrimental to the health of consumers. It is well known that high concentrations of Cu^{2+} can be toxic to yeasts, inhibiting growth and activity, causing sluggish fermentation and reducing alcohol production. In this study, 47 *S. cerevisiae* strains were tested for copper tolerance by two different tests, growth on copper added medium and fermentative activity in copper added grape must. The results obtained by the two different tests were comparable and the high strain variability found was used to select four wild strains, possessing this characteristic at the highest (PP1-13 and A20) and the lowest level (MPR2-24 and A13). The selected strains were tested in synthetic and natural grape must fermentation for ability to reduce copper content in wine. The determination of copper content in wines and yeast cells revealed that at the lowest copper residual in wine corresponded the highest content in yeast cells, indicating a strong strain ability to reduce the copper content in wine. This effect was inversely correlated with strain copper resistance and the most powerful strain in copper reduction was the most sensitive strain, MPR2-24. This wild strain was finally tested as starter culture in cellar pilot scale fermentation in comparison to a commercial starter, confirming the behavior exhibited at lab scale. The use of this wild strain to complete the alcoholic fermentation and remove the copper from wine represents a biotechnological sustainable approach, as alternative to the chemical-physical methods, ensuring at the same time a completed alcoholic fermentation and organoleptic quality of wine.

Keywords: copper resistance, copper-reducing yeasts, wine, *Saccharomyces cerevisiae* biodiversity, biotechnological tools

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INTRODUCTION

In organic viticulture the control of downy mildew on grapes is based almost exclusively on copper, which is allowed to be used because considered a traditional fungicide in organic farming. The long-term use of copper led to an increased copper level not only in soil (Provenzano et al., 2010; Ash et al., 2012), but also in grape and must; copper salt addition for eliminating H_2S (García-Esparza et al., 2006; Tamasi et al., 2010) may also increase the copper content in must and consequently in wine.

In biological vineyards the increased intake of copper compounds has caused high levels of copper residues on the grapes (Brandolini et al., 2002). In winemaking, elevated concentrations of this metal can be toxic to yeasts, affecting cell growth and activity; high level in must of Cu^{2+}, such as 0.1 mM (Ohsumi et al., 1988) influences negatively yeast growth, inducing sluggish fermentation (Azenha et al., 2000) and a reduction in alcohol production (Mrvcić et al., 2007).

Moreover, the copper can influence wine strains activity in different ways: prevention or limiting of *Saccharomyces cerevisiae* growth, reduction of absorption of reducing sugars, which consequently causes a decrease on ethanol production. These effects were directly correlated with copper concentration and strain biodiversity (Sun et al., 2015). In *Saccharomyces cerevisiae*, the strains exhibit a wide variability in the level of copper tolerance (Capece et al., 2016) and in the cell capability to adsorb copper ions (Benítez et al., 2002; Mira et al., 2007; Schubert and Glomb, 2010). The adsorption of heavy metal in yeasts can be achieved by two ways, non-biological (dead cells) or biological (living cells) adsorption. Different studies reporting data on yeast biological adsorption of heavy metals are available, mainly addressed to the study of factors influencing the properties of heavy metal adsorption or the dynamic models of adsorption (Vasudevan et al., 2002, 2003). Furthermore, the adsorption by living cells can be subdivided as extracellular and intracellular adsorption (Chen et al., 2014). However, most of these results were related to industrial wastewater treatment systems, whereas few data on wine fermentation process are available. The studies on wine fermentation were based mainly on distinction between adsorption by dead or living cells, whereas Sun et al. (2016) reported results regarding extracellular or intracellular copper adsorption by living yeast cells. In this pathway, *S. cerevisiae* cells might firstly adsorb copper on cell surface, after the copper ions are moved into intracellular spaces.

In the first step, named as "passive biosorption" or extracellular, the interactions between metal-functional groups present on cell surface, such as carboxyl, phosphate, hydroxyl, amino, sulfur compounds, etc., capture metal ions to the cell surface. This process is independent from the metabolism, it starts very quickly (within several min) and it is a dynamic equilibrium of reversible adsorption–desorption, as the metal ions adsorbed on cell surface can be removed by different agents, such as other ions, chelating agent or acids.

During the second step, named as "active biosorption" or intracellular, metal ions enter in the cells by going through the cell membrane and it was an ongoing slow process.

It was recently reported that after copper adsorption, the cell surface and intracellular compartments of *S. cerevisiae* changed irregularly. A yeast strain copper resistant and able to accumulate this metal in the cell was patented with aim to clean copper from extracellular solutions (Abe and Horikoshi, 2001). Recent results (Sun et al., 2015) demonstrated that in *S. cerevisiae* the principal mechanism involved in copper adsorption during alcoholic fermentation was cell surface adsorption, which reaches saturation in 24 h.

Due to detrimental effects in high concentrations, "maximum residue levels" (MSL) of copper in European and South African regulations have been established in 20 mg L^{-1} in grape must and 1 mg L^{-1} in wine (García-Esparza et al., 2006). Nowadays the excessive copper ions in wines is removed by addition of adsorbent such as glue; recently OIV allowed to add some additives, such as potassium ferrocyanide, bentonite, gum Arabic, polyvinylimidazole, polyvinylpyrrolidone copolymers, chitin, chitosan etc., but these treated wines have a lower content of polyphenols and aromatic compounds, which is reflected in the organoleptic properties of wine (Benítez et al., 2002). Anyway copper is unavoidable in winemaking and the adverse effects of long-term copper fungicide use can be just diminished by reducing the number of applications and doses of conventional copper fungicides and by combining this strategy with increasing use of biological preparations.

In this work, the variability for copper adsorption among wild *S. cerevisiae* strains allowed to select strains able to reduce excessive copper content in wine. The aim was to promote the utilization of a biotechnological method, alternative to chemical removal, ensuring at the same time a completed alcoholic fermentation and organoleptic quality of wine.

MATERIALS AND METHODS

Yeast Strains

In this study 47 *S. cerevisiae* strains were used (**Table 1**): 44, belonging to the collection of the University of Basilicata, were isolated during spontaneous fermentation of grapes sampled in different areas and previously characterized for enological parameters, and three are commercial starters. Yeast cells were maintained on slants in YPD medium (1% w/v yeast extract, 2 w/v% bacto peptone, 2 w/v% glucose, 2 w/v% agar) at 4°C.

Strain Resistance to Copper

The strain resistance to copper was assessed both by evaluating the influence of copper, added as copper sulfate ($CuSO_4$), on growth and fermentative activity of strains.

The copper influence on strain growth was tested by inoculating approximately 1×10^6 cells/ml on solid synthetic medium, containing 6.7 g L^{-1} YNB (Yeast Nitrogen Base without amino acids and sulfate), 20 g L^{-1} glucose, added with increasing levels of $CuSO_4$ (50, 100, 200, 300, 400, and 500 μmol L^{-1}), in comparison to the control (the same medium without copper addition). After incubation at 26°C for 24 h, the strain resistance level to copper was defined as the lowest concentration of the metal allowing strain growth.

TABLE 1 | *Saccharomyces cerevisiae* strains used in this study.

Strain	Origin	References
ND-14; CD2-6SC2; ND7; RB3-7SC2; TA8-4SC2; CB1-7SR3	Nero d'Avola variety, Sicily region	Capece et al., 2010
5TB8-60	Bosco variety, Liguria region	Capece et al., 2012
M1-47; M3-60; M3-59; M3-80;	Aglianico variety, Basilicata region	Capece et al., 2014
B7; A13; A14; A20; A21; 10_1; 10_2; B51	Aglianico del Vulture variety, Basilicata region	This study*
4LB; AGME	Aglianico del Vulture variety, Basilicata region	Capece et al., 2011b
PP1-1; PP1-15; PP1-31; PP2-22; PP1-13; MPR2-18; MPR2-42; MPR2-43; MPR2-28; MPR2-24; MPR2-26; BP1-29; BP2-17; BP2-33; BP1-13; BP1-33	Primitivo variety, Basilicata region	This study*
SC2-37; SB5-15; SB5-18; SA7-13	Sangiovese variety, Tuscany region	Capece et al., 2013
BA-215	Sangiovese variety, Tuscany region	Capece et al., 2011b
SN41	Sangiovese variety, Tuscany region	Brandolini et al., 2002
TA4-10	Inzolia variety, Sicily region	Capece et al., 2011a
EC1118	Commercial strain	Lallemand
796 AWRI	Commercial strain	Maurivin
FI5	Commercial strain	Laffort
ES 454	Commercial strain	Enartis

**These strains were characterized in this study.*

To evaluate the effect of copper on fermentative activity, each strain was inoculated (10^7 cell/mL from pre-cultures grown for 24 h in YPD) in 10 mL of pasteurized grape must (100°C for 20 min), supplemented with 300 mg L^{-1} of $CuSO_4$. As control, pasteurized grape must without copper addition was used. The copper resistance (FVR) was expressed as ratio between strain fermentative vigor in copper-added fermentations (Cu-FV) and the fermentative vigor without Cu addition (C-FV).The fermentative vigor was measured as the amount of CO_2 produced at the third day of fermentation.

Strain Ability to Reduce Copper Content in Synthetic Wine

On the basis of previous results, four wild strains were selected (MPR2-24, A13, PP1-13, A20) and tested in fermentation of synthetic grape must (SGM) in order to evaluate the strain ability to reduce the copper content in winemaking. As SGM, the medium reported by Henschke and Jiranek (1993) was used. Fermentations were conducted at 26°C in 130-mL Erlenmeyer flasks, equipped with Müller valves containing sulphuric acid and filled with 100 ml of SGM. The synthetic must was added with 300 μmol L^{-1} of $CuSO_4$; as control, SGM without copper addition was used. The SGM was inoculated with 10^7 cells mL-1, from a pre-culture grown in SGM for 24 h, and the fermentations were daily monitored by analyzing the weight loss. All the experiments were performed in triplicate. At the end of the process (when weight loss was less than 0.02 g for 2 days), the samples were centrifuged at 4.000 rpm for 10 min at 4°C. Both the obtained fractions (fermented samples and yeast cells) were stored at −20°C until required for analysis.

For copper determination in synthetic and natural wines, the samples, previously filtered through a 0.45 μm membrane filter, were degassed using an ultrasonic bath, while the yeast cells were submitted to the acid digestion prior filtration and analysis. Successively, each sample was added with HNO_3 solution and mixed with internal standard (2 ppm Yttrium) by means of a fitting (T) positioned after the peristaltic pump. The copper level was determined according to EPA 6020A. Standard was purchased from Sigma-Aldrich (USA) and all analytical solvents used during the analysis were furnished from Levanchimica (Bari, Italy). The copper quantification in the alcohol matrix was carried out using an ICP-MS ICAP TM 7400 of Thermo Scientific (USA), equipped with an automatic sampler. The operating conditions used were: power 1.2 kW, gas flow 15.0 L/min, gas flow 2.25 L/min, spraying pressure 220 kPa, pump speed 18 rpm, wavelength of Cu 327.395 nm. Three replications were performed on each sample.

The strain ability to reduce copper content in synthetic wine (RCuSW) was calculated on the basis of the following equation: RCuSW = CuSW-CuSC, where CuSW and CuSC are copper content in copper added and control synthetic wine, respectively. The copper adsorption by strain (AsCuY) was calculated on the basis of the following equation: AsCuY = YCuSW-YCuSC, where YCuSW and YCuSC were copper content in yeast cells from copper added and control synthetic wine, respectively.

Strain Ability to Reduce Copper Content in Wine

The four wild selected strains were tested in inoculated fermentation at laboratory scale in pasteurized natural grape must (NGM). The NGM used was "Aglianico del Vulture," presenting the following characteristics: pH 3.7; total soluble solids 227 g L^{-1}; yeast assimilable nitrogen 281 mg L^{-1}. The fermentations were performed following the protocol previously reported for SGM. The experimental wines and yeast cells recovered at the end of the process were analyzed for copper content, by using the protocol previously described. The strain ability to reduce copper content in wine (RCuW) was calculated

on the basis of the following equation: RCuW = CuW-CuC, where CuW and CuC are copper content in copper-added and control wine, respectively. The copper adsorption by strain (AwCuY) was calculated on the basis of the following equation: AwCuY = YCuW-YCuC, where YCuW and YCuC were copper content in yeast cells from copper-added and control wine, respectively.

Analytical Profiles of Experimental Wines

Experimental wines obtained from NGM fermentation were analyzed for conventional chemical parameters, such as total and volatile and total acidity, residual sugars, alcohol, were measured using Fourier Transfer Infrared WineScan (FOSS, Hillerød, Denmark). The content of the main secondary compounds influencing wine aroma, such as higher alcohols (*n*-propanol, isobutanol, amyl alcohols), acetaldehyde, ethyl acetate, were determined by direct injection gas chromatography, whereas other volatile compounds, such us esters, volatile fatty acids, alcohols, aldehydes, ketones, were analyzed by SPME-GC-MS, following the methods described by Capece et al. (2013).

Pilot Scale Fermentations

Pilot scale fermentations were performed by using the selected indigenous starter (MPR2-24) in comparison to the commercial strain ES 454 (ENARTIS), commonly used by the producer. The trials were performed in a cellar using grapes from vineyard following organic farming system. The fermentations were performed in sulphited (50 mg L^{-1}) grape must (240 g L^{-1} sugar, pH 3.5) in 100 L stainless steel and inoculated with 1×10^7 cells ml^{-1}. The fermentation processes were daily monitored by determining sugar consumption. The final wines were analyzed for content of secondary compounds, conventional chemical parameters and the copper content, following the protocols previously reported. The copper removal ratio (CuRR) was calculated following this equation: CuRR = CuM-CuW/CuM, where CuM is copper concentration in grape must and CuW is copper level in wine. The implantation ability of each starter was evaluated by yeast isolation on WL medium (Pallmann et al., 2001) from wine samples, collected at the end of the process from each fermentation vessel; a representative number of yeast colonies (at least 20), randomly chosen from each sample, were submitted to amplification of inter-delta region, in comparison to inoculated starters. The starter implantation level was calculated as previously reported (Capece et al., 2012).

Data Analysis

Statistical software (PAST software ver. 1.90; Hammer et al., 2001) was used for analyzing all data. Data of volatile compounds and copper content in wines and yeast cells were analyzed using one-way Analysis of Variance (ANOVA) to compare the mean values between fermentations with and without copper addition.

RESULTS

Evaluation of Strain Resistance to Copper

Forty-seven *S. cerevisiae*, 44 wild and three commercial strains, were tested for copper sensitivity, in particular for tolerance to copper sulfate, the copper formulation applied as a fungicide to treat powdery mildew in vineyards. The evaluation of copper influence on yeast growth revealed a wide strain variability on YNB medium. In particular, about 60% of the strains exhibited low copper tolerance (the maximum tolerated doses were 100–200 μmol L^{-1}), 15% of the strains were high copper tolerant (growing on the maximum tested dose), whereas the remaining strains grew at concentrations ranging between 300 and 400 μmol L^{-1} of $CuSO_4$.

The copper influence on strain fermentative activity was evaluated as strain ability to tolerate this compound, preserving its fermentative performance. As reported in **Figure 1** and Table S1, six strains resulted very high copper tolerant, as the fermentative vigor was not affected by the presence of the compound (FVR values equals to or higher than 1), numerous strains (23) were slightly affected by the copper addition (FVR was about 0.96), whereas few strains exhibited a very low copper tolerance as the fermentative vigor was reduced at about 50% (or more) by copper addition (FVR values ranging between 0.32 and of 0.64).

It has be underlined that the results obtained by evaluating copper influence on fermentative activity confirmed those obtained by testing the copper effect on strain growth in synthetic medium; in fact, in both the tests, the most sensitive strain was MPR2-24.

On the basis of these results, four wild *S. cerevisiae* strains, exhibiting the lowest (MPR2-24, A13) and the highest copper sensitivity (PP1-13, A20) were selected for further characterization.

Evaluation of Strain Ability to Reduce Copper Content in Synthetic Wine

The four selected strains were tested in SGM fermentation added with $CuSO_4$, in comparison to the control, in order to test the strain ability to reduce copper content of wine. The monitoring

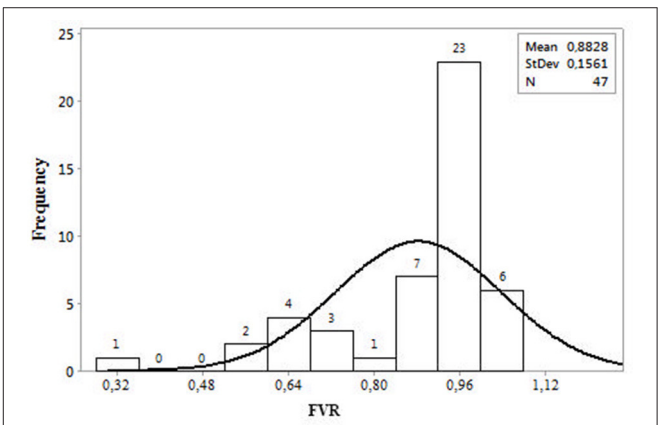

FIGURE 1 | Distribution of 47 strains in function of copper resistance (FVR). FVR is expressed as ratio between strain fermentative vigor in copper-added fermentations (Cu-FV) and the fermentative vigor without Cu addition (C-FV). The fermentative vigor was measured as the amount of CO_2 produced at the third day of fermentation.

of fermentative process revealed that all the fermentations (copper-added and controls) were completed, although the copper affected significantly the fermentative performance of sensitive strains and the duration of fermentation process (data not shown). Samples obtained at the end of the fermentations (synthetic wines and yeast cells recovered after centrifugation) were analyzed for copper content. The strain influence on copper content of synthetic wine is reported in **Figure 2A**. MPR2-24 exhibited the highest strain ability to reduce copper content in synthetic wine (RCuSW); in fact the lowest copper residual was detected in synthetic wine fermented by this strain, which was significantly different from all the other samples. On the contrary, the lowest RCuSW was shown by the two resistant strains, PP1-13 and A20, which determined the highest copper residual in the samples.

The determination of copper residual in yeast cells (**Figure 2B**) showed that the highest residual content was detected in the cell pellet of the sensitive strain MPR2-24, which contained about 2.050 mg kg^{-1}, whereas the copper residual content detected in cells of the other strains ranged between 1.300 and 1.530 mg kg^{-1}. As a consequence, MPR2-24 resulted the strain with the highest RCuSW and AsCuY (copper adsorption by strain).

Evaluation of Strain Ability to Reduce Copper Content in Natural Wines

In order to confirm the strain ability to reduce copper content also in wines from natural grape must, the experimental wines and yeast cells (separated by centrifugation from final samples) were analyzed for copper content. The strain ability to reduce copper content in wines (RCuW) is reported in **Figure 3A**. A different behavior was found in function of strain copper sensitivity: the highest reduction level was obtained by the sensitive strain, MPR2-24, followed by the other copper sensitive strain A13, whereas the highest copper content was detected in wines produced by the two resistant strains (PP1-13 and A20),

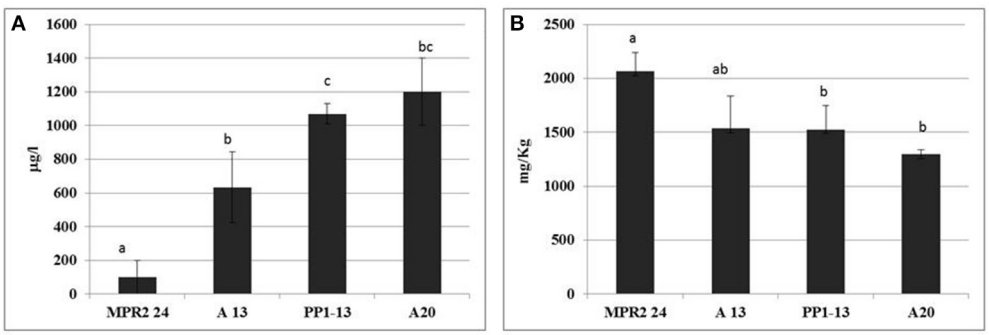

FIGURE 2 | Strain ability to reduce copper content in synthetic grape must (SGM) fermentation. **(A)** Residual copper content in synthetic wine (RCuSW), calculated on the basis of the following equation: RCuSW = CuSW-CuSC, where CuSW and CuSC are copper content in copper added and control synthetic wine. **(B)** Copper adsorption by strain (AsCuY), calculated on the basis of the following equation: AsCuY = YCuSW-YCuSC, where YCuSW and YCuSC were copper content in yeast cells from copper added and control synthetic wine. Data are means ± SD of three independent experiments; different superscript letters indicate significantly different values (one-way ANOVA, $p < 0.05$).

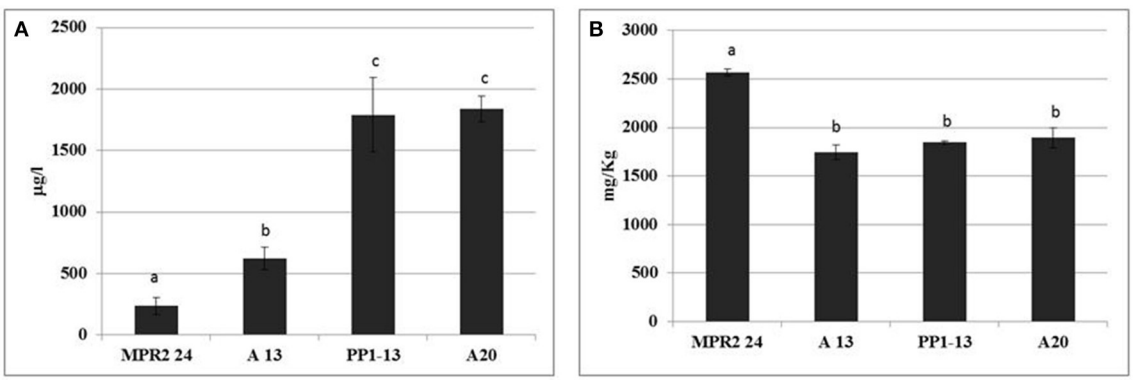

FIGURE 3 | Strain ability to reduce content in natural grape must (NGM) fermentation. **(A)** Residual copper content in wine (RCuW), calculated on the basis of the following equation: RCuW = CuW-CuC, where CuW and CuC are copper content in copper added and control synthetic wine. **(B)** Copper adsorption by strain (AwCuY), calculated on the basis of the following equation: AwCuY = YCuW-YCuC, where YCuW and YCuC were copper content in yeast cells from copper-added and control wine, respectively. Data are means ± SD of three independent experiments; different superscript letters indicate significantly different values (one-way ANOVA, $p < 0.05$).

which exhibited a behavior very similar, with a copper content ranging between 1.790 and 1.830 mg L^{-1}. The residual copper content adsorption by yeast cells (AwCuY) recovered at the end of the fermentative process (**Figure 3B**) revealed that the highest level was detected in MPR2-24 cells (about 2.600 mg kg^{-1}), with a significantly higher level than those found in the other strain cells (values ranging between about 1.750 and 1.900 mg kg^{-1}).

It has be underlined that the lowest level of copper was found in wine obtained by inoculating MPR2-24 and the highest copper content was detected in yeast cells of the same strain, confirming the results obtained in SGM fermentations. These results outline the potential ability of MPR2-24 strain to remove copper content from wine.

Copper Influence on Fermentative Performance of Selected Strains in NGM

The evolution of fermentative process and chemical parameters detected in the experimental wines from NGM are shown in **Table 2**. All the data related to strain fermentative performance, such as fermentative vigor (FV) and power (FP), reflected the different copper sensitivity of the strains. In fact, statistically significant differences between values detected in fermentation with and without $CuSO_4$ addition were found for copper sensitive strains (MPR2-24 and A13). For these strains, a low fermentation activity was found in copper-added must, with a FP decrease of 23% (MPR2-24) and 35% (A13) and, consequently, high residual sugars in final wines were detected in fermentation with $CuSO_4$ addition than values detected in the control (**Table 2**). No influence of $CuSO_4$ supplementation on strain FV and FP was found for copper tolerant strains (PP1-13, A20). However, all the strains completed the fermentation (1.23 g L^{-1} maximal residual sugars), although the processes were delayed for sensitive strains in grape must containing copper.

Otherwise for all the strains, no significant differences between the two fermentations were found in the levels of total acidity, while the ethanol content (ranging between 8.45 and 9.84% v/v) was significantly higher in wines obtained from fermentation without $CuSO_4$ for all the strains, except for A20. In wines obtained by the two sensitive strains, the copper supplementation affected significantly the volatile acidity, determining a considerable increase.

Copper Influence on Analytical Profiles of Wines Produced by Selected Strains

The experimental wines obtained from the two fermentations were analyzed for content of by-products related to wine aroma, in order to evaluate the influence of copper on strain metabolic behavior. Among the compounds detected by gas-chromatography (**Table 3**), acetaldehyde was produced in the highest amounts in copper added fermentation (except for

TABLE 2 | Main technological characteristics of selected *S. cerevisiae* strains.

Strain	FT	FV	FP	Residual sugars gL^{-1}	Total acidity gL^{-1}	Volatile acidity gL^{-1}	Ethanol % v/v
MPR2-24	C	1.4 ± 0.21*	0.81 ± 0.05*	0.43 ± 0.12*	8.17 ± 0.15	0.30 ± 0.09*	9.39 ± 0.07*
	Cu	0.35 ± 0.25	0.63 ± 0.02	0.87 ± 0.06	8.44 ± 0.15	0.79 ± 0.03	9.06 ± 0.09
A13	C	1.63 ± 0.03*	1.11 ± 0.01*	0.83 ± 0.06*	8.68 ± 0.69	0.05 ± 0.08*	9.80 ± 0.02*
	Cu	0.53 ± 0.29	0.71 ± 0.15	1.23 ± 0.06	8.40 ± 0.07	0.28 ± 0.02	8.45 ± 0.47
PP1-13	C	1.45 ± 0.11	1.11 ± 0.03	0.57 ± 0.06	9.66 ± 0.14	0.59 ± 0.08	9.84 ± 0.10*
	Cu	1.53 ± 0.11	1.11 ± 0.05	0.60 ± 0.10	9.68 ± 0.14	0.65 ± 0.08	9.23 ± 0.14
A20	C	1.53 ± 0.08	1.05 ± 0.02	0.57 ± 0.12	9.53 ± 0.12	0.66 ± 0.02	9.54 ± 0.18
	Cu	1.50 ± 0.08	1.13 ± 0.10	0.40 ± 0.17	9.58 ± 0.36	0.48 ± 0.17	9.40 ± 0.06

FT, fermentation type; C, fermentation in grape must (control); Cu, fermentation in Cu-added grape must.
FV, strain fermentative vigor expressed as g CO_2/day measured at the second fermentation day.
FP, strain fermentative power expressed as g CO_2/day measured at the end of the fermentation.
Data are mean ± SD of three independent experiments. For each strain, the asterisk indicates significantly different values (one-way ANOVA, $P < 0.05$) between wines from control and Cu-added grape must.

TABLE 3 | By-products (mg L^{-1}) in experimental wines produced by the four *S. cerevisiae* strains fermentation with and without copper addition.

	MPR2-24		A13		PP1–13		A20	
	C	Cu	C	Cu	C	Cu	C	Cu
Acetaldehyde	34.88 ± 4.93	34.46 ± 2.46	48.61 ± 3.70	53.42 ± 2.76	33.71 ± 3.21*	50.50 ± 4.01	35.86 ± 2.29	41.66 ± 6.50
Ethyl acetate	14.67 ± 0.15*	20.89 ± 3.09	18.16 ± 1.01*	14.11 ± 1.06	26.23 ± 0.95	25.64 ± 2.26	27.54 ± 1.84	30.81 ± 4.57
n-Propanol	29.17 ± 0.87	39.51 ± 6.42	67.13 ± 2.86*	48.04 ± 2.45	52.19 ± 3.15	65.55 ± 0.63	69.13 ± 3.99*	112.52 ± 10.21
Isobutanol	48.19 ± 1.20*	34.45 ± 2.93	46.36 ± 3.09*	39.63 ± 2.98	54.89 ± 4.62	55.41 ± 2.65	41.12 ± 4.80	40.41 ± 1.31
Amyl alcohols	184.26 ± 0.42*	126.64 ± 1.90	205.64 ± 27*	148.48 ± 4.34	159.14 ± 2.14	160.97 ± 2.65	155.82 ± 4.80	164.28 ± 1.31

C, fermentation in grape must (control). Cu, fermentation in Cu-added grape must. Data are mean ± SD of three independent experiments For each strain, the asterisk indicates significantly different values (one-way ANOVA, $P < 0.05$) between wines from control and Cu-added grape must.

MPR2-24 strain), although statistically significant differences were found only for wines obtained by inoculating PP1-13 strain. The production levels of isobutanol, *n*-propanol and amyl alcohols were significantly affected by copper addition in fermentations with sensitive strains, mainly for A13, which produced a lower level of these by-products in wines obtained from copper-added must. Also the ethyl acetate production was significantly affected by copper addition for sensitive strains, although in different way in the two strains. The analysis of the volatile fraction by SPME-GC-MS of the experimental wines allowed the identification of 49 compounds, belonging to different chemical classes, such as esters, alcohols, aldehydes (**Table 4**). Among the esters, the compounds present in the highest amounts were ethyl propanoate, ethyl isobutyrate, isobutyl acetate, ethyl butyrate, isoamyl acetate, ethyl hexanoate, 2-phenylethyl acetate, whereas 1-hexanol, benzyl alcohol, 2-phenylethanol were the alcohols present in the highest concentration and furfural was the main aldehyde. By analyzing the influence of copper addition on production level of these compounds, no statistically significant differences were found in wines obtained with and without copper addition for the resistant strains A20 and PP1-13 (wines from PP1-13 significantly differed only for cis-3-Hexen-1-ol content) and for sensitive strain MPR2-24. On the contrary, wines obtained inoculating A13 strain with copper addition differed significantly from the control for numerous volatile compounds, such as ethyl butyrate, isoamyl acetate, ethyl valerate, isoamyl butyrate, methyl octanoate, ethyl 6-hydroxyhexanoate, 1-pentanol, 2-heptanol, benzyl alcohol, 2-phenylethanol, linalool, β-citronellol.

Fermentations at Pilot Scale in Cellar

Taking into account the potential ability of the strain MPR2-24 to remove copper content from wine, this strain was selected for pilot scale fermentation at cellar level in comparison to the commercial starter commonly used by the cellar (ES 454). The aim was to test the performance of this selected wild strain in real winemaking conditions. The tests were performed in must obtained by grapes collected in vineyard following the organic production system. Furthermore, the strain MPR2-24 was previously isolated during spontaneous fermentation of grapes collected in the same vineyard. The analysis of parameters correlated to a successful starter performance during fermentation, such as sugar consumption, ethanol production, reported in **Table 5**, showed that the wild strain possesses a fermentative performance comparable to the commercial starter. Also the content of some secondary compounds mainly involved in wine aroma, such as acetaldehyde and higher alcohols, detected in wine obtained by MPR2-24 was very similar to the level detected in wine produced by inoculating the commercial starter. The main differences between the two wines were related to the content of higher alcohols, mainly amyl alcohols, with higher content in wine obtained by wild strain than the level detected in wine fermented by commercial starter (344 and 293 mg L^{-1}, respectively). In any case, it should be pointed out that the quantities of main by-products produced by the two starters respected the threshold values. Both indigenous and commercial starters showed a high strain implantation ability (92 and 100% for MPR2-24 and ES 454, respectively).

As regards the strain ability to reduce copper content in wine (CuRR), both the starters induced a reduction of this compound, although the wild strain possessed this capability at higher level than commercial starter (71 and 50%, respectively).

DISCUSSION

In consequence of the recent significant increase of organic wine sector, it is frequent to find grape must containing high level of copper residues, which is one of the most important biopesticides used in organic farms as copper formulates are effective against a high number of crops pests. High copper residual in grape must can be detrimental for the wine-making process and wine quality (Mira et al., 2007; Li et al., 2008). In fact, if the yeast strains performing the fermentative process are copper-sensitive, high amounts of this compound in must can inhibit yeast growth and activity.

The screening of copper tolerance among forty-seven *S. cerevisiae* strains was performed by the two different tests, growth on copper-added medium and fermentative activity in copper added grape must. The results obtained by the two different tests were comparable: the strains tolerating the highest copper concentration in YNB medium were the same which kept a good fermentative activity also in copper added must. These tests were very useful tools to identify very sensitive and tolerant strains and revealed the existence of high strain variability for this parameter, confirming previous data reporting that natural isolates of *S. cerevisiae* vary in their sensitivity to copper sulfate (Cavalieri et al., 2000; Mortimer, 2000). Some authors report that the analysis of traits of yeast population from specific area encompassed phenotypes that may reflect man-directed selection, for example copper resistance has been classified as a domestication trait (Warringer et al., 2011) and it may be an acquired adaptation as a result of the application of copper sulfate as a fungicide to treat powdery mildew in vineyards. These results support the idea that the isolation environment can exert a selective pressure on natural microflora. In our study, conversely, strain possessing copper tolerance at very different level, such as PP1-13 (very high copper tolerant) and MPR2-24 (very low copper tolerant) were isolated from fermented grapes collected in the same vineyard; the same findings were found for A20 and A13, both isolated from Aglianico del Vulture fermented grapes. These results suggest that, although some traits can be affected by natural selective pressure, it is necessary to consider the strain genetic basis for natural trait variation. The strain variability found was used to select four wild strains possessing this characteristic at the highest (PP1-13 and A20) and lowest level (MPR2-24 and A13). Looking at the evaluation of strain influence on copper content in fermentation, the four selected strains were firstly tested in SGM, a fermentation synthetic medium in which all the physical-chemical parameters can be standardized. The determination of copper content in final synthetic wines and yeast cells revealed that at the lowest copper residual in wine corresponded the highest content in

TABLE 4 | Volatile compounds ($\mu g\ L^{-1}$) in experimental wines produced by the four *S. cerevisiae* strains in fermentation with and without copper addition.

Compounds	MPR2-24		A13		PP1–13		A20	
	C	Cu	C	Cu	C	Cu	C	Cu
ESTERS								
Ethylpropanoate	122.84 ± 13.2	102.44 ± 6.68	90.39 ± 14.44	119.97 ± 22.4	137.89 ± 2.91	139.68 ± 39.93	140.10 ± 18.41	153.03 ± 5.16
Ethylisobutyrate	189.98 ± 19.86	156.64 ± 10.21	130.73 ± 28.16	167.75 ± 7.84	210.35 ± 5.15	242.51 ± 31.48	214.18 ± 28.14	233.17 ± 8.25
Ethylbutanoate	1.23 ± 0.28	1.11 ± 0.37	0.99 ± 0.08	0.97 ± 0.30	1.08 ± 0.23	1.14 ± 0.46	1.22 ± 0.16	1.15 ± 0.33
Propyl acetate	47.03 ± 5.83	38.14 ± 2.90	32.59 ± 5.38	43.55 ± 7.94	50.34 ± 0.61	50.69 ± 14.38	50.78 ± 6.67	55.44 ± 1.88
Isobutyl acetate	96.56 ± 21.91	70.15 ± 4.56	60.45 ± 10.99	82.56 ± 14.49	94.20 ± 2.16	96.05 ± 26.57	95.83 ± 12.59	104.69 ± 3.53
Ethylbutyrate	97.74 ± 22.18	70.73 ± 4.66	49.89 ± 20.62*	87.70 ± 10.54	96.32 ± 1.06	101.34 ± 21.18	97.01 ± 12.75	105.88 ± 3.60
Ethyl 2-methylbutanoate	5.65 ± 1.28	5.08 ± 1.70	4.32 ± 0.24	4.55 ± 1.26	4.87 ± 1.23	5.34 ± 1.99	5.61 ± 0.74	5.27 ± 0.82
Ethyl 3-methylbutanoate	1.95 ± 0.44	1.75 ± 0.59	1.76 ± 0.27	1.47 ± 0.59	1.79 ± 0.24	1.75 ± 0.84	1.94 ± 0.25	1.82 ± 0.53
Ethyl 2-methylpropanoate	2.00 ± 0.45	1.80 ± 0.60	1.38 ± 0.13	1.67 ± 0.36	1.66 ± 0.54	1.95 ± 0.62	1.99 ± 0.26	1.87 ± 0.54
Ethylisovalerate	0.92 ± 0.21	0.83 ± 0.28	0.33 ± 0.31	0.88 ± 0.11	0.65 ± 0.46	1.01 ± 0.15	0.92 ± 0.12	0.86 ± 0.25
Butyl acetate	1.35 ± 0.31	1.21 ± 0.41	0.45 ± 0.49	1.30 ± 0.17	0.93 ± 0.69	1.48 ± 0.21	1.34 ± 0.18	1.26 ± 0.37
Isoamyl acetate	501.89 ± 113.89	363.35 ± 23.88	218.00 ± 138.62*	464.27 ± 50.90	495.26 ± 5.27	479.28 ± 12.99	498.10 ± 65.45	545.62 ± 18.09
Ethylvalerate	1.02 ± 0.23	0.92 ± 0.31	0.42 ± 0.30*	0.95 ± 0.11	0.73 ± 0.47	1.09 ± 0.18	1.01 ± 0.13	0.95 ± 0.28
Methylhexanoate	1.64 ± 0.37	1.48 ± 0.50	0.65 ± 0.51	1.54 ± 0.18	1.18 ± 0.77	1.77 ± 0.28	1.63 ± 0.21	1.53 ± 0.45
Ethylhexanoate	103.02 ± 23.38	74.95 ± 4.87	74.49 ± 3.62	101.56 ± 15.79	101.46 ± 1.15	115.91 ± 15.01	102.25 ± 13.44	111.38 ± 3.90
Isoamylbutyrate	3.15 ± 0.71	2.83 ± 0.95	1.25 ± 0.97*	2.96 ± 0.34	2.25 ± 1.48	3.40 ± 0.54	3.12 ± 0.41	2.94 ± 0.85
Hexyl acetate	6.67 ± 1.51	6.00 ± 2.01	4.31 ± 0.65	7.58 ± 2.60	5.44 ± 1.99	8.51 ± 1.95	6.62 ± 0.87	6.22 ± 1.81
Ethylheptanoate	1.07 ± 0.24	0.96 ± 0.32	0.43 ± 0.33	1.00 ± 0.11	0.77 ± 0.50	1.15 ± 0.18	1.06 ± 0.14	1.00 ± 0.29
Ethyl trans-2-hexenoate	1.36 ± 0.31	1.22 ± 0.41	0.36 ± 0.57	1.34 ± 0.21	0.90 ± 0.76	1.53 ± 0.20	1.35 ± 0.18	1.27 ± 0.37
Isobutylhexanoate	0.16 ± 0.04	0.14 ± 0.05	0.14 ± 0.02	0.48 ± 0.57	0.15 ± 0.02	0.50 ± 0.55	0.16 ± 0.02	0.15 ± 0.04
Methyloctanoate	0.91 ± 0.21	0.82 ± 0.27	0.44 ± 0.21*	0.83 ± 0.09	0.69 ± 0.37	0.95 ± 0.19	0.90 ± 0.12	0.85 ± 0.25
Ethyloctanoate	8.75 ± 1.98	7.87 ± 2.64	6.54 ± 0.35	9.05 ± 1.96	7.48 ± 2.00	10.26 ± 1.44	8.68 ± 1.14	8.16 ± 2.37
Isoamylhexanoate	4.29 ± 0.97	3.86 ± 1.29	1.96 ± 1.11	3.94 ± 0.43	3.17 ± 1.84	4.54 ± 0.83	4.26 ± 0.56	4.00 ± 1.17
Ethylnonanoate	0.11 ± 0.02	0.10 ± 0.03	0.04 ± 0.03	0.11 ± 0.03	0.08 ± 0.05	0.13 ± 0.02	0.11 ± 0.01	0.10 ± 0.03
Methyldecanoate	0.13 ± 0.03	0.11 ± 0.04	0.10 ± 0.01	0.10 ± 0.03	0.11 ± 0.02	0.12 ± 0.05	0.13 ± 0.02	0.12 ± 0.03
Isoamyloctanoate	3.06 ± 0.70	2.76 ± 0.92	1.67 ± 0.55	2.71 ± 0.36	2.37 ± 1.13	3.14 ± 0.72	3.04 ± 0.40	2.86 ± 0.83
Ethylphenylacetate	7.89 ± 1.79	7.09 ± 2.38	4.47 ± 1.29	6.92 ± 0.98	6.18 ± 2.79	8.02 ± 1.92	7.83 ± 1.03	7.36 ± 2.14
2-Phenylethyl acetate	93.25 ± 21.16	67.88 ± 4.40	77.93 ± 7.99	96.36 ± 20.71	82.97 ± 15.75	109.34 ± 15.24	92.54 ± 12.16	100.95 ± 3.46
Ethyl 6-hydroxyhexanoate	0.88 ± 0.20	0.79 ± 0.27	0.25 ± 0.35*	0.91 ± 0.20	0.59 ± 0.48	1.04 ± 0.15	0.87 ± 0.11	0.82 ± 0.24
ALCOHOLS								
1-Pentanol	2.00 ± 0.45	1.80 ± 0.60	0.66 ± 0.74*	1.93 ± 0.25	1.38 ± 1.03	2.21 ± 0.31	1.99 ± 0.26	1.87 ± 0.54
4-Methyl-1-pentanol	1.24 ± 0.28	1.12 ± 0.37	0.85 ± 0.08	1.03 ± 0.22	1.03 ± 0.34	1.21 ± 0.38	1.23 ± 0.16	1.16 ± 0.34
2-Heptanol	0.83 ± 0.19	0.74 ± 0.25	0.41 ± 0.18*	0.75 ± 0.09	0.62 ± 0.33	0.86 ± 0.17	0.82 ± 0.11	0.77 ± 0.22
1-Hexanol	112.99 ± 25.64	81.45 ± 5.48	75.56 ± 9.05	97.93 ± 15.35	111.35 ± 1.22	113.70 ± 9.19	112.14 ± 14.73	105.36 ± 30.66
cis-3-Hexen-1-ol	0.01 ± 0.00	0.01 ± 0.00	0.04 ± 0.05	0.01 ± 0.01	96.89 ± 11.37*	33.46 ± 17.93	0.01 ± 0.00	0.01 ± 0.00
1-Octen-3-ol	0.31 ± 0.07	0.28 ± 0.09	0.34 ± 0.09	0.31 ± 0.06	0.31 ± 0.00	0.36 ± 0.05	0.31 ± 0.04	0.29 ± 0.08
1-Heptanol	0.17 ± 0.04	0.15 ± 0.05	0.09 ± 0.03	0.15 ± 0.02	0.13 ± 0.06	0.17 ± 0.04	0.17 ± 0.02	0.16 ± 0.05
Benzylalcohol	148.82 ± 33.77	107.16 ± 7.27	72.02 ± 34.76*	134.97 ± 15.34	111.67 ± 61.03	155.73 ± 30.46	158.14 ± 3.66	161.25 ± 5.47
2-Phenylethanol	414.82 ± 51.50	325.47 ± 21.83	267.63 ± 63.65*	422.01 ± 47.21	358.13 ± 151.28	439.80 ± 4.36	480.85 ± 10.71	490.57 ± 16.27
linalool	11.82 ± 2.68	10.63 ± 3.56	5.69 ± 2.79*	10.73 ± 1.22	11.61 ± 0.16	12.38 ± 2.41	11.73 ± 1.54	11.02 ± 3.21
trans-Linalooloxide	0.02 ± 0.00	0.02 ± 0.01	15.22 ± 0.82	8.23 ± 1.00	11.24 ± 1.10	6.44 ± 3.74	0.02 ± 0.00	0.02 ± 0.01
cis-Linalooloxide	0.00 ± 0.00	0.00 ± 0.00	7.56 ± 3.57	2.76 ± 1.78	3.00 ± 5.19	2.75 ± 4.77	0.00 ± 0.00	0.00 ± 0.00
a-Terpineol	13.02 ± 2.95	11.71 ± 3.93	10.37 ± 0.75	10.32 ± 3.14	11.38 ± 2.55	12.14 ± 4.83	12.92 ± 1.70	13.44 ± 1.33
b-Citronellol	10.72 ± 2.43	9.64 ± 3.23	4.92 ± 2.74*	9.82 ± 1.08	10.55 ± 0.12	11.32 ± 2.08	10.64 ± 1.40	10.00 ± 2.91
nerol	0.10 ± 0.02	0.09 ± 0.03	0.12 ± 0.05	0.06 ± 0.05	0.10 ± 0.01	0.07 ± 0.06	0.09 ± 0.01	0.09 ± 0.03
Geraniol	7.69 ± 1.74	6.91 ± 2.32	1.93 ± 0.34	4.71 ± 4.15	0.11 ± 0.01	8.45 ± 1.19	7.63 ± 1.00	8.31 ± 0.29
exo-2-Hydroxy-1.8-cineole	0.03 ± 0.01	0.03 ± 0.01	0.38 ± 0.31	0.17 ± 0.24	0.36 ± 0.10	0.17 ± 0.24	0.03 ± 0.01	0.03 ± 0.01
ALDEHYDES								
Benzaldehyde	26.70 ± 6.06	24.01 ± 8.05	19.67 ± 2.76	22.18 ± 4.89	26.37 ± 0.28	25.91 ± 8.31	26.50 ± 3.48	29.03 ± 0.96
Hexanal	3.98 ± 0.90	3.58 ± 1.20	3.06 ± 0.16	3.99 ± 0.71	3.44 ± 0.84	5.25 ± 1.44	3.95 ± 0.52	3.71 ± 0.08
Furfural	99.07 ± 22.48	74.70 ± 6.55	74.45 ± 8.23	81.27 ± 19.69	97.53 ± 1.13	95.11 ± 32.46	98.32 ± 12.92	107.84 ± 3.57

C, fermentation in grape must (control); Cu, fermentation in Cu-added grape must. Data are mean ± SD of three independent experiments. For each strain, the asterisk indicates significantly different values (one-way ANOVA, $P < 0.05$) between wines from control and Cu-added grape must.

TABLE 5 | Fermentation performance at cellar level by the selected indigenous *S. cerevisiae* strain in comparison to the commercial one.

Parameters	Indigenous strain (MPR2-24)	Commercial strain (ES 454)
Total acidity[a]	7,56	7,82
Volatile acidity[a]	0.18	0.22
Ethanol[b]	12.59	11.72
Acetaldehyde[c]	15,72	19,28
Ethylacetate[c]	63.25	64.61
n-Propanol[c]	33.77	46.63
Isobutanol[c]	31.03	48.86
Amyl alcohols[c]	344.17	292.98

a, g L^{-1}*; b, % v/v; c, mg L*$^{-1}$.

yeast cells, indicating a strong strain ability to reduce the copper content in wine. This effect was inversely correlated with copper resistance: the most powerful strain in copper reduction was the most sensitive strain, MPR2-24. These results confirm the data previous reported by Sun et al. (2015), who demonstrated that "copper tolerance and copper adsorption ability of strains showed a negative correlation." It's well known that a strict regulation of Cu homeostasis is required for *S. cerevisiae* cell survival and one of the mechanisms protecting cells from excess of copper is the reduction in copper uptake and its overload. Brady et al. (1994) found that the copper content in copper-tolerant yeast was lower than other strains when exposed to similar conditions, demonstrating that the mechanism for copper-resistance in *S. cerevisiae* was to reduce the intracellular uptake of copper (Wang and Chen, 2006). Adamo et al. (2012) hypothesize that one of the mechanisms of robustness toward copper might rely on hindering metal uptake. Some authors suggested a central role of the plasma membrane (Avery et al., 1996; Fernandes and Sa-Correia, 2001; Vagabov et al., 2008) and of the cell wall (Abbott et al., 2007) in the onset of tolerance to heavy metals. By our opinion, the high copper reduction ability of MPR2-24 strain might be most probably correlated to a biosorption mechanism. Factors affecting the metal biosorption in yeasts, such as status of biomass (living or non-living), types of biomaterials, properties of metal-solution chemistry, environmental conditions, were widely studied (reviewed in Wang and Chen, 2006), whereas studies reporting the influence of different *S. cerevisiae* strains on copper biosorption are very limited. Sun et al. (2015) reported that different *S. cerevisiae* strains are able to adsorb different quantity of copper during wine fermentation. These authors demonstrated that the main copper adsorption mechanism in *S. cerevisiae* during alcoholic fermentation was cell surface adsorption, as no copper was detected inside the yeast cells. It has been reported (Vinopal et al., 2007) that metallosorption capacity of the yeast wall is largely dependent on the outer mannoprotein layer. Park et al. (2003) reported that Cd^{2+} sorption capacity is proportional to thickness of the mannoprotein layer. The enzymatic removal of mannoproteins from the *S. cerevisiae* cell wall decreased the amount of sorbed Cd^{2+}, Co^{2+}, and Cu^{2+} (Brady et al., 1994). The enrichment of the *S. cerevisiae* cell wall with α-agglutinin derived mannoprotein enhanced the sorption capacity of genetically modified yeast for Cd^{2+} and Zn^{2+}. Our results show that copper reduction was strain specific, with MPR2-24 strain exhibiting a very high ability to reduce copper content in wine, probably in consequence of high biosorption ability. We can speculate that this strain behavior can be correlated to a different cell wall composition of MPR2-24 in comparison to the other tested strains. In order to validate strain behavior in conditions that mimic wine fermentation, the strains were tested in NGM fermentation. The ability of MPR2-24 strain to reduce copper content in wine was confirmed also in fermentation of NGM. Other than the evaluation of strain ability to reduce copper content of natural wine, the aim of this trial was to evaluate the effect of copper addition on metabolic activity of copper sensitive and tolerant strains. As expected, copper affects the fermentative performance of sensitive strains; in particular, these strains started and completed the fermentative process later than copper tolerant strains, although all the fermentations were concluded with final very low residual sugars. The copper strain sensitivity affected wine volatile acidity; in fact copper sensitive strains in fermentation of copper-added must yielded wines with higher volatile acidity than wines obtained without copper. This result could be related to the stressful conditions suffered by sensitive strains in copper supplemented fermentation as an increase of volatile acidity after alcoholic fermentation is generally associated to a yeast stress signal (Bely et al., 2005; Cavazza et al., 2013). As regards the copper influence on strain metabolic activity, the determination of the secondary compounds affecting organoleptic quality of experimental wines showed that the production levels of these compounds were affected in sensitive strains, mainly in A13 (**Tables 3**, **4**). Also this effect could be a consequence of mechanisms triggered as response of copper sensitive strains to metal stress. Since copper is a strong oxidizing agent (Adamo et al., 2012), the changing of metabolic activity of sensitive strains in copper added must fermentation can be a consequence of the reconfiguration of the glycolytic flux, a mechanism reported to regulate the response to oxidative stress in yeast cells and other eukaryotic organisms, such as human and plant (Morigasaki et al., 2008; Romano et al., 2015). Although the influence of copper on metabolic activity of sensitive strains, it has be underlined that all the secondary compounds detected in experimental wines were present at acceptable level (Swiegers et al., 2005).

On the basis of very interesting traits of MPR2-24 strain as biotechnological tool to reduce the copper content in wine, this wild strain was finally tested as starter culture in cellar pilot scale fermentation in comparison to a commercial starter. Also in real winemaking conditions this strain confirmed the traits exhibited during lab scale fermentation. Although MPR2-24 is a copper sensitive strain and the fermentations were performed in a grape must from organic vineyard, it completed successfully the fermentative process and showed high implantation ability, at a level comparable with fermentation performed by commercial starter culture. Therefore, this strain was able to survive and ferment in presence of copper, highlighting its good efficiency as starter culture. As reported by other authors, the choice of the right starter culture is crucial when there is a risk of high copper content in the grape must (Ferreira et al., 2006; Cavazza

et al., 2013). The analysis of copper content in the two wines revealed that both the starters were able to reduce the copper content, although the indigenous starter at higher level than commercial one.

Our results showed that the study of copper adsorption in *S. cerevisiae* strains is an important tool to select starter strains able to conduct efficiently the fermentation process also in grape must containing too high copper residual. This situation is quite frequent in the last years, as a consequence of worldwide increase of organic wine sector, but it's well known that high copper residual in final wine, particularly existence with other heavy metals such as iron, manganese, zinc, nickel, lead, can cause some unaccountable risks for health consumers if metal concentrations are not kept under allowable limits (Naughton and Petróczi, 2008). Furthermore, copper content affects also wine quality since metallic ions have important role in oxide-reductive reactions resulting in wine browning, turbidity, cloudiness, and astringency. The wild strain MPR2-24, in addition to its ability to complete the fermentation and give acceptable flavor to the wine, possesses copper binding abilities and does therefore have great potential to be utilized as starter culture at industrial level. The use of this wild strain, that at the same time is able to perform successfully the alcoholic fermentation and reduce copper content in wine, represents an useful tool to assure not only the wine quality, but also to preserve the original color and flavor of wine.

Biotechnological reduction of copper content in wine is potentially a sustainable approach, as alternative to the chemical-physical methods, currently allowed by the official organizations, such as OIV. Continuing advances in yeast biology provide many opportunities for innovation and adaptation to a changing market. These will enable the development of new oenological practices based on the exploitation of new strains (Comitini et al., 2017). These new biotechnological tools can satisfy the increasing environmental pressures for a wine industry that is more efficient and more sustainable.

AUTHOR CONTRIBUTIONS

AC contributed to the design of the work, to the acquisition and analysis of data, to draft the work and revising it, and ensured that questions related to the accuracy or integrity of any part of the work were appropriately investigated and resolved. RR contributed to the design of the work, to the management of experimental fermentation, to the statistical elaboration of data, to draft the work. LS contributed to the design of the work, to the chemical analysis of wine samples, to the interpretation of data for the work. GS contributed to the management of experimental fermentation, to the interpretation of data for the work. PR contributed to the design of the work, to the interpretation of data, to draft the work and revising it.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2017.02632/full#supplementary-material

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Wild Grape-Associated Yeasts as Promising Biocontrol Agents against *Vitis vinifera* Fungal Pathogens

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The increasing level of hazardous residues in the environment and food chains has led the European Union to restrict the use of chemical fungicides. Thus, exploiting new natural antagonistic microorganisms against fungal diseases could serve the agricultural production to reduce pre- and post-harvest losses, to boost safer practices for workers and to protect the consumers' health. The main aim of this work was to evaluate the antagonistic potential of epiphytic yeasts against *Botrytis cinerea*, *Aspergillus carbonarius,* and *Penicillium expansum* pathogen species. In particular, yeast isolation was carried out from grape berries of *Vitis vinifera* ssp sylvestris populations, of the Eurasian area, and *V. vinifera* ssp *vinifera* cultivars from three different farming systems (organic, biodynamic, and conventional). Strains able to inhibit or slow the growth of pathogens were selected by *in vitro* and *in vivo* experiments. The most effective antagonist yeast strains were subsequently assayed for their capability to colonize the grape berries. Finally, possible modes of action, such as nutrients and space competition, iron depletion, cell wall degrading enzymes, diffusible and volatile antimicrobial compounds, and biofilm formation, were investigated as well. Two hundred and thirty-one yeast strains belonging to 26 different species were isolated; 20 of them, ascribed to eight species, showed antagonistic action against all molds. Yeasts isolated from *V. vinifera* ssp sylvestris were more effective (up to 50%) against *B. cinerea* rather than those isolated from *V. vinifera* ssp vinifera. Six strains, all isolated from wild vines, belonging to four species (*Meyerozyma guilliermondii*, *Hanseniaspora uvarum*, *Hanseniaspora clermontiae,* and *Pichia kluyveri*) revealed one or more phenotypical characteristics associated to the analyzed modes of antagonistic action.

Keywords: yeasts, molds, *V. vinifera* ssp sylvestris, biocontrol, fungal diseases

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INTRODUCTION

Plants provide over 80% of the human diet. Just three cereal crops (i.e., rice, maize, and wheat) and two fruit crops (grape-berries and citrus fruits) provide 70% of energy intake and cope the production of 80% of the fermented beverages in the world (FAO, 2011). Since the 1900s, around 75% of crop diversity has been lost from farmers' fields. Regarding harvest products, many losses (up to 25% of total production in industrialized

countries and more than 50% in developing countries) are attributed to decay fungi, such as the *Botrytis*, *Penicillium, Aspergillus,* or *Cholletotrichum* genera, which are also the source of mycotoxins, harmful compounds to humans (FAO, 2011). The control of fungal diseases and mycotoxins in food and feed chains is principally based on the use of synthetic fungicides. In 2015, Spain, France, Italy, and Germany together made up 70.5% of the European Union-28's pesticide sales. Fungicides are also increasing the level of hazardous residues in the environment, they are becoming less effective due to both the increasing of resistant fungal strains, and the use of restrictions carried out by the European authorities (Directive 2009/128 /EC). Natural diversity and ecosystems provide agricultural production in many different ways (Power, 2010), but not all are well-known. Although animal and plants have received considerable attention as a resource for natural-product discovery, the microbiological component of this natural richness remains relatively unexplored.

Yeasts are unicellular fungi that have been isolated from different ecosystems and sources both natural and in connection with human activities. They can be found on/in fruits, including *Vitis vinifera* ssp vinifera cultivars and *V. vinifera* ssp. sylvestris, plants, insects, animal intestinal tracts, soils, and marine environments (Kurtzman et al., 2011). In the past 35 years, there have been extensive research activities to explore and develop the potential of yeasts as antagonists to biologically control harvest pathogens and as an alternative to chemical pesticides (Liu et al., 2013). Representing an eco-friendly alternative to synthetic pesticides, the use of antagonist yeasts as biocontrol agents has generated a great enthusiasm (Wisnieswski et al., 2007; Droby et al., 2009; Sipiczki, 2016; Spadaro and Droby, 2016). However, yeasts often show a lower and non-comparable effectiveness against pathogenic fungi (*Botrytis cinerea, Aspergillus carbonarius, and Penicillium expansum*) in comparison to chemical fungicides (Liu et al., 2013), thus reducing their practical applications and leaving the problem of plant fungal disease still unsolved. Considerable progress has been made in increasing knowledge and commitment to elucidate some modes of action of few yeast strains against pathogenic fungi (Sipiczki, 2006; Sharma et al., 2009; Jamalizadeh et al., 2011; Spadaro and Droby, 2016). The described mechanisms are; nutrient or space competition (Suzzi et al., 1995), iron depletion (Sipiczki, 2006; Parafati et al., 2015), extracellular lytic enzymes production (Bar-Shimon et al., 2004), volatile organic compounds (Fredlund et al., 2004), reactive oxygen species (ROS) tolerance (Jamalizadeh et al., 2011; Liu et al., 2011), biofilm formation (Giobbe et al., 2007; Wisnieswski et al., 2007), or inducing host-plant resistance throughout the accumulation of phytoalexins (Arras, 1996; Jeandet et al., 2002) and the synthesis of pathogenesis-related proteins (Chan and Tian, 2006). Inhibition capabilities on mycelial growth or conidia germination in molds have been reported by some yeast strains of species living in vineyards, overwintering grapes, and cellar ecosystems (Elmer and Reglinski, 2006; Nally et al., 2012; Sipizcki, 2016). Nevertheless, all the scientific strategies focused on looking at different components of such interactions separately or taking into consideration binary or ternary trophic levels of the host-pathogen-antagonist interplay (Droby et al., 2009; Spadaro and Droby, 2016). In general, interactions are not between two single microorganisms and the host; they also involve the native microbiota of the host and the environmental factors (i.e., the variation of the climatic conditions and other abiotic factors such as the soil, plant emplacement, or nutrient availability for the plant). In the case of the vineyards, efforts to understand the influence of different agronomic parameters on yeast populations associated to grape-berries have been published (Cordero-Bueso et al., 2011a,b, 2014) but there is still a lack of bibliography. Moreover, there are unexplored ecosystems such as wild vines like the protected species *V. vinifera* ssp sylvestris (Gmelin) Hegi which could represent a great reservoir of novel and promising yeast species to be used in the food industry, as well as a substitutive of agrochemicals.

The main aim of this work was to evaluate the antagonistic potential of yeasts isolated from grape berries collected from *V. vinifera* ssp sylvestris populations in the Mediterranean and Black Sea basins and from *V. vinifera* ssp vinifera cultivars managed under three different farming systems: organic, biodynamic, and conventional. The mode of action and the grape-berry population associate to grape-berries were investigated as well.

MATERIALS AND METHODS

Yeast Strain Identification

Yeast strains were isolated between 2013 and 2016 from grape berries collected in Georgia, Italy, Romania, and Spain from *V. vinifera* ssp. sylvestris populations as stated in Cordero-Bueso et al. (2017) and in Italy from *V. vinifera* ssp. vinifera cv. Pinot Noir cultivated in three different farming systems: organic, biodynamic, and conventional in 2014 (**Figure 1**). Grape samples were treated following the protocol of Vigentini et al. (2016). All yeasts used in this work were stored in YPD medium (20 g/L peptone, 10 g/L yeast extract, 20 g/L glucose) added with 20% (v/v) glycerol at −80°C. Fresh yeast cultures were obtained by inoculation 1% (v/v) glycerol stocks in YPD broth at 25°C for 3 days in aerobic conditions. Isolates were also plated onto Wallerstein Laboratory Nutrient Agar (WL) to evaluate colony diversity as suggested by Pallmann et al. (2001). DNA extraction from the yeast isolates was performed according to Querol et al. (1992). The patterns belonging to the different species were obtained by Restriction Fragment Length Polymorphism (RFLP) analysis of the amplified ITS1-5.8S-ITS2 region; the primers used for DNA amplification were ITSY1 (5′-TCCGTAGGTGAACCTGCGG-3′) e ITSY4 (5′-TCCTCCGCTTATTGATATGC-3′) as described by White et al. (1990). PCR products were digested by *Cfo*I, *Dde*I, *Hae*III, and *Hinf*I restriction enzymes (Thermo Fisher Scientific, Massachusetts, U.S.A.). *Meyerozyma guilliermondii* (anamorph *Candida guilliermondii*) and *Meyerozyma caribbica* (anamorph *Candida fermentati*) are closely related species. Thus, to avoid misidentification these species of yeasts were also subjected to RFLP analysis using the enzyme *Taq*I as stated by Romi et al. (2014). Amplification products and their fragments were separated on 1.4% (w/v) and 2.5% agarose gel, respectively, added with 0.05 μg/L of ethidium bromide in TAE buffer

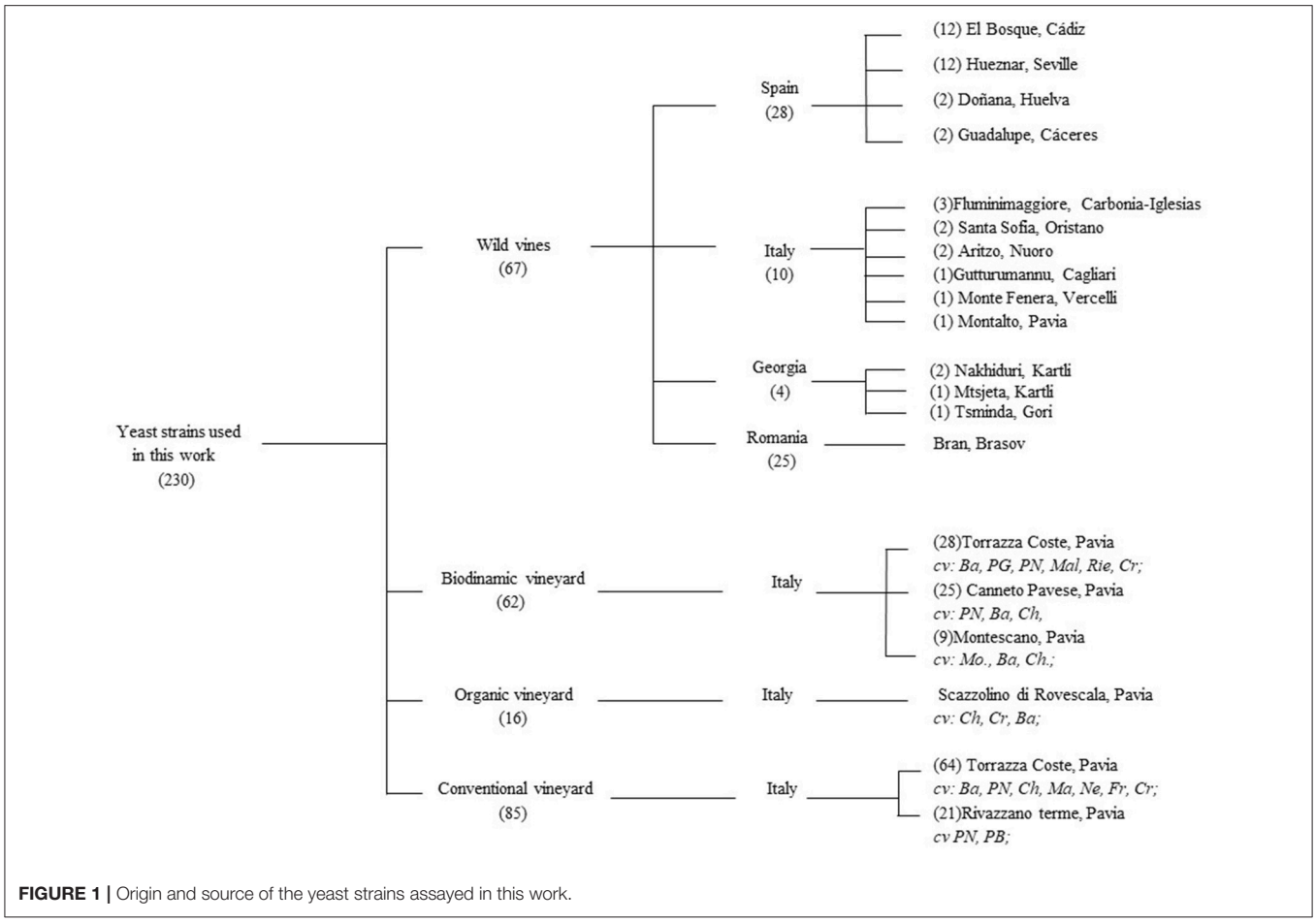

FIGURE 1 | Origin and source of the yeast strains assayed in this work.

(Tris-acetate 40 mM, EDTA 1 mM, pH 8) at 100 V for 90 min. The agarose gels were visualized using UV and photographed (1000 System, Bio-Rad Laboratories, California, U.S.A.). At least two representative members from each ITS-RFLP genotype group were randomly selected for sequencing LSU sRNA gene D1/D2 domain. Certain database sequences of several species such as *Aureobasidium pullulans* and *Rhodotorula nothogafi*, have identical D1/D1 sequences with other species. Thus, when necessary, we included the ITS1-5.8S-ITS2 region sequences. Amplification of D1/D2 region was carried out using primers NL1 (5′-GCATATCAATAAGCGGAGGAAAAG-3′) and NL4 (5′-GGTCCGTGTTTCAAGACGG-3), as previously described Kurtzman and Robnett (1998). Purification and sequencing of PCR products were performed by Macrogen Inc. facilities (Seoul, South Korea) using an ABI3730 XL automatic DNA Analyzer. The obtained sequences were aligned using ClustalX algorithm. The Basic Local Alignment Search Tool (BLAST) (http://www.ebi.ac.uk/blastall/nucleotide.html) was used to compare the sequences obtained with databases from the European Molecular Biology Laboratory (EMBL). As proposed Sipiczki (2016), the sequences of the strain types were also determined by pairwise Blast alignment using the bl2seq algorithm available at the website of the NCBI (http://www.cbs.knaw.nl). We considered an identification as "correct" when the gene sequence showed an identity $\geq$ 98% and a good query cover with the exception of the species *Vishniacozyma carnescens* and *V. victoriae* which D1/D2 sequences of their type strains differ only by 1.8%. Moreover, yeast strains were tested for the fermentation or assimilation of the different compounds as sole carbon, nitrogen, and others sources, with the exception of the hexadecane, vitamin-free, 5-keto-D-glucanase, saccharate, cadaverine, and CoQ component, as stated in Kurtzman et al. (2011) but using a 96-well microtiter plate technology.

Mold Strains and Growth Conditions

The mold strains used in this work were *P. expansum* UCAF0034 (Colección de la Universidad de Cádiz, Spain), *B. cinerea* BO5.10 (*Colección Española de Cultivos Tipo,* Burjassot, Valencia, Spain), and *A. carbonarius* UCAF0012 (Colección de la Universidad de Cádiz, Spain). Molds were selected based on their virulence by artificial inoculation on wounded grapes (data not shown).Mold cultures were plated on a Potato Dextrose Agar medium (Conda Laboratories, Torrejón de Ardoz, Madrid, Spain). Plates were incubated at 25°C under constant white light for at least 10 days. After incubation, spores were collected in a solution of 0.1% (v/v), Tween 20 (SIGMA). The concentration of the conidial suspension was adjusted to give 6×10^6 spores/mL according

to Comménil et al. (1999). Mold strains were stored as conidial suspensions added with 20% (v/v) glycerol at −80°C.

In Vitro Assays for Antagonistic Activity

Dual Screening of Antagonistic Activity on Agar Media

The antagonistic activity of the 241 yeast isolates against *A. carbonarius, B. cinerea,* and *P. expansum* molds was investigated by *in vitro* assay. In the *first* screening, 5 μL of a fresh conidial suspension of the molds, one for each plate, were inoculated in the center of the PDA plate. Then, 5 μL of six fresh yeast cultures were positioned at 2.5 cm from the center of each Petri dish. The plates were incubated at 25°C for 10 days under constant white light and 80% relative humidity. A clear zone around the yeast colonies was interpreted as total inhibition of the growth of the mold. The strains showing an inhibitory activity were chosen for the second step of selection. In this case the PDA plates were prepared as follows: 10 mL of PDA were first included in each plate; afterwards, 5 mL of soft PDA (7 g/L agar) containing a final concentration 10^6 CFU/mL of yeast cells, one for each strain, were inoculated in the plates. Subsequently, when the plates were solidified, 5 μL of fresh conidial suspensions of the tested molds were inoculated upon them. The plates were incubated at the same conditions of first screening. After incubation, the radial growth was measured and the inhibition percentage was calculated as follows: inhibition (%) = (DC – DA)/DC x 100, where DC is the diameter of the growth area without the antagonistic yeast (control), DA is the diameter of growth area with the antagonistic yeast (Ruiz-Moyano et al., 2016). The experiments were repeated three times to confirm reproducibility of the results.

Evaluation of the Minimum Inhibiting Concentration

An estimation of the starting concentration of yeast cells capable to inhibit the mold growth was carried out by the following test. Fresh cultures of the yeasts that overcome the second step of selection were grown in YPD broth at 25°C for 3 days. PDA plates were prepared for each strain containing a different cell concentration, from 10^3 to 10^6 CFU/mL. When the plates solidified, 10 μL of conidial suspensions (3×10^5 spores/mL) of *B. cinerea*, *A. carbonarius,* and *P. expansum* were spotted on the center of the Petri dish. The plates were incubated at 25°C for a week under constant light. The results were considered positive when the yeast was able to inhibit the total mold growth within the time of incubation. Control tests without inoculated yeast cells were carried out. The experiments were repeated three times to confirm reproducibility of the results.

Killer Character Assay

The killer character assay was performed according to Stumm et al. (1977). Plates containing YPD-agar and 0.003% (w/v) of methylene blue that was buffered to pH 4.5 with 0.1 mol/L of citrate-phosphate buffer were used. Yeast strains were cultured in liquid YPD until their exponential growth phase. Then, yeast strains were diluted in YPD and spread onto the plates at a concentration of 10^5 cells per plate and incubated at 25°C for 48–96 h. Killer activity was scored positive when the killer strain was surrounded by a region of bluish-stained cells, or by a clear zone of growth inhibition bounded by stained cells.

Test for Lytic Enzymes Activity

In order to investigate the reason of the observed inhibitory effect, the previous selected strains were examined taking in consideration the production of cell wall lytic enzymes. Yeast fresh cultures were adjusted at a final concentration of 1×10^6 CFU/mL. To evaluate the proteolytic activity, 20 μL of the yeast suspension were spotted onto Skim Milk agar (Merck, Darmstadt, Germany); the formation of a clear halo around the colony after incubation at 25°C for 5 days indicated the enzymatic activity. Glucanase and chitinase activities were determined by replica plating technique. In this case, 20 μL of the yeast suspension were spotted onto YPD plates containing 0.2% β-glucan (Sigma, Town, Nation) and YPD plates containing 0.2% chitin (Sigma). Petri dishes were incubated at 30°C for 5 days. Colonies were rinsed off the plates with distilled water before staining the plates with 0.03% (w/v) Congo Red. A clear zone around the colony meant the presence of glucanase activity. Yeasts were screened for polygalacturonase production with the method described by Strauss et al. (2001) as well; they were spotted onto polygalacturonate Agar Medium containing 12.5 g/L polygalacturonic acid (Sigma), 6.8 g/L potassium phosphate (pH 3.5), 6.7 g/L yeast nitrogen base without ammonium sulfate (YNB, Difco), 10 g/L glucose, and 20 g/L agar. Plates were incubated at 30°C for 5 days. Colonies were rinsed off the plates with deionized water before staining the plates with 0.1% (w/v) Ruthenium Red. Colonies showing a purple halo were considered positive. β-glucosidase activity was tested by plating the yeast onto a selective medium containing 6.7 g/L yeast nitrogen base (YNB, Difco), 5 g/L arbutin (Sigma), and 20 g/L agar (pH 5.0). Two milliliters of a filter-sterilized 1% (v/v) ammonium ferric citrate solution was added to 100 mL media before pouring onto the plates. Petri dishes were incubated at 30°C for 3 days. Positive colonies were identified by the discoloration of the media to a brown color.

Production of Volatile Organic Compounds (VOCs) and Hydrogen Sulfide Release

Selected yeast strains were also evaluated for their production of VOCs and hydrogen sulfide released against the molds *B. cinerea, A. carbonarius,* and *P. expansum*. Four-part Petri dishes containing 3.5 mL of PDA for each sector were used. In one part, 20 μL of 10^6 CFU/mL of yeast suspension were inoculated. The plates were incubated at 25°C for 3 days. Then, 20 μL of conidial suspension (6×10^6 spores/mL) of each mold were inoculated in the other three sectors of each plate. Plates without the inoculation of yeasts were utilized as control. Finally, the plates were double wrapped with sterile HDPE film (Parafilm, Neenah, U.S.A) to prevent air escape and incubated for 3 days at 25°C under constant white light. Radial growth reduction, in relation to the control test, was calculated after 6 days. All experiments were performed in triplicate. Data were analyzed by one-way ANOVA. The means were separated at the 5% significance level using Tukey's test. The yeast strains slowed or inhibited the

mold growth were also tested for the production of acetic acid and hydrogen sulfide. Ten microliters of yeast cell suspensions (10^6 CFU/mL) were spotted on Biggy Agar (Oxoid, Bakingstoke, U.K.) and in a $CaCO_3$ agar medium (5.0 g/L yeast extract; 20 g/L glucose; 10 g/L $CaCO_3$; 20 g/L agar). The plates were incubated at 30°C for 3 days. The qualitative amount of H_2S production on this indicator medium was determined by the color of the colonies, which ranged from white (no release) through brown to near black, depending on the extent of production (high release). In the case of the acetic acid production, a clear zone around the colony meant the presence of acetic acid. A halo greater than 3 mm of radius meant a high acid release, if the halo was between 2 and 3 mm meant low acid release, if the halo was between 1 and 2 mm meant slight acid formation, and if the halo was less than 1 mm meant traces.

Biofilm Formation

The capability to produce biofilm was evaluated following the protocol of Jin et al. (2003) partially modified. Ten microliters of fresh yeast suspension as previously described were inoculated in 1 mL of Yeast Nitrogen Base (YNB, Difco, Swedesboro, U.S.A.) added with 100 mM glucose and incubated overnight at 28°C. Subsequently, the tubes were centrifuged at 4,000 rpm for 5 min (Rotina 380 R, Hettich Zentrifugen, Tuttlingen, Germany), the cells were washed twice with a 1X phosphate-buffered saline (10X PBS: NaCl 1.37 M, KCl 27 mM, Na_2HPO_4 100 mM, KH_2PO_4 18 mM), pH 7.2) and re-suspended in YNB + glucose (100 mM) medium to obtain 10^7 CFU/mL. A control test was prepared with the medium without yeast cells added. One hundred microliters of the cell suspension were inoculated in triplicate into 96-well polystyrene plate with flat bottom (Starlab, Hamburg, Germany) at 28°C in a shaker at 75 rpm for 3 h. After the adhesion phase, the wells were washed twice with 150 μL of PBS, and then 100 μL of same medium were added into each well and incubated at 28°C in a shaker at 75 rpm for 72 h. The medium was sucked up daily and, then, 100 μL of fresh YNB were put into each well. After incubation, the wells were washed twice with 150 μL of PBS then 100 μL of crystal violet 0.4% (w/v) were put into each well. After 45 min, the wells were washed again for four times with 150 μL of distillate sterile water and immediately 200 μL of 95% (v/v) ethanol were added. After 45 min, 100 μL of solution were transferred to a new polystyrene 96-well plate and then the solution was measured at 590 nm. The absorbance values were subtracted for the control test values.

Effect of Iron Concentration on the Inhibitory Activity of the Yeast Strains

In order to investigate the influence of iron concentration on the inhibitory activity of the selected yeasts the following test was carried out. PDA plates without added iron and plates with 5 and 20 μg/mL of $FeCl_3$ were prepared spreading on plates a conidial suspension (3×10^5 spores/mL) of *B. cinerea, A. carbonarius,* and *P. expansum.* Then, 10 μL of yeast suspensions (10^6 CFU/mL) were dropped on Petri dishes in triplicate. Three plates for each mold without yeast addition were used as control. The plates were incubated at 25°C for 1 week under constant white light. The width of reddish halos developing around the yeast colonies were measured according to Parafati et al. (2015). The results of the role of competition for iron on the antagonistic activity of the yeasts were obtained measuring the width of inhibition zones around the yeast colonies after a week.

Effect of Other Metabolites Released by Yeast Strains on Mold Growth

In order to examine the effect of other potential metabolites derived from the primary or secondary metabolism of yeasts produced by antagonistic yeasts, the molds were grown in a medium containing the supernatant of a yeast culture. The yeast cultures were grown in 50 mL YPD broth at 25°C for 5–7 days in a shaker at 125 rpm. The cell growth was monitored by spectrophotometer measurements at 600 nm (Jenway 7315, Staffordshire, U.K.). When yeast cultures attained the stationary phase the supernatants were collected by centrifugation at 3,500 rpm for 5 min at 4°C (Rotina 380 R, Hettich Zentrifugen, Tuttlingen, Germany) and filtered by a 0.45 μm sterile membrane (Minisart, Goetting, Germany). Five, 0.5, and 0.05 mL of supernatants were mixed with warm (<45°C) and concentrated 5X PDA medium by adjusting the volume with sterile distilled water and poured in Petri dishes. When the plates solidified, 10 μL of conidial suspensions (3×10^5 spores/mL) of *B. cinerea*, *A. carbonarius,* and *P. expansum* were inoculated. The plates were incubated at 25°C for a week under constant light. The test was considered positive if the tested molds did not grow or if a severe growth inhibition was observed with respect to the control.

In Vivo Assays for Inhibitory Activity

Efficacy of Yeast Strains in Controlling Grapes Infected by Molds

The yeast strains showing an evident inhibitory activity by *in vitro* assays were selected for the *in vivo* test. Fresh yeast cultures were collected by centrifugation at 3,000 rpm (Rotina 380 R, Hettich Zentrifugen, Tuttlingen, Germany) for 5 min at 4°C and washed twice with sterile distilled water. The yeast suspensions were adjusted at 10^6 CFU/mL. Healthy berries of table grapes (cultivar Superior Seedless, Egypt) were used for the test. Grape berries surface was disinfected by dipping them in a solution 1% (v/v) sodium hypochlorite for 5 min and rinsed three times with sterile distilled water. Afterwards, three berries for treatment were cut with a sterile scalpel (one wound of 5 mm for each berry) and submerged in the yeast cells suspensions for 5 min. The berries were put into sterile 50 mL Falcon tubes (Sigma-Aldrich, Darmstadt, Germany) and incubated for 24 h at 25°C. Then, the wounds were inoculated with 20 μL of conidial suspension (6×10^6 spores/mL) of *B. cinerea, A. carbonarius,* and *P. expansum* (three berries for each mold and for each yeast) and incubated at 25°C under constant light for a week. Three berries for each mold without yeast cells were used as control. The disease severity was evaluated by a visual score "1-to-4" (1: no visible symptoms; 2: soft rot; 3: formation of mycelium; 4: sporulation of mold) according to Parafati et al. (2015).

Inhibitory Effect of Yeasts vs. a Chemical Pesticide by *in Vivo* Tests

The inhibiting activity of strains, that showed the best results in the previous tests, were compared to the commercial pesticide Switch®, Syngenta (37.5% *Cyprodinil* and 25% *Fluodioxinil*). The fresh yeast cultures were prepared as above described. The pesticide was used at the suggested concentration of 1 g/L, according to the manufacturer's instruction, and it was dissolved in 25 mL of distilled sterile water. Healthy berries of table grape (cultivar Sugarone, Chile) for each yeast strain, pesticide, and control, repeated for the three tested molds, were used in this trial. The berries were treated and disinfected as above described. Afterwards, the berries were submerged in the solutions containing the yeast cells and in the solution containing the chemical pesticide for 5 min. Three berries for each mold without yeast cells and pesticide were used as control. The berries were included in six-well plate (Starlab, Hamburg, Germany) at 25°C for 24 h. Then, 10 μL of conidial suspension (6×10^6 spores/mL) of *B. cinerea*, *A. carbonarius,* and *P. expansum* were inoculated on the berries, in the correspondingwound points. The plates were incubated at 25°C for a week under constant light. The results were evaluated by a visual score previously stated.

RESULTS

Identification of Yeasts

Two hundred and thirty-one yeast strains were isolated from grape berries samples of different vines: 85, 62, and 16 from a conventional, a biodynamic, and an organic vineyard, respectively. Sixty-seven yeasts were collected from *V. vinifera* ssp. sylvestris. The sampling plan and the distribution of the isolates are reported in Supplementary Material 1. Sixteen different morphologies were observed on WL-agar plates (data not shown). Three distinct colony subtypes were also identified within the pink-halo producers. Molecular identification by using amplification and restriction analysis of ITS1-5.8S-ITS2 region revealed 26 different patterns. The D1/D2 region of the 26S rDNA gene of at least two yeast strains, for each potential species was sequenced to identify the species. **Table 1** shows the number of strains ascribed to each different species. The accession number of the sequences deposited at GenBank and the most similar CBS strain numbers are shown in **Tables 1**, **3**. *Aureobasidium pullullans* can easily be confused with *Aureobasidium subglaciale*, *Kabatiella microsticta,* or *Columnospaeria fagi* because many database sequences of these species have identical D1/D2 sequences (Brysch-Herzberg and Siedel, 2015; Sipiczki, 2016). Moreover, *R. nothofagi* is difficult to distinguish from *C. pallidicorallinum* because certain database of sequences of these species have identical D1/D2 sequences (Sampaio, 2011; Sipiczki, 2016). Therefore, we analyzed the ITS region of *A. pullulans* and *R. nothofagi* as well (**Table 1**). Since mating partners of the type strains of these species exhibited the most similar ITS sequences and the most similar D1/D2 sequences it's justified to assign the yeast strains of this study to *A. pullulans* and *R. nothofagi*. Furthermore, our strain of *R. nothofagi* did not grow on maltose, trehalose, and inulin, which are usually assimilates by *C. pallidicorallinum* (Sipiczki, 2016). The D1/D2 sequence of our strain identified as *V. carnescens* totally fits with the sequences of type strains found in the explored databases.

Unfortunately, we encountered the problem that isolates ROMA1A, ROM10, CABM7C, and CABM9C (**Table 1**) which seem to belong to *Metschnikowia*-like strains, did not show sequence identity of their D1/D2 to any of the type strains despite they were fairly similar to one species of the *Metschnikowia pulcherrima* clade. It happened also with the ITS sequences. In agreement with Lachance (2011), Sipiczki et al. (2013), Brysch-Herzberg and Siedel (2015), Lachance (2016), and Sipiczki (2016), species belonging to the *M. pulcherrima-like* strains cannot be unequivocally assigned to one of the species of this clade after rDNA analysis because some species such as *M. fructicola* or *Metschnikowia andauensis* have a non-homogenized rDNA array. Moreover, these yeast strains cannot be easily separated by phenotypical and physiological tests. Efforts to clarify the taxonomic situation of the *Metschnikowia* clade are required. Although was impossible to assign our strains to one of the currently described species in the *M. pulcherrima* group, we showed in **Tables 1, 3**, the most probable species related to this genus according to the results obtained after the analysis performed.

In Vitro Tests

In Vitro Dual Assays to Show the Antagonist Yeast-Mold Interactions

All yeast isolates were subjected to a preliminary *in vitro* assay for the detection of an antagonistic activity against *B. cinerea*, *P. expansum,* and *A. carbonarius*. Sixty out of the 231 yeast strains showed an effect of slowing down or inhibiting growth of the three tested molds. Thirty-six out of 60 selected antagonistic yeasts were isolated from *V. vinifera* ssp. sylvestris, 9 from the biodynamic vineyard, 1 from the organic vineyard, and 4 from the conventional one (**Table 2**). The majority of the strains with antagonistic activity were isolated from wildlife vines (53%), followed by those isolated from the biodynamic (14.5%), the organic farming system (6.2%), and the conventional (4.7%) vines (**Table 2**).

After the preliminary assay, a second *in vitro* test was performed. It consisted of a test on solid medium where Petri-dishes were plated with a yeast cell-top agar suspension and the mold spores were spotted on the center of the plate. The percentage of the mycelium growth was calculated for each yeast strain against each mold (Table S1, Supplementary Material 1). Twenty yeast strains (plus the control) out of 60, which passed the first screening, inhibited the 100% of hyphal growth of the three tested molds in comparison with the control. Among these, 18 strains were isolated from the wild vines and belonged to *H. uvarum* (9), *M. guilliermondii* (2), *P. kluyveri* (2), *S. cerevisiae*, *H. clermontiae*, *M. fructicola*-like yeast strain, *M. viticola,* and *C. californica* species, and two strains were isolated from the biodynamic vines and were ascribed to *A. pullulans* and *V. carnescens* species (**Table 2**). These 20 yeast strains were selected for the successive tests in order to understand the nature of antagonistic activities.

TABLE 1 | Yeast species occurrence and distribution of the isolated and identified from *V. vinifera* ssp sylvestris and from the different vine cultivars of *V. vinifera* ssp vinifera (conventional, biodynamic, and organic), GenBank accession numbers of the deposited sequences and The Centraalbureau voor Schimmelcultures (CBS) and D1/D1 Genbank accession numbers of the most similar types.

Isolate			Most similar type/reference strain		Source			
Strain code	**D1/D2 accession no.**	**ITS accession no.**	**Taxonomic name**	**D1/D2 accession number**	**Conventional vineyard**	**Biodynamic Vineyard**	**Organic Vineyard**	***Vitis vinifera* ssp. sylvestris**
FZ02	MF926292	MF783894	*Aureobasidium pullulans* CBS584.75	KT361587.1	46	15	9	1
CABMC2A	MF927682	MF770161	*Candida californica* CBS989	KY816896	–	–	–	1
FZ03a	MF783064	–	*Filobasidium stepposum* CBS10265	KY107724.1	2	–	–	–
HB09c	MF783066	–	*Filobasidium wieringae* CBS1937	KY107733	–	–	–	1
CABMB1A	MF783060	–	*Hanseniaspora clermontiae* CBS8821	EU272040	–	–	–	1
HURM6B	MF926297.1	–	*Hanseniaspora* ssp CBS276	KY107853	–	–	–	4
CAMB9A	MF783054	–	*Hanseniaspora uvarum* CBS9790	KJ794689	17	34	1	28
NUR3AM	MF926296	–	*Hyphopichia pseudoburtoni* CBS2455	KU609072	–	–	–	1
ROMA10*	MF783057	–	*Metschnikowia fructicola* CBS8853	AF360542	–	–	–	5
CABM7C*	MF783068	–	*Metschnikowia pulcherrima* CBS5833	JN083816	9	8	1	1
CABM9C*	MF783069	–	*Metschnikowia* spp CBS5536	KM350710	–	–	–	5
ROMAM1A*	MF783062	–	*Metschnikowia viticola* CBS9950	KC859919	–	–	–	2
SEHMA2	MF783056	–	*Meyerozyma caribbica* CBS2829	KX507035	–	–	–	1
SEHIB8	MF783055	–	*Meyerozyma guilliermondii* CBS8105	KY108543	–	–	–	4
HB01a	MF926291	MF783893	*Papiliotrema flavescens* CBS942	AB035042	4	–	1	–
CABM8C	MF926294	MF783895	*Pichia fermentans* CBS5663	EF550234	–	–	–	1
SEMA6B	MF783059	–	*Pichia kluyveri* CBS7274	KY108823	–	–	–	4
SEHM2A	MF927685	MF783892	*Rhodosporidium babjevae* CBS322	AF387771	–	–	–	1
EP02c	MF783058	MF927679	*Rhodotorula glutinis* CBS2889	KY109044	3	4	1	–
HURM4A	MF783067	MF927680	*Rhodotorula mucilaginosa* CBS482	KY109140	–	–	–	1
SEHUM7B	MF783065	MF784281	*Rhodotorula nothofagi* CBS9091	AF444736	–	–	–	1
ARIM1B	MF926295	MF783896	*Rhodotorula paludigena* CBS4477	KY109146.1	–	–	–	1
CABMA3A	MF783053	–	*Saccharomyces cerevisiae* CBS2963	KF214442	–	–	–	1
SEHM1C	MF770267	–	*Scheffersomyces stipitis* CBS7126	KY109584.1	–	–	–	1
PIEM5B	MF783061	–	*Schwanniomyces polymorphus* CBS6456	KY109627	–	–	–	1
HB02b	MF926293	MF783891	*Vishniacozyma carnescens* CBS973	AB035054	4	1	3	–
			Total:		85	62	16	67

** This table shows the most probable yeast strain according to the compared databased belonging to the Metschnikowia clade, but these yeast strains cannot be assigned unequivocally to one of the species in the clade.*

TABLE 2 | *In vitro* dual assays of yeast strains against mycelial growth of *B. cinerea*, *P. expansum,* and *A. carbonarius*.

Source	Isolates from grapes	Isolates with inhibitory capacity at preliminary *vitro* assaying	% of isolates with inhibitory capacity at preliminary *vitro* assaying	Isolates with inhibitory capacity at second *vitro* test	% of isolates with inhibitory capacity at second *vitro* test	% of isolates with inhibitory capacity
Wildlife vines	67	42	62.7	18	42.9	26.9
Biodynamic vineyard	62	11	17.7	2	18.2	3.2
Organic vineyard	16	1	6.2	0	0	0
Conventional vineyard	85	6	7.1	0	0	0
Total isolates	230	60	26.1	20	33.3	8.7

In the first in Vitro assaying, all isolates are present. At second in Vitro test only the positive at first are shown.

Evaluation of the Minimum Inhibiting Concentration (MIC)

MICs were determined in triplicate for all yeast strains selected after dual assays against the different molds. The evaluation of the MIC revealed that the 20 yeasts significantly reduced the progress of hyphal growth of *B. cinerea* and *P. expansum* at a concentration of 10^5 cells/mL, and 10 (5 *H. uvarum*, 1 *P. kluyveri*, 1 *M. guilliermondii*, 1 *H. clermontiae,* and 1 *S. cerevisiae*) at a concentration of 10^3 cells/mL both under the mentioned growth conditions (**Table 4**). However, the occurrence of *A. carbonarius* was completely reduced by only 14 yeast strains at a concentration of 10^6 cells/mL. Only two yeast strains (1 *H. uvarum* and 1 *S. cerevisiae*) were able to protect grapes or to compete for the nutrients against *A. carbonarius* at a concentration of 10^3 cells/mL and under the same growth conditions of *B. cinerea* and *P. expansum* (**Table 4**). The yeasts that were able to protect grapes or to exhaust the medium from all the assayed molds were those isolated from *V. vinifera* ssp. sylvestris.

Killer Character Assay

From over the 20 yeast strains assayed for the killer character, only *S. cerevisiae* displayed a slightly killer phenotype (**Table 3**).

Enzymatic Tests

All yeasts that passed the dual test were evaluated for extracellular enzymatic activities (β-1, 3-glucanase, proteolytic, and pectinolytic activities). Twelve out of the 20 yeast strains were able to hydrolyze at least one of the assayed compound (milk proteins, pectin, glucan, and chitin). Only five yeast strains (4 *M. fructicola*-like yeast strains and 1 *P. kluyveri*) showed all the enzymatic activities (**Table 3**).

Production of Volatile Organic Compounds (VOCs) and Hydrogen Sulfide Release

Percentage data concerning production of VOCs and hydrogen sulfide release among the 20 yeast strains selected showed that 10 yeast strains (3 *H. uvarum*, 4 *M. fructicola*-like yeast strains, 2 *M. guilliermondii,* and 1 *S. cerevisiae*) evidenced the highest values of growth inhibition. These values significantly differed ($p < 0.05$) from the control and the other yeast strains analyzed (**Table 3**).

Biofilm Formation

Only yeast strains of *H. uvarum* (1), *P. kluyveri* (1), *V. carnescens,* and *A. pullulans* proved to be able to form biofilm by the adhesion to polystyrene 96-well plate surface (O.D. > 0.1) after 3, 48, and 72 h of incubation (**Table 3**).

Effect of Iron Concentration on the Inhibitory Activity of the Yeast Strains

Antagonistic activity of most of the selected strains were not significantly influenced by tested $FeCl_3$ concentrations showing that inhibition activity of these yeasts against *B. cinerea* and *A. carbonarius* were not related with iron competition (**Table 3**). On the other hand, the activity of the *P. kluyveri* strains resulted iron-sensitive at a concentration of 20 μg/mL of $FeCl_3$. The potential yeast strain ROMA10 (*presumably M. fructicola*) always produced red pigments in absence or presence of $FeCl_3$ at different concentrations on PDA plates without affecting the pigment coloration or the inhibition of the mold. Regarding the species *A. pullulans,* depending on the concentration of iron, yeast colonies, and haloes pigmentation turned from pale white to maroon, but in absence of $FeCl_3$ colonies were not pigmented and the halo was not visible. These findings will be argued in the discussion section.

Effect of Other Metabolites Released by Yeast Strains on Mold Growth

Yeast primary or secondary metabolism generates numerous compounds as products of the transformation of the carbon, nitrogen, or sulfur sources. Two of the most common substances released are acetic acid and hydrogen sulfide that have antimicrobial effect. **Table 3** shows that *M. fructicola*-like strain, *H. uvarum* (2 strains), *M. guilliermondii* (1 strain), *S. cerevisiae,* and *C. californica* species are able to produce these compounds probably affecting the mold development.

In Vivo Assays for Inhibitory Activity

Efficacy of Yeast Strains in Controlling Mold Infection on Grape Berries

The results of the efficacy of the 20 selected strains in reducing molds berry rots are reported in **Table 3**. *P. kluyveri* (2 strains), *H. uvarum* (2 strains), *H. clermontiae* (1 strain), and *M. guilliermondii* (1 strain) revealed the highest efficacy in

TABLE 3 | Phenotypical assaying for yeast antagonistic activity against molds and their volatile organic compounds (VOCs) referred to mycelial growth reduction of *B. cinerea*, *P. expansum,* and *A. carbonarius*.

Species	Strain	D1/D2 Accession no.	VOCs[a] (%)	Protease	Pectinase	Glucanase	Chitinase	Glucosidase	Killer activity	Acetic acid production[b]	H_2S released	Iron depletion[c]	Biofilm formation[d]
A. pullulans	FZ02a	MF926292	28.0	−	+	+	+	−	−	0.3	+	Positive with *Botrytis*	0.110
C. californica	CABMC2A	MF927682	45.0	−	−	−	−	−	−	0	+	Positive with *Botrytis*	0.030
H. uvarum	SEHMA6A	MF783054	31.0	−	+	−	−	−	−	0	−	Positive with *Botrytis*	0.042
H. uvarum	CABM8A	MF926284	44.5	−	+	−	−	−	−	0.1	+	Positive with *Botrytis* and *Aspergillus*	0.010
H. uvarum	CABCM1A	MF926285	35.8	−	+	−	−	−	−	0.2	−	Positive with *Botrytis*	0.100
H. uvarum	CAMM3A	MF926286	34.8	+	+	−	−	−	−	0.1	−	Positive with *Botrytis*	0
H. uvarum	CAMM6A	MF926287	40.5	−	−	−	−	−	−	0.3	−	Negative	0.010
H. uvarum	SEHI3C	MF927683	25.8	−	−	−	−	−	−	0.1	−	Positeive with *Botrytis*	0.030
H. uvarum	SEHI1C	MF926288	21.0	−	+	−	−	−	−	0	+	Positive with *Botrytis*	0.080
H. uvarum	SEHM7C	MF926289	26.3	−	−	−	−	−	−	0.1	−	Positive with *Botrytis* and *Aspergillus*	0.150
H. uvarum	CAMB9A	MF926290	27.7	−	−	−	−	−	−	0	−	Negative	0.034
H. clermontiae	CABMB1A	MF783060	18.7	+	−	−	−	−	−	0	−	Positive with *Botrytis*	0.011
H. uvarum	Control	MF801365	28.7	+	−	−	−	−	−	0.3	+	Negative	0.033
*M. fructicola**	ROMA10	MF783057	28.3	+	−	−	−	−	−	0	−	Positive with *Botrytis*	0.070
M. guilliermondii	CABM1A	MF927684	44.5	−	+	−	−	−	−	0.2	+	Negative	0.010
M. guilliermondii	SEHIB8	MF783055	37.0	+	+	−	−	−	−	0.2	+	Positive with *Botrytis*	0.027
*M. viticola**	ROMMA1A	MF783062	46.5	+	−	−	−	−	−	0	−	Positive with *Botrytis*	0.050
P. kluyveri	SEHMA6B	MF783059	26.7	−	−	−	−	−	−	0	+	Positive with *Botrytis* and *Aspergillus*	0.014
P. kluyveri	CABMC6C	MF926283	29.5	+	−	−	−	−	−	0	−	Positive with *Botrytis*	0.360
S. cerevisiae	CABMA3A	MF783053	40.0	−	−	−	−	+	+	0.1	+	Positive with *Botrytis*	0.010
V. carnescens	HB02b	MF926293	28.0	−	−	−	−	−	−	0	−	Positive with *Botrytis*	0.110

**This table shows the most probable yeast strain according to the compared databased belonging to the Metschnikowia clade, but these yeast strains cannot be assigned unequivocally to one of the species in the clade.*

[a]*The percentage is calculated: (M – Mwy)/M*100 where M is the mold growth (cm) without antagonistic yeast on the plate and Mwy is the mold growth in presence of the antagonistic yeast on septet plates (cm). The percentage represents the reduction of mold grown caused by yeast VOCs.*

[b]*Values are expressed in centimeters (diameter of the halo of the positive acetic acid-producing yeast strains on the plate) a strain of Acetobacter was used as positive control.*

[c]*Positive is when in presence of iron the yeast decreases its antagonistic activity; Negative is when the antagonistic activity of the yeast is the same in presence or in absence of iron.*

[d]*The values are expressed as the average of the absorbance at 590 nm of three well-subtracted for the control test values.*

TABLE 4 | Disease incidence by *A. carbonarius*, *B. cinerea,* and *P. expansum* after simultaneous inoculation with different concentrations of yeast strains on PDA-agar after 5 days at 25°C under constant light.

Species	Strains	*A. carbonarius*				*B. cinerea*				*P. expansum*			
		10^{6*}	10^5	10^4	10^3	10^6	10^5	10^4	10^3	10^6	10^5	10^4	10^3
A. pullulans	FZ02a	−	−	−	−	+	−	−	−	+	+	+	+
C. californica	CABMC2A	−	−	−	−	+	−	−	−	+	+	+	+
H. clermontiae	CABMB1A	+	−	−	−	+	+	+	+	+	+	+	+
H. uvarum	SEHMA6A	+	−	−	−	+	+	+	+	+	−	−	−
H. uvarum	CABM8A	+	−	−	−	+	+	+	+	+	+	−	−
H. uvarum	CABCM1A	+	+	−	−	+	+	+	+	+	+	+	+
H. uvarum	CAMM3A	+	+	−	−	+	+	+	+	+	+	+	+
H. uvarum	CAMM6A	+	−	−	−	+	+	−	−	+	+	+	+
H. uvarum	SEHI1C	+	−	−	−	+	−	−	−	+	+	+	+
H. uvarum	SEHM7C	+	−	−	−	+	+	−	−	+	+	+	−
H. uvarum	CAMB9A	+	+	+	+	+	+	+	+	+	+	+	+
H. uvarum	SEHIC3	−	−	−	−	+	+	+	+	+	+	+	+
H. uvarum	Control	−	−	−	−	−	−	−	−	−	−	−	−
M. guilliermondii	CABM1A	+	+	−	−	+	+	+	+	+	+	+	−
M. guilliermondii	SEHIB8	+	−	−	−	+	+	+	+	+	+	+	+
P. kluyveri	SEHMA6B	+	−	−	−	+	+	+	−	+	+	+	+
P. kluyveri	CABMC6C	+	+	−	−	+	+	+	+	+	+	+	+
S. cerevisiae	CABMA3A	+	+	+	+	+	+	+	+	+	+	+	+
V. carnescens	HB02b	−	−	−	−	+	−	−	−	+	+	+	+

*Values are expressed as (+) if yeast strains were able to inhibit the total growth of the mold over a particular concentration and (–) if yeast strains were not able to inhibit mold growth. Values were obtained from three trials.*The values are expressed in CFU/mL.*

TABLE 5 | Comparative *in vivo* test of the most suitable yeast strains against molds vs. a commercial chemical fungicide.

Species	Strains	*A. carbonarius*			*B. cinerea*			*P. expansum*			Mean
H. uvarum	SEHMA6A	3	3	3	3	3	3	3	3	3	3.00
H. uvarum	CABMB9A	2	3	3	3	3	3	3	3	3	2.89
P. kluyveri	SEHMA6B	2	2	2	2	1	1	1	3	2	1.78
Commercial fungicide		1	2	2	3	3	3	2	2	3	2.33
Control		4	4	4	4	4	4	3	3	3	3.67

The disease severity was evaluated by a visual score "1-to-4" (1: no visible symptoms; 2: soft rot; 3: formation of mycelium; 4: sporulation of mold) according to Parafati et al. (2015).

reducing mold infection and growth caused by *B. cinerea*, *A. carbonarius,* and *P. expansum*. On the contrary, a strain of *M. guilliermondii* showed the worst result in controlling molds decay on grape-berries.

Comparison of the Inhibitory Effect with Chemical Pesticide by *In Vivo* Test

The three yeast strains which showed a better antagonistic effectiveness against the studied molds taking into account the above described experiments, were subjected to a comparative *in vivo* test with a commercial chemical fungicide used against *B. cinerea* and other molds including *P. expansum* and *A. carbonarius* (**Table 5**). In this case, the strain *P. kluyveri* SEHMA6B proved to be more effective than the chemical fungicide used under the proposed growth conditions.

DISCUSSION

The control of fungal diseases and mycotoxins contamination during grape maturation and post-harvesting is currently based on treatments with chemical fungicides. However, the environmental dispersion, the progressive loss of effectiveness, the emergence of resistant strains, and the increasing level of residues in table grape and wine (Marssat et al., 2016), have led the European Union to restrict the use of these compounds, addressing the researchers toward innovative and eco-friendly protocols to face the problem. In agreement with the recommendations pursued by UE Directive 128/2009, this work has been focused on the exploration of the natural antagonistic potential of 241 yeasts isolated from grape samples of *V. vinifera* ssp. sylvestris and *V. vinifera* ssp. vinifera against *B. cinerea*, *A. carbonarius,* and *P. expansum*. These molds are spoilage agents of the berries, both in vineyard after the veraison and

during the over-ripening practices, by rotting the grape bunches that cause the falling of the fruit quality and, in the case of *Aspergillus* and *Penicillium* genera, a threat to food safety due to the release of mycotoxins. According to Wilson and Wisniewski (1989), biocontrol is the application of selected microorganisms with antagonistic activity against other ones and their usage at large-scale to reduce the impact of chemical synthesis pesticides on human health and environment. Many papers report the discovering of novel microbial strains with antifungal properties, proposing them as biocontrol strains against certain molds (Marssat et al., 2016). Although some natural fungicides have been marketed, they can fail in field practices since climatic conditions affect the establishment, survival and activity of the biocontrol agents (Benbow and Sugar, 1999). Yeasts are structurally and functionally heterogeneous because of their differential expression of genes, in a way that epigenetic factors, such as the host environment or abiotic external factors influence the down/up regulation of the gene expression, changing the behavior of yeast populations and their interactions (Spadaro and Droby, 2016). The present investigation shows that yeast strains isolated from various environments have significant differences on the effectiveness against three potentially harmful fungi. To our knowledge, this is the first report in which yeasts isolated from *V. vinifera* ssp. sylvestris and from biodynamic or organic grapevines have been assessed for potential antagonist ability against *A. carbonarius*, *B. cinerea,* and *P. expansum*.

Our results pointed out that there is a greater number of species found on wildlife vines (23), compared to cultivated ones, with only seven species. This is in line with other studies, which demonstrated that the biodiversity level of yeasts community is influenced by human activities (Cordero-Bueso et al., 2011a,b, 2014, 2017; Martins et al., 2014; Drumonde-Neves et al., 2016). In addition, *S. cerevisae* was also isolated on wildlife grape surfaces. Previous studies on yeast diversity from cultivars or overwintering vines show that *Saccharomyces* genus is either absent on grapes or found in a small number and incidence (Mortimer and Polsinelli, 1999; Torija et al., 2001; Sipiczki, 2016). The results obtained from the preliminary *in vitro* dual assay have clearly disclosed how most isolates collected from wildlife vines (18 strains) are able to inhibit the mold growth vs. the isolates from managed cultivars (only two strains in biodynamic farming). Interestingly, yeast strains, which passed the preliminary tests, have been isolated in two ecosystems where the microbial antagonism against molds could only be produced by the associate microbiota onto grape-berries or natural barriers of the plant that hinder the entry of fungal pathogens. Consequently, *H. uvarum*, *H. clermontiae*, *M. guilliermondii,* and *Pichia kluyveri* strains, all of them isolated from *V. vinifera* ssp. sylvestris, could play a pivotal role as biocontrol agents in the natural environment. These data cannot be compared with the current literature since this is the first time that isolates from wildlife vines are studied with this aim. It is possible to hypothesize that the observed differences in microbiota structure between grapes from wildlife vines and cultivated ones can be due to the use of synthetic or natural pesticides in vineyards or the isolation from overwintering vineyards, resulting in a diverse selective pressure on resident microorganisms (Sipiczki, 2006, 2016; Cordero-Bueso et al., 2011a, 2014; Brysch-Herzberg and Siedel, 2015). The higher yeast biodiversity found in samples from native conditions, highlighted in this work, might have been because the natural environment is hostile for the mold development. Moreover, it seems reasonable to think that molds exposed to repetitive doses of synthetic fungicides can acquire, modify, or adjust genetic characters that provide them an increase in the resistance.

The minimum inhibitory concentrations (MICs) assays, defined as the lowest concentrations of yeasts resulting in complete growth inhibition of the molds, have shown that a concentration of 10^5 cells/mL is enough to reduce the progress of *B. cinerea* and *P. expansum* by all yeast strains. The mold *A. carbonarius* needed a concentration of 10^6 cells/mL to be inhibited. These concentrations are considerably lower than those found for other antagonistic yeasts (Chanchaichaovivat et al., 2007; Zhang et al., 2007; Nally et al., 2012). However, further experiments are required to evaluate the influence of the growth condition on the MIC values on field.

Since several mechanisms of action are involved in the biocontrol activity of the antagonistic yeasts, we have examined the main modes of actions, such as iron depletion, cell wall degrading enzymes, diffusible, and volatile antimicrobial compounds, and biofilm formation on the 20 selected yeast strains. Within this group *M. guilliermondii*, *H. clermontiae*, *P. kluyveri*, *H. uvarum*, *A. pullulans,* and the yeast strain ROMA10 (*M. fructicola*-like strain*)* strains proved to release lytic enzymes potentially capable of hydrolyzing the fungal cell wall. Among these species, it is well-known that *A. pullulans* is able to produce β-1,3 glucanase, and chitinase active on *Monilinia laxa*, *B. cinerea,* and *P. expansum*, especially when the mold wall represents the sole carbon source (Zhang et al., 2009).

The yeast metabolism leads to the formation of acetate and ethyl acetate, which are by-products with inhibitory action against molds in storing cereals (Fredlund et al., 2004). Furthermore, some yeasts can emit volatile compounds that inhibit the development of molds, as described by Parafati et al. (2015) where the growth of *B. cinerea* was counteracted by *S. cerevisiae*. In our experimental conditions, the species *H. uvarum*, *S. cerevisae,* and *M. guilliermondii* were able to release sufficient levels of acetic acid and hydrogen sulfide (evaluated qualitatively) to cause inhibition to mold growth. Likewise, some *M. fructicola-like* strains were capable of preventing the development of molds through the emission of volatile compounds. Regarding this species there are no examples in the literature, despite the report of a commercialized product used as biocontrol agent (Shemer, Bayer CropScience, AG, Germany).

Little is known about the role of biofilms in the biocontrol activity of yeast used to control fungal diseases and the mechanisms involved in their formation. In this work, *H. uvarum*, *P. kluyveri*, *V. carnescens,* and *A. pullulans* strains revealed the capability to form biofilm. Previous studies carried on the species *S. cerevisiae* showed that the ability to adhere to a surface was related to the production of extracellular polysaccharides and molecules belonging to glycoproteins family implicated in this action and in the grape wounds protection (Reynolds and Fink, 2001; Parafati et al., 2015). Yeasts cells with

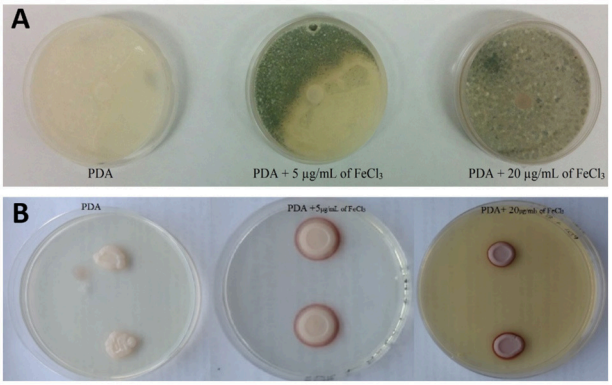

FIGURE 2 | (A) Biocontrol activity of *P. kluyveri* SEHMB8A against *P. expansum* in PDA at different concentration of iron. The activity of this yeast strain is iron-sensitive in presence of an excess of iron the strain loses its antagonism. **(B)** Depending on the concentration of iron added to the PDA medium, the species *A. pullulans* turned from pale white to maroon, but in absence of $FeCl_3$ the halo was not visible.

the ability to form biofilm are recognized as most effective in limiting pathogen growth being able to colonize more efficiently the inner of grape wounds (Ianiri et al., 2013).

Iron is essential for fungal growth and pathogenesis, thus, competition for this metal is functional for counteracting of pathogenic molds. Sipiczki (2006) and Spadaro and Droby (2016) reported this action on strains belonging to the genus *Metschnikowia* that were capable of stopping mold development in crop areas through an iron deficiency mechanism. In the tests we carried out, the presence of iron in growth medium modified the inhibitory properties of the antagonist yeasts (**Figure 2A**). In particular, for *B. cinerea*, when an excess of iron was present the mold was able to develop contrary to what was happening in growth media without $FeCl_3$, where the action of yeast prevented its development. Spadaro and Droby (2016) affirmed that some *M. fructicola* strains were able to produce the red pigment pulcherrimin surrounding its colonies in presence of $FeCl_3$ in the growth medium. However, in accordance to Sipiczki (2006), Sipiczki et al. (2013), Brysch-Herzberg and Siedel (2015), Lachance (2016), and Sipiczki (2016) these yeast strains could not be suitable for the delimitation of the species *M. fructicola*. This species is not distinguishable from *M. andauensis* and other species of the *M. pulcherrima* clade because of a possible heterogeneity of the rRNA repeats. Thus, we will consider that these yeast strains are inside of the *M. pulcherrima* clade but not as confirmed *M. fructicola* species. Previous studies investigating the mechanism of antifungal antagonism of pulcherrimin-producing *Metschnikowia* strains claimed that iron immobilization by pulcherrimin (and thus antifungal activity) was suppressed by iron depletion (Sipiczki, 2006). However, in our study, yeast strain ROMA10 (presumably identified as *M. fructicola*) was able to produce pulcherrimin-like substances in presence of $FeCl_3$ at the studied concentrations. This result was also previously observed on apple fruits (Saravanakumar et al., 2008). Interestingly, our yeast strain FZ02 identified as *A. pullulans*, did not show halo without the $FeCl_3$ addition on the medium, but colonies showed a pink halo at low iron concentration and then they turned to red-maroon at high iron concentrations (**Figure 2B**). This observation is in accordance with Chi et al. (2013) that reported that in a medium supplemented with iron, the colonies of *A. pullulans* turned to brown. They supposed that the iron was chelated by the secreted siderophores and considerable amount of the intracellular siderophores was responsible for brown colonies. However, further studies are necessary to elucidate both findings described above. The antagonistic potential of the 20 yeast strains selected after *in vitro* tests was further proven on wounded grape berries inoculated with *A. carbonarius*, *B. cinerea,* and *P. expansum*, *P. kluyveri*, *H. uvarum*, *H. clermontiae,* and *M. guilliermondii* strains exhibited the best efficacy in reducing the development of tested mold diseases. As reported by Parafati et al. (2015), *S. cerevisiae* species reveals to be less efficient than the non-*Saccharomyces* to hamper the fungal growth, probably due to its difficulty to multiply on grape wounds. Nevertheless, these results display that the cumulative effects of different antagonistic activities detected by the *in vitro* tests are not sufficient to explain the outcome of the most performant strains on grape berries (*in vivo* experiments). The efficacy of the yeast strains which showed the greatest *in vivo* action on grape berries, were also compared with a fungicide formulation (37.5% Cyprodinil and 25% Fludioxonil) normally used against *Botrytis* and as secondary rots *Aspergillus* spp. and *Penicillium* spp., according to the supplier's recommendations. We decided to exclude those isolates that show the VOCs production and that release extracellular enzymes, taking into account that the emission of certain compounds, and hydrolytic enzymes by yeasts could alter the balance of the resident microbiota and destabilize the microbial composition of the must. Surprisingly, *P. kluyveri* strain SEHMA6B was more effective than the commercial fungicide, particularly against *Botrytis* (**Figure 3**). Considering that gray mold decay is the main problem of pre-harvesting, the application of this yeast strain in the field could be even more interesting. Moreover, in a recent study (Sipiczki, 2016) a grape-born *P. kluyveri* strain was tested against *Botrytis* and *S. cerevisiae*. It was active against *Botrytis* but no detectable inhibitory effect on *Saccharomyces*. Other studies have demonstrated that this species is unable to compete with *S. cerevisiae* during fermentation (Cocolin and Ciani, 2014), thus, *P. kluyveri* could be used as biocontrol without alter the fermentation processes. Interestingly, the *P. kluyveri* strain tested by Sipiczki (2016) was isolated from mummified grapes which indicates that it prefers harsh conditions. This fact makes us hypothesize that *P. kluyveri* would be able to cope in the different conditions in field. Nevertheless, further studies are needed to test the antagonistic activity of *P. kluyveri* in field to verify if in the conditions that occur in the vineyard such as temperature swings, high humidity, water, solar radiation, and interaction with the resident microbiota it is able to be effective in counteracting the growth of molds.

Actually, several yeast strains tested in the *in vitro* trials, when air exchange was limited, proved to be effective against molds, while under the *in vivo* outdoor conditions turned out to be ineffective. The main studies on volatile substances are aimed at storing, packaging, and transporting fruit and vegetables

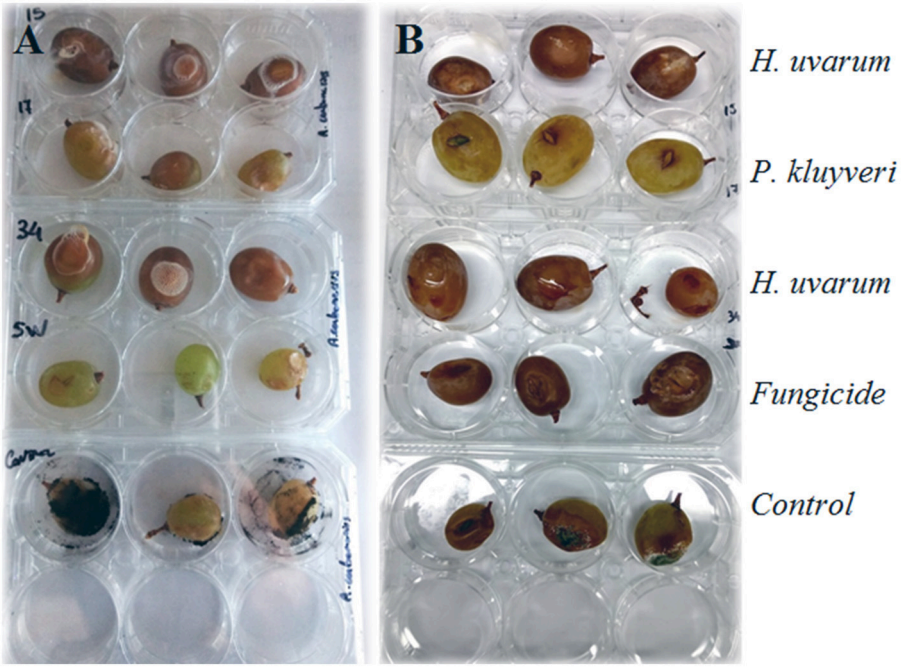

FIGURE 3 | Comparison of the three selected antagonistic yeast strains against *A. carbonarius* **(A)** and *B. cinerea* **(B)** and the commercial fungicide. Line 1: Grapes soaked with *H. uvarum* strain 1, Line 2: Grapes soaked with *P. kluyveri* SEHMB8A, Line 3: Grapes soaked with *H. uvarum* SEHMA61 strain 2, Line 4: Grape soaked with commercial fungicide, Line 5: Grapes without treatment.

(Gomes et al., 2015). From a commercial point of view, it is important to understand the ways in which yeast acts to develop an appropriate formulation and method of application (Spadaro and Droby, 2016). The ability to compete with some nutrient yeast, for example for iron or biofilm formation, is the desired interaction. For these reasons, two isolates of *H. uvarum* and one of *P. kluyveri*, which do not produce hydrolytic enzymes, have been used for the final test with the phytopoietic drug.

Though variable performances in field can be a significant constraint for its practical implementation (Stewart, 2001; Elmer and Reglinski, 2006), the interest in the use of bio-control is renewed because of the recent normative (Directive 2009/128/EC), by matching the specific requirements of International Organization of Vine and Wine for the sustainable production of wine.

In conclusion, this investigation on antagonism patterns in new yeast isolates, over all from *V. vinifera* ssp. sylvestris, can constitute a promising source of knowledge and experience to set strategies in preventing or reducing harvested commodity damages and to test the use of selected yeast strains as a substitutive of the chemical fungicide.

AUTHOR CONTRIBUTIONS

GC contributed to the design of the work, to the yeast isolation, and identification, to the *in vitro* assays for antagonistic activity, to the analysis and to the interpretation of data for the work, to draft the work and revising it, NM contributed to the *in vitro* assays for antagonistic activity, to *in vivo* assays for inhibitory activity, to draft the work, and revising it, DM to the samples collection for yeast isolation, RF and JC contributed to draft the work and revising it, FV contributed to the yeast identification, IV contributed to the design of the work, to the interpretation of data for the work, to draft the work, and revising it for important intellectual content, and ensured that that questions related to the accuracy or integrity of any part of the work were appropriately investigated and resolved.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2017.02025/full#supplementary-material

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Distribution of Native Lactic Acid Bacteria in Wineries of Queretaro, Mexico and Their Resistance to Wine-Like Conditions

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Native lactic acid bacteria (LAB) are capable of growing during winemaking, thereby strongly affecting wine quality. The species of LAB present in musts, wines during malolactic fermentation (MLF), and barrels/filters were investigated in wineries from the emerging wine region of Queretaro, México using multiplex PCR and culture. The resistance to wine-like conditions (WLC): ethanol (10, 12, and 13%), SO_2 (30 $mg{\cdot}l^{-1}$), and low pH (3.5) of native LAB strains was also studied. Five species were detected within 61 samples obtained: *Oenococcus oeni*, *Lactobacillus plantarum*, *Pediococcus parvulus, Lactobacillus hilgardi*, and *Lactobacillus brevis*. Four species (excepting *L. brevis*) were found in must; *O. oeni* and *P. parvulus* were ubiquitous in wine and *L. plantarum* and *L. brevis* were mainly present at the initial stage of MLF, while *L. hilgardii* was mostly detected at the advanced stage. Furthermore, some species detected in barrel/filter, prove them to be hazardous reservoirs. From 822 LAB isolates, only 119 resisted WLC with 10% ethanol; the number of strains able to grow in WLC with 13% ethanol decreased approximately by 50%, *O. oeni* being the most versatile species with 65% of resistant isolates, while *Lactobacillus* spp. and *P. parvulus* were the most strongly affected, especially those recovered from barrel/filter, with less than 10% of resistant isolates. This study evidences the presence of local strains able to be used as starter cultures, and also enabled the assessment of the risks derived from the presence of spoilage LAB strains resistant to WLC.

Keywords: malolactic fermentation, multiplex PCR, *Oenococcus oeni*, starter cultures, wine spoilage

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INTRODUCTION

The conversion from grape must into wine is a complex process that involves the development of various microorganisms, including lactic acid bacteria (LAB). However, wine is considered an unsuitable environment for microbial growth due to its low pH, high concentrations of ethanol and sulfur dioxide (SO_2), and other limiting factors (Spano and Massa, 2006). The LAB capable of overcoming these conditions mainly belong to *Oenococcus*, *Lactobacillus*, *Pediococcus*, and *Leuconostoc* genera (Lonvaud-Funel, 1999).

In order to have any effect on wine quality, LAB should be able to not only survive, but also to grow within wine (Renouf et al., 2008), and the effect produced therein will depend on the major species present and their ability to overcome the harsh environment of winemaking (du Toit and Pretorius, 2000). The specie *Oenococcus oeni* is known as the main one responsible for malolactic fermentation (MLF), a process in which L-malic acid is decarboxylated into L-lactic acid, causing a partial deacidification, conferring microbial stability, and improving wine flavor profile (Lerm et al., 2010). However, some other LAB, such as *Pediococcus* spp. and some species of *Lactobacillus,* are widely associated with wine spoilage, often producing biogenic amines, off-odors, and other undesirable metabolites (Bartowsky, 2009).

Moreover, LAB can enter wine from vineyard or winery equipment (Fleet, 1993), and their diversity is influenced by grape variety and geographic region (Bokulich et al., 2013b). Therefore, it is advisable to study the autochthonous LAB of a particular winemaking area in order to detect potential starter cultures or species that represent risks of wine spoilage (Pérez-Martín et al., 2014). The use of molecular techniques to achieve this porpoise is currently preferred; some of them, such as ARDRA (Rodas et al., 2003), DGGE (Cocolin et al., 2013), or new generation sequencing (Bokulich et al., 2013a), display all the diversity of bacteria present in a sample. Meanwhile, other techniques, such as multiplex PCR described by Petri et al. (2013), are aimed at those bacteria of particular interest in winemaking. This particular technique allows the identification of 13 of the principal LAB associated with winemaking in a simple PCR assay, facilitating data processing or subsequent analyses to complete the identification of an amplicon.

Several studies intending to elucidate the presence, distribution, and adaptation of wine associated LAB have already been performed in wineries from regions with an extensive winemaking tradition, such as Mentrida (Pérez-Martín et al., 2014), La Rioja (González-Arenzana et al., 2015), Patagonia (La Hens et al., 2015), and Apulia (Garofalo et al., 2015). However, this kind of studies are missing in areas where the development of this industry is recent, like Queretaro State in Mexico. This region is considered nowadays the second most important within the Mexican territory. Located in the central area of the country, the climate is semi dry and temperate, the soils are deep with either a clayey loam texture or lightly calcareous. In 2013, above 350 ha of vineyards were censed and wine production was estimated in 1.5 millions of liters (Consejo Mexicano Vitivinícola A.C [CMV], 2014). To date, the main varieties established are 'Merlot,' 'Cabernet Sauvignon,' 'Syrah,' and 'Tempranillo' as well as the white varieties 'Macabeo' and 'Chardonnay' (Asociación de Vitivinicultores de Querétaro [AVQ], 2011). Wines possess low ethanol contents (from 9 to 12%) and a total titratable acidity around 7 g/L tartaric acid (De la Cruz-de Aquino et al., 2012). Wineries usually use commercial yeasts to guarantee an optimal alcoholic fermentation, but MLF is almost always carried out spontaneously, which makes it very unpredictable.

The aim of this research was to elucidate the principal LAB species present in strategic materials in wineries established in Queretaro and to determine their resistance to wine-like conditions (WLC), including high ethanol concentrations and low pH, in order to assess risks and detect possible starter cultures within local strains.

MATERIALS AND METHODS

Experimental Site and Sampling

This study was conducted in four wineries named A, B, C, and D, located in Queretaro State, Mexico. Wineries A, B, and C have the respective vineyards and are located in the municipality of Ezequiel Montes, approximately 205 km from Mexico City. Winery D lacks a vineyard and is located 21 km from the others, in the municipality of Tequisquiapan. At winery C commercial cultures of LAB are used to induce MLF after finishing alcoholic fermentation; at winery B a commercial inoculum of LAB was used for the first time the year of the study, and at wineries A and D, MLF is left to occur spontaneously.

Depending on the availability at the wineries, different types of samples were collected, their characteristics are described in **Table 1**. Must, wine and barrel/filter samples were taken at winery A; must and wine at winery B; only must at winery C and only wine at winery D. Each type of sample was collected in triplicate as follows:

(i) Must: Four mature bunches of grapes from the varieties: 'Cabernet Sauvignon,' 'Tempranillo,' and 'Syrah' at wineries A and B, and only 'Macabeo' at C, were randomly sampled in triplicates using plastic bags (20 cm × 30 cm). Also, 500 ml of must were taken from the stemmer of wineries A and B (one and two batches, respectively). Once they reached the laboratory, bunches were manually crushed inside their bags, the musts obtained from grapes and those collected from the stemmers were transferred to sterile flasks (500 ml) and left to spontaneously ferment at 25°C. For 15 days, aliquots of fermenting must were obtained every 5 days for molecular and microbial analyses.

(ii) Wine: Samples were taken once the alcoholic fermentation had ended. At wineries A and D, 100 ml of wine were sampled from three fermentation tanks, in three stages of MLF: (a) beginning, (b) intermediate, and (c) advance. At winery B, only the beginning stage was sampled, before a commercial strain inoculation. At each winery three types of wines were collected: two single-variety, one 'Cabernet Sauvignon,' another 'Tempranillo,' and the third a blend of 'Grenache,' 'Carignan,' 'Syrah,' and 'Nebbiolo.'

(iii) Barrel/filter: The inside of a barrel was rinsed with 500 ml of peptone diluent (0.1%, pH 5), which was swirled five times; afterward the diluent was recovered in a sterile flask. Three filters were also individually collected in plastic bags. Once they reached the laboratory, 100 ml of peptone diluent was added to each filter and then homogenized in a Stomacher® 400 (Seward Ltd.) at medium speed for 1 min.

TABLE 1 | Principal characteristics of the samples collected.

Winery	Sample type	N[1]	Sugar content (°Bx)	pH	Ethanol (%, v/v)	SO_2 Total ($mg{\cdot}L^{-1}$)
A	Must	12	23	3.8	–	–
	Wine-i	3	5	3.4	12.1	31.5
	Wine-m	3	5	3.6	12.1	31.5
	Wine-a	3	5	3.7	12.1	31.5
	Barrel/filter	4	–	–	–	–
B	Must	15	22	3.8	–	–
	Wine-i	3	5	3.4	11.9	29.8
C	Must	9	21	3.7	–	–
D	Wine-i	3	4	4.1	12.6	33.1
	Wine-m	3	4	3.7	12.6	33.1
	Wine-a	3	4	3.8	12.6	33.1

[1] *Total number of samples.*
Data reported as mean of three replicates per sample analyzed.

LAB Enumeration and Isolation

Must, wine and barrel/filter rinse aliquots (1 mL) were taken for serial dilutions and plated in three culture media: Man Rogosa Sharpe (MRS; DIBICO), MRS added to tomato juice (10%, v/v; Ruiz et al., 2008) or to apple juice (15%, v/v; Solieri et al., 2010). All media were adjusted to pH 4.8 and supplemented with natamycin ($100\ mg{\cdot}l^{-1}$) and sodium azide ($50\ mg{\cdot}l^{-1}$) to prevent yeast and acetic acid bacteria growth, respectively (Reguant et al., 2005). Incubation was carried out at 30°C for 8 days. As bacterial population is a non-normal data, the results were statistically analyzed using the non-parametric Kruskal – Wallis with Dunn's *post hoc* test using the software JMP 9.0.

From culture plates, approximately 5% of the colonies were isolated and purified. Gram stain and catalase tests were performed to confirm the isolates belonging to LAB group. Isolates were preserved in MRS broth with glycerol 20% at −80°C until subsequent identification and resistance tests.

Isolates Resistance to Wine-Like Conditions

The isolates' ability to grow in the presence of ethanol, SO_2, and low pH (WLC) was assessed through automatic readings of optical density (OD; every 20 min, for 72 h, at 30°C) using a Bioscreen© analyzer (Miranda-Castilleja et al., 2015). Approximately 5 Log $CFU{\cdot}ml^{-1}$ (OD = 0.2) of each LAB isolate were inoculated in individual wells containing 200 μL of synthetic medium similar to wine (SW, Carreté et al., 2002) added to $53\ mg{\cdot}l^{-1}$ of potassium metabisulfite (equivalent to $30\ mg{\cdot}l^{-1}$ SO_2), pH 3.5, and ethanol (10, 12, and 13%). As positive control, the isolates were also inoculated in the SW medium (pH 4) without the inhibitors. Detection time (DT), an indirect measure of the lag phase, was used as a response variable, considering the strain to be resistant to each condition when its DT value was lower than the total incubation time (72 h).

Detection of LAB Species in Wineries

The detection of species present in the wineries' samples (must, wine, and barrel/filter) and the identification of LAB isolates capable of growing in WLC were both carried out using a multiplex PCR (Petri et al., 2013).

DNA Extraction

Must, wine, and barrel/filter rinse aliquots (15 mL) were centrifuged (5000 × *g*, 10 min). From a cell pellet, DNA was extracted using the commercial kit Powersoil (MoBio Laboratories, Inc.) and the bench bead-top homogenizer PowerLyzer (MoBio Laboratories, Inc.) at 4500 rpm for 4 min, following the manufacturer's instructions.

DNA extraction of LAB isolates was performed as follows: The strains were grown in 1 ml of MRS broth at 30°C for 3 days. The cell pellet obtained through centrifugation (13000 × *g*, 2 min) was re-suspended in 300 μl of lysis buffer (200 mM Tris–HCl, pH 8.5, 250 mM NaCl, 25 mM EDTA, 0.5% w/v SDS) with powdered glass (0.2 g). The suspension was shaken in a PowerLyzer (MoBio) at 4500 rpm for 1 min. After centrifugation at 13000 × *g* for 5 min, 150 μl of 3 M sodium acetate (pH 5.2) was added to the supernatant, which was stored at −20°C for 30 min and then centrifuged (13 000 × *g*, 10 min). The supernatant was transferred to a new tube and nucleic acids were precipitated with 400 μl of isopropanol and then washed with ethanol (70%). Finally, the DNA was re-suspended in 25 μl of TE buffer (Soto-Muñoz et al., 2014).

Multiplex PCR

The multiplex PCR was done using Multiplex Mastermix (Qiagen) with 1 μL of sample DNA, following the procedure described by Petri et al. (2013) with some modifications: 95°C for 15 min for initial denaturation, six cycles consisting of 30 s at 94°C, annealing for 3 min beginning at 69°C with a reduction of 1°C each cycle and an elongation step of 1.5 min at 72°C; then 25 cycles of 30 s at 94°C, 3 min at 62°C, and 1.5 min at 72°C, followed by a final extension step of 10 min at 72°C. The primers used are listed in **Table 2**. The PCR products were analyzed by electrophoresis on 1.8% agarose gels with TBE buffer (90 V for 45 min). Gels were stained with ethidium bromide ($0.5\ \mu g{\cdot}ml^{-1}$) and visualized with an EDAS 290 digital imaging system (Kodak). Trackit™ 100 bp (Invitrogen) was used as the standard molecular weight marker.

TABLE 2 | Primers used for the identification of lactic acid bacteria (LAB) by multiplex PCR.

Primer	Sequence	Target
Primer mixture I		
SCAR-OENI-F	GGTAGATTAACCCGCGACG	*O. oeni*
SCAR-OENI-R	GGAATCGGTAGCATCCTG	
SCAR-LBR-F	GGAAGATCAAGAATATCGGTG	*L. brevis*
SCAR-LBR-R	GCGTCTCTAATTCACTGAGC	
SCAR-LPL-F	GAAGATTTGCCCATCGGTG	*L. plantarum*
SCAR-LPL-R	CGTTTGATGGTAGCGTTGC	
SCAR-LEU-F	GTGGTCATGGGTCTTAGC	*Leuconostoc*
SCAR-LEU-R	GGATCAAGACTAGCCAATGG	*mesenteroides*
SCAR-WPA-F	GCTGATGAACCCATACCTC	*Weissella paramesenteroides*
SCAR-WPA-R	GACCTGATTCGCTCGTTG	
SCAR-PDA-F	GTCTAAACTGGTGGTTAAACG	*P. damnosus*
SCAR-PDA-R	ATCGCACCTGGTTCAATGC	
SCAR-PPA-F	GCATGAATCACTTTTCGCTC	*P. parvulus*
SCAR-PPA-R	CAAAGATTGTGACCCAGTTG	
Primer mixture II		
SCAR-LBU-F	CTATCTTTAACCGCATTGCCG	*L. buchneri*
SCAR-LBU-R	GACACGCTTCTCATGATTGTC	
SCAR-PAC-F	ATGATGGACAGACTCCCTG	*P. acidilactici*
SCAR-PAC-R	CGAGCTGCGTAGATATGTC	
SCAR-LBH-F	TTCCTTGGTAATGTGCTTGC	*L. hilgardii*
SCAR-LBH-R	AATGGCAATCGCAATGGACG	
SCAR-PIN-F	CTATCCTTACAATGTGCATCG	*P. inopinatus*
SCAR-PIN-R	TGGTGCGTCAGTAAATGTAAG	
SCAR-LCU-F	CCAGATCCATCAGAAGATACG	*L. curvatus*
SCAR-LCU-R	GCTAACTTACCACTAACGACC	
SCAR-PPE-F	GGGAACGGTTTTAGTTTTATACG	*P. pentosaceus*
SCAR-PPE-R	CTAAGAGCGGTGATGATAAG	

TABLE 3 | Number of samples handled and isolates obtained from the four wineries located in Queretaro, Mexico.

Winery	Sample type	Total samples	Total isolates
A	Must	12	23
	Wine	9	213
	Barrel/filter	4	156
B	Must	15	96
	Wine	3	89
C	Must	9	103
D	Wine	9	142
Total		**61**	**822**

RESULTS

Enumeration and Isolation of LAB in Different Samples and Stages of MLF

A total of 822 isolates were recovered from the counting plates of the 61 samples collected at the four wineries (**Table 3**). Three culture media were used in this study to improve LAB recovery; however, contrary to previous reports (Solieri et al., 2010; Schillinger and Holzapfel, 2012), the population, the morphology of the colonies observed and species identified were very similar in the different media (**Supplementary Figure S1**). Therefore, in **Figure 1**, the LAB populations are shown, independent of culture media, involving six replicates of each sample analyzed (two per culture media). The LAB counts in musts from wineries A and B were rather low (10^1–10^3 CFU·ml^{-1}) and no bacterial growth (<10 CFU·ml^{-1}) was observed in several samples (5/12 in A and 6/15 in B). By contrast, higher counts (10^4–10^5 CFU·ml^{-1}) were observed in musts from winery C, being this winery the one with the highest populations observed. In wine, the LAB populations ranged from 10^2 CFU·ml^{-1} at the beginning of the process, to 10^9 CFU·ml^{-1} at the second stage (climax of MLF), with intermediate values at the advanced stage. Finally, in barrel/filters rinse, the LAB population was around 10^8 CFU·ml^{-1} being superior comparing to must but similar to the populations observed in wine.

Detection and Distribution of LAB Species through the Wineries

Five species (*O. oeni*, *Pediococcus parvulus*, *Lactobacillus plantarum, Lactobacillus hilgardii*, and *Lactobacillus brevis*) were detected among the wineries' samples (**Table 4**). In most of the cases, the detection by culture confirmed what was observed with the molecular detection (culture-independent). However, some discrepancies between detection approaches were observed: *L. brevis* in wines (from A and B) and barrel/filter was only detected by culture. Conversely, the presence of *O. oeni* at winery C was only determined by direct multiplex PCR.

In several must samples (18/33), the LAB species investigated were not detected, and in the remaining ones, *L. plantarum* was widely detected at wineries A (58%) and C (100%). *O. oeni* was found in 67% of the samples from B and 56% from C. Finally, *P. parvulus* was only found in 8% of the samples from winery B and *L. hilgardii* only in 22% from C.

In wine samples, the five species were detected and *O. oeni* and *P. parvulus* were found in all samples. *L. plantarum* was detected in several samples from three wineries (22–56%). *L. hilgardii* was only found at winery A (22%), whereas, *L. brevis* was present at wineries A and B at 11 and 33%, respectively. Additionally, *L. brevis* and *L. plantarum* were mainly detected at the first stage of MLF, and *L. hilgardii* predominated at the advanced stage. Finally, in barrel/filter samples, all the five species were found. Winery A showed the greatest diversity of LAB species and at winery B the presence of *O. oeni* was remarkable.

LAB Resistance to Increasing Ethanol Concentrations with SO_2 and pH of 3.5

As expected, the number of resistant isolates falls as ethanol concentration increases (**Figure 2**). In some samples (must from C; wine from A and D), the diversity of resistant species remained, with fewer individual ones capable of growing with 13% ethanol, evidencing strain variation. Moreover, the number of resistant *O. oeni* isolates remained unchanged, even with higher ethanol concentrations, which is particularly notable at winery B. Conversely, *P. parvulus* was strongly affected by higher ethanol levels, particularly those isolates obtained from

TABLE 4 | Percentage of incidence of LAB species detected by culture (C) and molecular assay (M) in samples of must, wine in three stages of malolactic fermentation (MLF): Initial (i), middle (m), and advanced (a) and barrel/filter; obtained in wineries A, B, C, and D.

Winery	Sample type	N[1]	*O. oeni*		*P. parvulus*		*L. plantarum*		*L. hilgardii*		*L. brevis*	
			M	C[2]	M	C	M	C	M	C	M	C
A	Must	12	0	0	0	0	58	17	0	0	0	0
	Wine-i	3	100	100	100	100	67	67	0	0	0	33
	Wine-m	3	100	100	100	100	0	0	0	0	0	0
	Wine-a	3	100	100	100	100	0	0	67	67	0	0
	Barrel/filter	4	100	100	100	100	0	50	100	100	0	50
B	Must	15	0	67	0	8	0	0	0	0	0	0
	Wine-i	3	100	100	100	100	0	100	0	0	0	100
C	Must	9	56	0	0	0	100	100	0	22	0	0
D	Wine-i	3	100	0	100	100	100	0	0	0	0	0
	Wine-m	3	100	33	100	100	67	0	0	0	0	0
	Wine-a	3	100	33	100	100	0	0	0	0	0	0

[1]*N: number of samples analyzed.*
[2]*Culture-dependent approach: identifying isolates resistant to wine-like conditions (WLC) with 10% ethanol.*

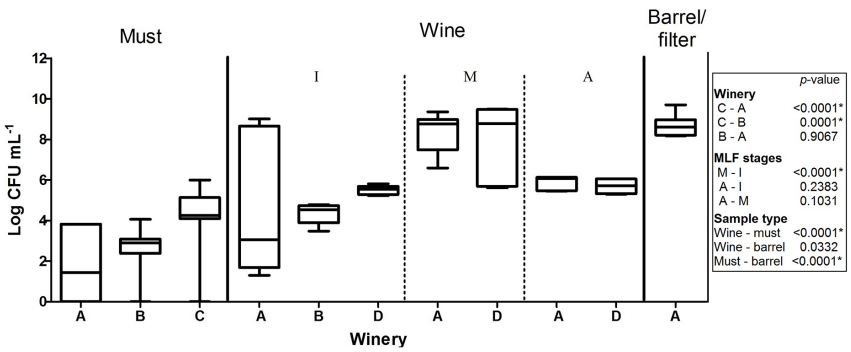

FIGURE 1 | Population of lactic acid bacteria in samples of must, barrel/filter and wine throughout three stages of malolactic fermentation: I beginning, M intermediate, A advanced; obtained from wineries (A–D). Kruskal–Wallis analysis and Dunn's *post hoc* test among wineries, MFL stages and sample type. The box indicates the 25th and 75th percentiles, line across the box shows the median value and the whiskers caps represent the maximum and minimum values.

barrel/filter, of which around 90% did not resist 13% ethanol. The *Lactobacillus* spp. in this study were also affected by 13% ethanol, with only 37% of the isolates being resistant to this condition. Finally, the high number of isolates (10 of 19) from must from winery C resistant to 13% ethanol is remarkable, given their origin.

DISCUSSION

LAB Populations

The low LAB populations found in musts are consistent with the fact that they are minor constituents of grape microbiota, the populations usually reported being around 10^2 CFU·g^{-1} (Barata et al., 2012). Meanwhile, higher populations found in must from winery C could be associated with grape variety; must from winery C was obtained from a white variety ('Macabeo'), while musts from wineries A and B derived from red varieties ('Tempranillo,' 'Syrah,' and 'Cabernet Sauvignon'). Higher numbers of LAB obtained from white varieties compared to red ones were also reported by Bae et al. (2006), which has been attributed to the fact that some phenolic compounds only present in red varieties can produce a toxic effect on bacteria (Reguant et al., 2000). The fluctuating populations of LAB observed in wine at different stages of MLF coincides with Saguir et al. (2009) and González-Arenzana et al. (2012), who reported that lower counts of LAB at the beginning of MLF increased throughout the process, reaching up to 8 Log CFU·ml^{-1}.

Furthermore, barrel/filter samples were considered together in this study since the barrel contained the wine in which the filters were used, and only a few samples of each material could be collected. In particular, the LAB population found in barrels (10^3 CFU·ml^{-1}) was similar to that reported by González-Arenzana et al. (2013). Barrels are recognized as microbial reservoirs in cellars, since they offer shelter where microorganisms can remain. However, this material also represents a stressful environment, which could explain the low populations encountered therein (Renouf et al., 2007; Bokulich et al., 2013a).

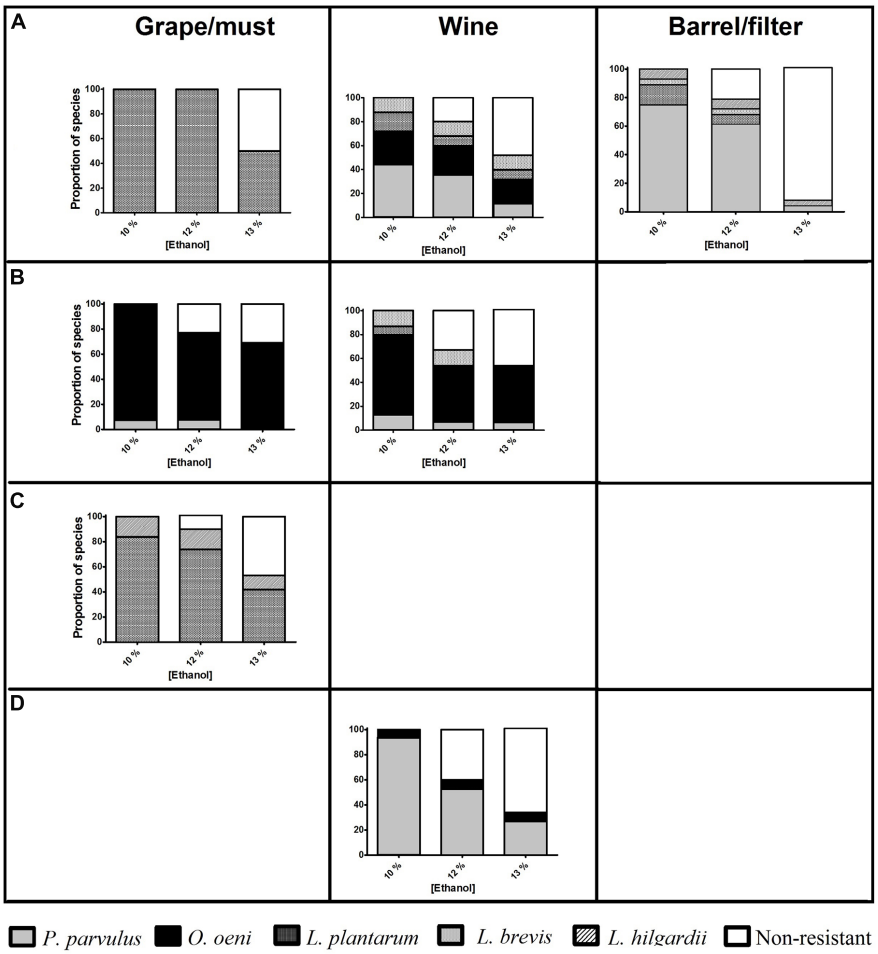

FIGURE 2 | Proportion of isolates of lactic acid bacteria (LAB) species capable of growing in a synthetic medium similar to wine (SW) with SO_2 30 mg l^{-1} pH 3.5 and different ethanol concentrations (% v/v, *x*-axis), from different wineries (horizontal boxes, A–D) and types of samples taken (vertical boxes). The number of resistant isolates is indicated above the column.

Detection and Distribution of LAB Species

The multiplex PCR assay was efficient in detecting the principal LAB species in the winery samples (**Figure 3**), however, it was hampered when low LAB populations were present, as in musts and wines at the first stage of MLF. The detection limit reported for this technique is 10^4 CFU·ml^{-1} (Petri et al., 2013), and the samples were concentrated 15 times, therefore, populations under 10^3 were not detectable in this study. This detection limit could also explain the lack of recognition of *L. hilgardii, L. plantarum*, and *L. brevis* through this approach in some samples. Another known bias that could explain the lack of detection of certain species is preferential amplification, in which the abundance of certain species, such as *O. oeni* and *P. parvulus*, may have caused reagents to exhaust without amplifying scarce species (Sint et al., 2012).

The species mainly detected in musts (*L. plantarum, P. parvulus*, and *L. hilgardii)* are widely associated with wine grapes (Renouf et al., 2005; Bae et al., 2006; Barata et al., 2012). The last two are known to produce off-odors (Costello and Henschke, 2002) and biogenic amines in wine (Lonvaud-Funel, 2001), while *L. plantarum* has been recently regarded as starter culture for MLF (Lerm et al., 2011; Bravo-Ferrada et al., 2013), and has even shown additional advantages due to its capacity of degradation of biogenic amines (Capozzi et al., 2012) as well as better performance in co-inoculation with *Saccharomyces cerevisiae* (Berbegal et al., 2016). Moreover, the detection of *O. oeni* in musts is remarkable, given its importance in MLF and since this species is rarely found therein (Bae et al., 2006; Mesas et al., 2011).

In wine, the fact that *O. oeni* and *P. parvulus* were frequently found together suggests some type of association between them, as has been previously posited by Renouf et al. (2007) and Pérez-Martín et al. (2014). Nevertheless, it is important to point out that *P. parvulus* is the species most often involved in ropiness, a major bacterial alteration in wines (Dols-Lafargue et al., 2008). Moreover, the detection of *L. brevis* and *L. plantarum* only at the beginning of MLF shows a decrease in their populations at advanced stages,

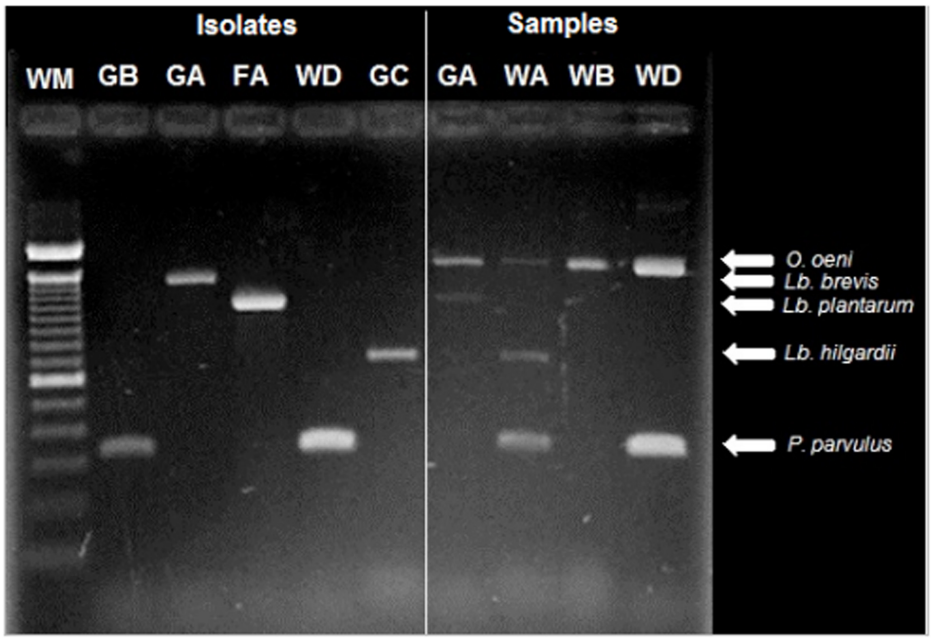

FIGURE 3 | Identification of LAB isolates (lines 2–5) and detection of species present in wine-related (6–9) samples using multiplex PCR. The origin of the sample is indicated above as follows: The first letter corresponds to the type of sample: G, must; F, banel/filter; W, wine. The second letter corresponds to the winery: A, B, C, or D. The first line corresponds to the molecular weight marker Trackit^{TM} 100 bp (Invitrogen). Moreover, the species corresponding to that molecular weight is specified (Petri et al., 2013).

probably due to a low resistance to the modified medium. Finally, the fact that *L. hilgardii* was only found at the advanced stages of MLF suggests a contamination of the wine, probably through the barrels where this bacterium was also found; this emphasizes the need to implement effective disinfection methods during the winemaking process (González-Arenzana et al., 2013).

Even if the presence of some of these species can lead to wine spoilage, this problem has not been perceived in the local wines; only certain delays or inhibitions of the MLF are apparent. The spoilage features of these bacteria are usually strain-dependent, and for spoilage phenotypes to be produced, not only is the presence of the responsible bacteria required, but also the conducive environmental conditions, for instance, several stress conditions (ethanol, SO_2, and low pH) promote the production of β-glucan responsible for ropiness by *P. parvulus* (Dols-Lafargue et al., 2008).

Resistance to Wine-Like Conditions

In this study, LAB species were challenged with scarce nutrients combined with ethanol, SO_2, and low pH, simulating a more realistic representation of what LAB face during winemaking. One of the principal changes in this process is ethanol concentration, which affects each LAB species differently, and the resistance of each isolate could also vary, depending on its origin (Arroyo-López et al., 2010).

The species showing more tolerant isolates to WLC was *O. oeni*, which is expected, since this species stands out for its ability to overcome the harsh conditions of wine, enabling it to dominate this media and establish itself in the cellars (Lonvaud-Funel, 1999). Conversely, higher ethanol levels significantly affected *P. parvulus,* an undesirable, but apparently prevalent species at these wineries. This high susceptibility could be due to the more stressful conditions found in barrels, which could lead to more sensitive strains.

Although *L. plantarum* has been previously reported with better adaptability to wine than *O. oeni* (G-Alegría et al., 2004), the isolates evaluated in this study did not show a remarkable performance. Even if *Lactobacillus* species are considered highly resistant to ethanol (Shane Gold et al., 1992), wines elaborated in Queretaro seldom reach more than 12% ethanol (De la Cruz-de Aquino et al., 2012), which could explain the lack of adaptation of local strains to 13% ethanol. Moreover, the fact that a high number of isolates (10 of 19) belonging to *Lactobacillus* spp. and obtained from winery C resisted 13% ethanol was surprising, since they were isolated from must, where they had not been previously exposed to alcohol. Winery C is the oldest one sampled (about 30 years old), which could have enabled some strains to adapt to both environments, vineyard and cellar. This allowed the identification of resistant strains that could eventually be used as starter cultures, as well as the detection of more hazardous species (and materials) with regard to spoilage.

CONCLUSION

This is the first report related to the diversity of wine associated LAB in Mexico, and particularly in the wine region of Queretaro.

Throughout the four wineries studied, five species (*O. oeni, P. parvulus, L. plantarum, L. hilgardii*, and *L. brevis*) were detected in must, wine, and barrel/filter samples. The species *O. oeni* and *L. plantarum* were detected at all the wineries and *P. parvulus* was only absent at winery C. *L. plantarum* and *L. brevis* were mainly found in musts and at the initial stages of MLF in wines, while *L. hilgardii* was principally detected at the end of MLF. The highest ethanol concentration tested (13%) combined with 30 $mg{\cdot}l^{-1}$ of SO_2 and pH of 3.5 diminished the number of resistant isolates by around half, regardless of materials origin, with *O. oeni* being the species with a greater proportion of resistant isolates. In contrast, *P. parvulus* and *Lactobacillus* species obtained from barrel/filters were the most affected by high concentration of ethanol.

AUTHOR CONTRIBUTIONS

All the authors have revised the present draft, contributed important intellectual content and approved the final version to be published. They have also agreed to be accountable for the content of the work. DM-C: Conception of work, acquisition, analysis and interpretation of data, draft development. RM-P: Conception of work and experimental design, consultation on wine quality aspects. JA-T: Support with data acquisition, molecular techniques, and microbial diversity. MI: Consultation on microbial diversity and microbial kinetic studies. LS-M: Consultation on molecular techniques and data interpretation. JP-A: Consultation on sampling at wineries and microbial metabolism in vineyards. SA-M: Conception and design of work, expertise in lactic acid bacteria characterization, data analysis and interpretation, draft development, and financial support management.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2016.01769/full#supplementary-material

FIGURE S1 | Proportion of LAB species recovered in three media from must, wine and barrel/filter rinse: MRS, Man Rogosa Sharpe; MRS-T, MRS added to tomato juice; and MRS-AJ, MRS added to apple juice.

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Oenococcus oeni in Chilean Red Wines: Technological and Genomic Characterization

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The presence and load of species of LAB at the end of the malolactic fermentation (MLF) were investigated in 16 wineries from the different Chilean valleys (Limarí, Casablanca, Maipo, Rapel, and Maule Valleys) during 2012 and 2013, using PCR-RFLP and qPCR. *Oenococcus oeni* was observed in 80% of the samples collected. Dominance of *O. oeni* was reflected in the bacterial load (*O. oeni*/total bacteria) measured by qPCR, corresponding to >85% in most of the samples. A total of 178 LAB isolates were identified after sequencing molecular markers, 95 of them corresponded to *O. oeni.* Further genetic analyses were performed using MLST (7 genes) including 10 commercial strains; the results indicated that commercial strains were grouped together, while autochthonous strains distributed among different genetic clusters. To pre-select some autochthonous *O. oeni*, these isolates were also characterized based on technological tests such as ethanol tolerance (12 and 15%), SO_2 resistance (0 and 80 mg l^{-1}), and pH (3.1 and 3.6) and malic acid transformation (1.5 and 4 g l^{-1}). For comparison purposes, commercial strain VP41 was also tested. Based on their technological performance, only 3 isolates were selected for further examination (genome analysis) and they were able to reduce malic acid concentration, to grow at low pH 3.1, 15% ethanol and 80 mg l^{-1} SO_2. The genome analyses of three selected isolates were examined and compared to PSU-1 and VP41 strains to study their potential contribution to the organoleptic properties of the final product. The presence and homology of genes potentially related to aromatic profile were compared among those strains. The results indicated high conservation of malolactic enzyme (>99%) and the absence of some genes related to odor such as phenolic acid decarboxylase, in autochthonous strains. Genomic analysis also revealed that these strains shared 470 genes with VP41 and PSU-1 and that autochthonous strains harbor an interesting number of unique genes (>21). Altogether these results reveal the presence of local strains distinguishable from commercial strains at the genetic/genomic level and also having genomic traits that enforce their potential use as starter cultures.

Keywords: wine, malolactic fermentation, malolactic bacteria, *Oenococcus oeni*, terroir, genome, bacterial

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INTRODUCTION

Malolactic fermentation (MLF) is a process performed by lactic acid bacteria (LAB) that transforms malic acid into lactic acid and CO_2, which causes a decrease in the total acidity and improvement of the taste, flavor, and microbial stability of wine (Henick-Kling, 1995; Capozzi et al., 2010). Those bacteria are naturally present in grapes, musts and wines. The predominant genera are *Leuconostoc, Pediococcus*, *Lactobacillus,* and *Oenococcus* (Lonvaud-Funel, 1995).

Previous studies investigating autochthonous LAB in winemaking have reported the presence of *Oenococcus oeni* strains in spontaneous MLF (Marques et al., 2011; Nisiotou et al., 2015; Cafaro et al., 2016). The genetic diversity of *O. oeni* has been shown in studies from different winemaking regions worldwide. Bartowsky et al. (2003) determined that *O. oeni* strains that originated from the same winery in Australia were either indistinguishable or closely related to each other. In Castilla-La Mancha, Spain, Cañas et al. (2009) showed that MLF was dominated by a variable number of *O. oeni* genotypes. The same observation of the genetic diversity in these bacteria was reported in La Rioja, Spain, and Apulia, Italy (González-Arenzana et al., 2012; Garofalo et al., 2015).

Bacterial dynamics during MLF have been studied using culture-dependent techniques. The major drawback of this strategy is the impossibility of correctly obtaining the diversity and dynamics of LAB during MLF (Spano et al., 2007). However, culture-independent analysis methods have been developed and are commonly used to detect and identify microorganisms directly from wine by analyzing their DNA. Ilabaca et al. (2014) designed a 16S rRNA Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (16S rRNA PCR-RFLP) culture-independent strategy that was a reliable tool for the identification and differentiation of winemaking LAB strains isolated during the MLF process. González-Arenzana et al. (2012) studied LAB populations in red wine (La Rioja, Spain) and compared two strategies (culture-dependent and culture-independent methods). Both methods were complementary during MLF. However, the culture-independent methods allowed the detection of a vaster number of species than the culture-dependent methods. Therefore, *O. oeni* was the most frequently detected bacterium during MLF. The conclusions drawn from these molecular studies indicate that LAB populations are diverse during the early stages of MLF; however, *O. oeni* subsequently becomes the most dominant bacterial population during the MLF process. This result is consistent with the previous observations derived using culture-dependent approaches (Rodas et al., 2003; López et al., 2007).

The genetic characterization of *O. oeni* has permitted the evaluation of differences between *O. oeni* isolates from diverse winemaking locations using several molecular strategies, including multilocus sequence typing (MLST) (de las Rivas et al., 2004; Bilhère et al., 2009; Bridier et al., 2010; Bordas et al., 2013; Wang et al., 2015). Several studies reported a high level of allelic diversity and a combination of alleles among *O. oeni* isolates (Bilhère et al., 2009; Bon et al., 2009). However, genetic differences between *O. oeni* strains must be studied and understood because they might affect the quality of the wine, especially in terms of organoleptic properties. Recently, the genome sequences of *O. oeni* strains have been made available from different enological locations (Mills et al., 2005; Borneman et al., 2010; Capozzi et al., 2014; Lamontanara et al., 2014; Jara and Romero, 2015). Jara and Romero (2015) suggested that genomic analyses might provide insights into the adaptation of strains to wine-hostile conditions and their contributions to the organoleptic properties of the final product. Cappello et al. (2017) proposed an association between inter/intra-species diversity and bacterial metabolic traits that impacted the wine's organoleptic characteristics. Additionally, these authors showed evidence of the importance of the enzymatic potential of LAB to enrich the wine aroma.

In Chile, most MLF processes are conducted spontaneously by the resident LAB microbiota in the cellars. However, spontaneous MLF has drawbacks, including stuck fermentation and contamination by microorganisms, which risk altering the wine quality. To solve this problem, the use of microbial starters has been introduced with commercial strains isolated from other wine-producing countries. However, in Chile some of these starters have produced poor results due to their insufficient imposition during MLF (Ilabaca et al., 2014). Among the many factors that impede the development of *O. oeni*, the most important is the presence of inhibiting factors, such as a low pH, high ethanol content, and low malic acid content.

This study reports the first genetic and technological characterization of *O. oeni* strains retrieved from spontaneous MLF in different Chilean valleys. Additionally, the genomes of selected isolates were examined and compared them to PSU-1 and VP41 strains to study their potential contributions to the organoleptic properties of the final product. These characteristics could be the basis for obtaining autochthonous isolates to serve as starters capable of improving the typicity of Chilean wines.

MATERIALS AND METHODS

Samples

Spontaneous MLF samples (58) were collected in 2012 and 2013 from 16 wineries, including cultivars (cvs.) Cabernet Sauvignon and Carménère, located in four Chilean valleys: Limarí (30°38′S–71°24′W), Maipo (33°45′S–70°46′W), Rapel (34°15′S–72°00′W), and Maule (35°58′S–72°19′W), sampling four wineries per valley. All the tested wineries carried out spontaneous MFL without commercial starter; sampling was performed at the end of the MLF. The winemaking process was initiated with healthy grapes harvested from March to May, followed by the traditional vinification practices of each winery. As a general rule of each winery alcoholic fermentation (AF) using commercial freeze-dried yeast was performed stainless steel tanks at 22–25°C. Spontaneous MLF was carried out immediately after AF in stainless steel tanks at 18–22°C for 30–40 days. Samples were aseptically collected at the end of MLF, where the wines showed average chemical parameters: ethanol (14.1%v/v) and pH (3.7). The criterion for defining the end of MLF in each winery is the reduction in the content of the L-malic acid (<0.3 g/L) in the wines determined using an enzymatic

test (Boehringer Manheim; Mannheim, Germany). Samples were stored at 4°C until being processed.

Bacterial Isolation

The bacterial isolation was carried out using medium for *Leuconosctoc oenos* (MLO), following indications by Blasco et al. (2003). This medium was supplemented with 2 ml L^{-1} sodium azida (Winkler, Chile) and 3 ml L^{-1} cyclohexamide (Sigma-Aldrich) to eliminate yeasts and acid acetic bacteria, respectively (Ruiz et al., 2008). Serial dilutions were plated onto the MLO media and incubated for 5–7 days at 28°C, under anaerobic conditions. After count colonies (CFU mL^1) 10 colonies per sample were randomly chosen. This selection was realized according to the phenotypic characterization (Mesas et al., 2011). Each selected colony was transferred and purified through two rounds of streak plating onto fresh agar plates. The isolates were maintained in a cryobank at −80°C.

Reference and Commercial Strains

Lactic acid bacteria commercial strains for comparison in genetic diversity study were used. This included Lallemand (Lalvin VP41®, Lalvin 31®, uvaferm Alpha®, uvaferm Beta®, Lalvin Elios®, PN4®, Lalvin MTO®); Lamothe Abiet (Oeno 1, Oeno 2); Laffort (Lactoenos B28 PreAc®). Lalvin VP41® was included in the technological evaluation.

DNA Extraction from Wine

The initial step for our culture independent approach was the extraction of DNA directly from wine with MLF, according to Jara et al. (2008) and Ilabaca et al. (2014). This DNA was used to quantify bacterial load (see below).

DNA Extraction from Bacterial Isolates

In the case of LAB isolates, each of the colonies selected was suspended in 200 mL PBS, with vigorous agitation, followed by centrifugation at 5,000 × g for 5 min. Subsequently, 20 μL aliquot of 20 mg mL^{-1} Lysozyme (Sigma) was added to the pellets, which were subsequently incubated at 37°C for 30 min. The Wizard Genomic DNA Purification Kit (Promega) was used for DNA extraction according to the protocol for isolating genomic DNA from Gram-positive bacteria the manufacturer's instructions. DNA obtained was frozen at −20°C until processed. The identification de LAB from samples MLF wine was done by 16S rRNA PCR-RFLP, according to Ilabaca et al. (2014).

Amplification *rpo*B gen

To distinguish *O. oeni* isolates, RNA polymerase ß subunit (*rpo*B) were PCR amplified using methodology described by Bridier et al. (2010). DNA sequencing was performed by Macrogen (USA) and the analyses were done by BLAST (basic Alignment Search Tool). From the *rpo*B sequence results of autochthonous *O. oeni* isolates and commercial strain (VP41), were analyzed with its differences about nucleotides among them by used BioEdit version 7.1.9, generating signature groups (Drancourt and Raoult, 2002). The signature sequences corresponded to part of a coding sequence of a gene which, because it is shared by different isolates, is thought to be evolutionarily conserved and therefore can serve to trace taxonomic relationships among these isolates (Gupta, 1998).

Quantitative PCR (qPCR) and Total Bacteria and *O. oeni*

Both total bacteria and load of *O. oeni* were quantified by qPCR reactions based on detection of SYBR fluorescence. The qPCR reactions were performed using an AriaMx real-time PCR System (Agilent Technologies), using primers and programs described in **Table 1**. PCR amplification was performed in 10 μL of mix containing 1 μL of DNA 0.5 *p*mol/mL of each respective primer 5 μL of LightCycler 480 SYBR Green I Master (Roche) and 3.5 μL of Milli-Q sterile H_2O. All samples were analyzed in triplicate. The statistical analyses among bacterial loads valleys were determined by ANOVA using R Development Core Team (2008), and the *post-hoc* test was performed by pairwise.t.test.

Multilocus Sequence Typing (MLST) Analysis

Based on *rpo*B gen analysis (signature groups), the autochthonous *O. oeni* isolates were selected about two criteria. First, the different *rpo*B sequences to obtain maximal diversity. Second, *rpo*B sequences isolates from different Chilean Valleys.

To analysis of MLST seven housekeeping genes for this study were selected. These genes were *gyrB* (Gyrase Beta subunit), *g6pd* (Glucose-6-phosphate dehydrogenase), *mleA* (Malolactic enzyme), *pgm* (Phosphoglucomutase), *dnaE* (DNA polymerase III, alfa subunit), *pgm* (Phosphorybosylaminoimidazole), *purK* (Phosphoribosylamino-imidazole carboxylase), *rpoB* (RNA polymerase, Beta subunit). The *recP* gene was not included in our analysis because it was not present in our isolates. After the examination of a subset of 114 *O. oeni* genomes originated from diverse geographic locations, only 40 strains harbored this gene (Supplementary Table S1). The PCR mixes were performed according to de las Rivas et al. (2004) and Bridier et al. (2010).

The PCR products were sequenced by Macrogen (USA). The analysis of sequences obtained by MLST was realized using BioEdit version 7.1.9 software and a dendrogram was constructed by UPGMA (unweighted pair-group method with arithmetic mean) method. The evolutionary distances were computed using the Maximum Composite Likelihood method, using the Mega 6 (version 6.0) software from the website (Tamura et al., 2013).

The sequences of each gen of autochthonous *O. oeni* isolates selected and commercial strain (VP41), were analyzed with its differences about nucleotides among them by used BioEdit version 7.1.9. The base pair of each gen analyzed were *rpoB gen* 579 bp; *dnaE* gen 641 bp; *g6pd* gen 591 bp; *gyrB* gen 544 bp; *mleA* gen 355 bp; *pmg* gen 580 bp; *purK* gen 493 bp.

Evaluation of Technological Properties of *O. oeni* Isolates

Based on the MLST analysis, some strains were included in the technological tests. For the different technological tests, autochthonous *O. oeni* isolates were grown in MLO broth to early stationary phase. An inoculum of $1*10^8$ cells mL^{-1} of

TABLE 1 | Primers and programs for quantitative PCR

	Programs	Primer	Sequences 5′-3′	References
Total bacteria	95°C, 5 m; 95°C, 5 s; 55°C, 10 s; 72°C, 10 s	341	CCTACGGGAGGCAGCAG	Opazo et al., 2012
		788	GGACTACCAGGGTATCTAA	
Oenococcus oeni	95°C, 5 m; 95°C, 10 s; 55°C, 10 s; 72°C, 10 s	RpoB F	CGATATTCTCCTTTCTCCAATG	Bridier et al., 2010
		RpoB R	CTTTAGCGATCTGTTCCAATG	

each *O. oeni* isolates was used to inoculate wine-like medium (Bordas et al., 2015). Our criterion to select the isolates was a first test; wine-like medium was supplemented with two ethanol concentrations (12% v/v and 15% v/v), the isolates natives were incubated at 25°C for 10 days. Then, the best isolates were submitted to wine-like medium supplemented with malic acid 1.5 and 4g L^{-1} at 25°C for 10 days. Wine-like medium either at pH 3.1 and 3.6 and were incubated at 25°C for 24 h and 5 days. Finally, the isolates were incubated into this medium, utilizing potassium metabisulfite concentrations (0 and 80 ppm free SO_2) at 25°C for 7 days. All bacterial growth was per triplicate and monitored by culture dependent analyses. The significant differences among isolates of each tests were determined by Kruskal–Wallis test using R Development Core Team (2008), and the *post-hoc* test was performed by posthoc.kruskal.dunn.test belonging to the PMCMR package.

Genomic Analyses

Based on technological characteristics, the draft genome of three autochthonous *O. oeni* isolates were obtained and analyzed. Genome characteristics and the accession numbers about those isolates have been previously reported (Jara and Romero, 2015). Using the online tool at bioinformatics.psb.ugent.be, a Venn diagram was generated to compared genome composition of *O. oeni* autochthonous isolates with 2 reference strains: VP41 (ACSE00000000) and PSU-1 (NC_008528).

Three approaches to calculate average nucleotide identity from genome sequences of O. *oeni* autochthonous isolates and 2 reference strains: VP41 (ACSE00000000) and PSU-1 (NC_008528) were used. Those were: DNA–DNA hybridization (DDH), Average Nucleotide Identity (ANI) and Orthology (OrthoANI). The similarity obtained by DNA–DNA hybridization (DDH) to genome-sequence-based similarity according to Meier-Kolthoff et al. (2013) was determined. Second approach utilized was average nucleotide identity (ANI) according to Rosselló-Mora (2006). Finally, third approach was average nucleotide identity by orthology (OrthoANI) according to Lee et al. (2016).

The presence and homology of 21 (*abf*, *arcA*, *alsS*, *alsD*, *arcB*, *arcC*, *bgl*, *citD*, *citE*, *citF*, *estA*, *estB*, *estC*, *gshR*, *maeP*, *metB*, *metC*, *metK*, *mleA*, *pad*, *prtP)* genes potentially related to aromatic profile were analyzed from *O. oeni,* autochthonous isolates and reference strains VP41 and PSU-1 genome sequences. These genes were taken from literature (Sumby et al., 2009, 2013; Mtshali et al., 2012; Cappello et al., 2014). The orthologous clustering analysis were performed using Inparanoid (Fouts et al., 2012; Sonnhammer and Östlund, 2015). To analyze enzymes groups (glycosidases, esterases, proteases, citrate metabolism, and peptidases), that may play a role in the wine organoleptic properties into proteomes of *O. oeni,* autochthonous isolates and reference strains VP41 and PSU-1 genome sequences were realized using coding sequence for protein (CDS) by PfamScan (Finn et al., 2016).

RESULTS

Lactic Acid Bacteria in Different Chilean Valleys

A total of 60 wine samples from four Chilean valleys were used to characterize the bacterial population dynamics at the end of spontaneous MLF with 16S rRNA PCR-RFLP. *O. oeni* was observed in 80% of the wine samples. *Lactobacillus* and *Leuconostoc* were detected in 4.5% and 2.3% of the samples, respectively. Furthermore, *Oenococcus*/*Pediococcus* and *Oenococcus/Leuconoctoc* were found together at frequencies of 4.5 and 2.3%, respectively.

Both the total bacterial and *O. oeni* loads were explored by qPCR. **Figure 1** shows the number of microorganisms ($\log_{10}$ scale) represented by each valley ordered from north to south (Limarí, Maipo, Rapel, and Maule). Limarí and Rapel valleys showed the highest total bacterial load with 10^7 total bacteria per mL of wine. Significant differences were found in total bacterial loads among valleys; Limarí and Maipo valleys (p-value: 0.034); Maipo and Rapel valleys (p-value: 0.000013); Rapel and Maule valleys (p-value: 0.0014). On the other hand, Rapel and Maule valleys, showed the highest *O. oeni* loads, with 10^6 *O. oeni* per mL of wine. Significant differences were found in *O. oeni* load among valleys, Maipo and Maule valleys (p-value: 0.00048) and Maipo and Rapel valleys (p-value: 0.00012). In summary, the *O. oeni* loads and the total bacterial loads indicated a dominance of *O. oeni* at the end of MLF.

Identification and Characterization of LAB Isolates

A total of 158 autochthonous LAB isolates were retrieved from red wine collected at the end of spontaneous MLF in wineries located in the Limarí, Maipo, Rapel, and Maule Valleys and characterized using phenotypic tests (catalase and Gram staining). Among them, 75 strains were identified as *O. oeni* by PCR-*rpo*B sequencing. The analysis of a 579-bp *rpo*B sequence generated signature groups according to the different nucleotides and positions using the *rpo*B sequence from the VP41 reference strain. Based on this analysis, 46 autochthonous *O. oeni* isolates were found to differ from VP41, and 7 signature groups were observed. The signature groups are described in

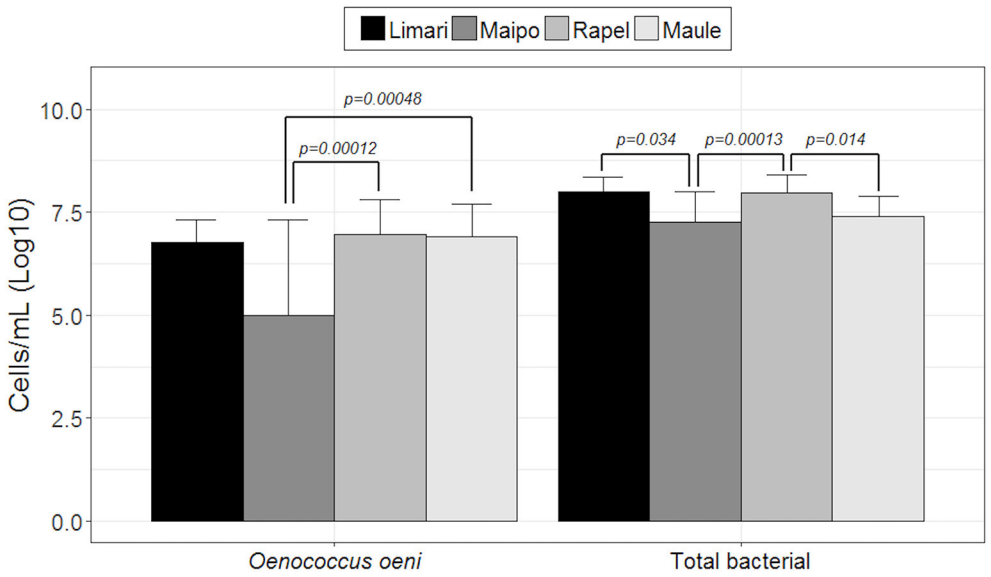

FIGURE 1 | Total bacterial and *O. oeni* load quantified by qPCR in wine from spontaneous MLF different valleys examined (log_{10} scale).

detail in **Table 2**, including the signature, number of isolates and origin. Based on these signature groups, 10 autochthonous *O. oeni* isolates for analysis using MLST typing were selected. The selection was based on two inclusion criteria of at least 1 isolate from each signature group and two strains per valley. Additionally, 10 *O. oeni* commercial strains were included in the MLST genetic analysis for comparison. Concatenation of the sequences for each of the seven genes formed a 3,783-bp sequence, which was examined using the MEGA software. The resulting dendrogram (**Figure 2**) showed two major genetic groups of *O. oeni* (M and A). Group M included all of the commercial strains and some autochthonous *O. oeni* isolates (13, 399, 565, and 74). Furthermore, the commercial strain MTO presented a transposon in *purK* from 423 to 1,282 bp. In contrast, group A was only composed of autochthonous *O. oeni*. Group M was composed of two subgroups that showed different distributions of autochthonous isolates based on the signature groups. Interestingly, two isolates obtained from geographically separated valleys (500 kilometers) grouped together in A.

Technological Properties of Autochthonous *O. oeni* Isolates

The ability of autochthonous strains to resist wine-like medium supplement with ethanol (12% v/v) was compared with strain VP41 (LABc). Three isolates were discarded because they were unable to survive in this medium. Therefore, only seven isolates were examined to assess their technological properties, including the kinetics of the transformation of malic acid, ethanol resistance, pH resistance, and SO_2 resistance. Supplementary Figures S1A,B shows the ability of the isolates to degrade malic acid at two initial concentrations. The strains 139 and 565 showed the best reduction of this acid. Similarly, all strains grew in the presence of 12% ethanol, but 139 showed the best survival in 12 and 15% ethanol (Supplementary Figures S1C,D). To study the association between bacterial growth and pH tolerance, autochthonous strains were examined in media with different pH values (3.1 and 3.6). Supplementary Figures S1E,F, showed that strain 17 and 74 did not survive these conditions; in contrast, strain 139 presented the highest potential for growth in restrictive pH media. Similarly, the effect of sulfur oxide (0 and 80 ppm) was examined (Supplementary Figures S1G,H). Sulfur oxide was deleterious for strains 17, 74, and 167 whereas isolate 139 presented a high viable count over the 10-day period. In summary, the best strains according to their technological properties were 139 and 565.

Genome-Based Phylogeny and Genome Comparison

To examine the relationships among the autochthonous *O. oeni* strains (565, 399, and 139) and the reference strains (VP41 and PSU-1), genome analyses were performed. First, the number of common genes shared by these *O. oeni* strains was evaluated; the results are shown in a Venn diagram in **Figure 3**. This figure revealed 470 common genes, of which 63% were uncharacterized proteins and 9% were ribosomal genes. Each strain presented unique genes, indicating that the autochthonous strains harbored an interesting number of unique genes. Strain 139 presented 28 unique genes, strain 565 presented 27 unique genes and strain 399 presented 21 unique genes. These genes encoded ABC transporters; galactose metabolism; phosphostransferase system (PTS); pentose phosphatase pathway; starch and sucrose metabolism; two component system; nicotinate and nicotinamide metabolism that might improve bacterial performance in the wine environment. Then, the autochthonous *O. oeni* genomes were compared to the reference strains using 3 methodologies (ANIb, DDH, and OrthoANI) (**Table 3**). The

TABLE 2 | Signature groups of autochthonous *O. oeni* isolates from different valleys.

Valleys	Different nucleotides in *rpo*B gen	Different position in *rpo*B gen	Number of signature groups
Maipo	G	23	1
Maipo	G	23	1
Maipo	G	23	1
Rapel	G	23	1
Rapel	G	23	1
Rapel	G	23	1
Rapel	G	23	1
Rapel	G	23	1
Maipo	G	23	1
Maipo	G	23	1
Maipo	G	23	1
Maule	G	23	1
Maule	G	23	1
Maipo	G	23	1
Rapel	G	536	2
Maipo	GT	23/43	3
Maipo	CTGT	38/116/234/347	4
Rapel	CTGT	38/116/234/347	4
Rapel	CTGT	38/116/234/347	4
Maule	CTGT	38/116/234/347	4
Maipo	CTGT	38/116/234/347	4
Maule	CTGT	38/116/234/347	4
Maipo	CTGT	38/116/234/347	4
Maipo	CTGT	38/116/234/347	4
Rapel	CTGT	38/116/234/347	4
Rapel	CTGT	38/116/234/347	4
Maule	CTGT	38/116/234/347	4
Maule	CTGT	38/116/234/347	4
Maule	CTGT	38/116/234/347	4
Maipo	CTGT	38/116/234/347	4
Maipo	CTGT	38/116/234/347	4
Maipo	CTGT	38/116/234/347	4
Rapel	CTGT	38/116/234/347	4
Rapel	CTGT	38/116/234/347	4
Maipo	CTGT	38/116/234/347	4
Maipo	CTGT	38/116/234/347	4
Maule	CTGT	38/116/234/347	4
Maule	CTGT	38/116/234/347	4
Maipo	CTGT	38/116/234/347	4
Rapel	CTGT	38/116/234/347	4
Rapel	CTGT	38/116/234/347	4
Maule	GTTTGT	38/42/43/116/234/347	5
Maipo	CGTGCCTGATTTTGCCAGTACCAGT CCAGTAAATATCCGCTGATCGTG	26/36/53/59/85/89/116/137/140/154/167/215/224/260/266/267/281/299/ 247/350/351/362/363/365/368/377/392/425/443/480/482/485/ 488/489/491/594/502/503/509/515/518/521/524/533/534/535/ 536/ 557	6
Limarí	CGTGCCTGATTTTGCCAGTACCA GTCCAGTAAATATCCGCTGATCGTG	26/36/53/59/85/89/116/137/140/154/167/215/224/260/266/267/281/299/ 247/350/351/362/363/365/368/377/392/425/443/480/482/485/ 488/489/491/594/502/503/509/515/518/521/524/533/534/535/ 536/ 557	6
Maipo	CGTGCCTGATTTTGCCAGTACCAGT CCAGTAAATATCCGCTGATCGT	26/36/53/59/85/89/116/137/140/154/167/215/224/260/266/267/281/299/ 247/350/351/362/363/365/368/377/392/425/443/480/482/485/ 488/489/491/594/502/503/509/515/518/521/524/533/534/535/ 536	7
Maipo	CGTGCCTGATTTTGCCAGTACCAG TCCAGTAAATATCCGCTGATCGT	26/36/53/59/85/89/116/137/140/154/167/215/224/260/266/267/281/299/ 247/350/351/362/363/365/368/377/392/425/443/480/482/485/ 488/489/491/594/502/503/509/515/518/521/524/533/534/535/ 536	7

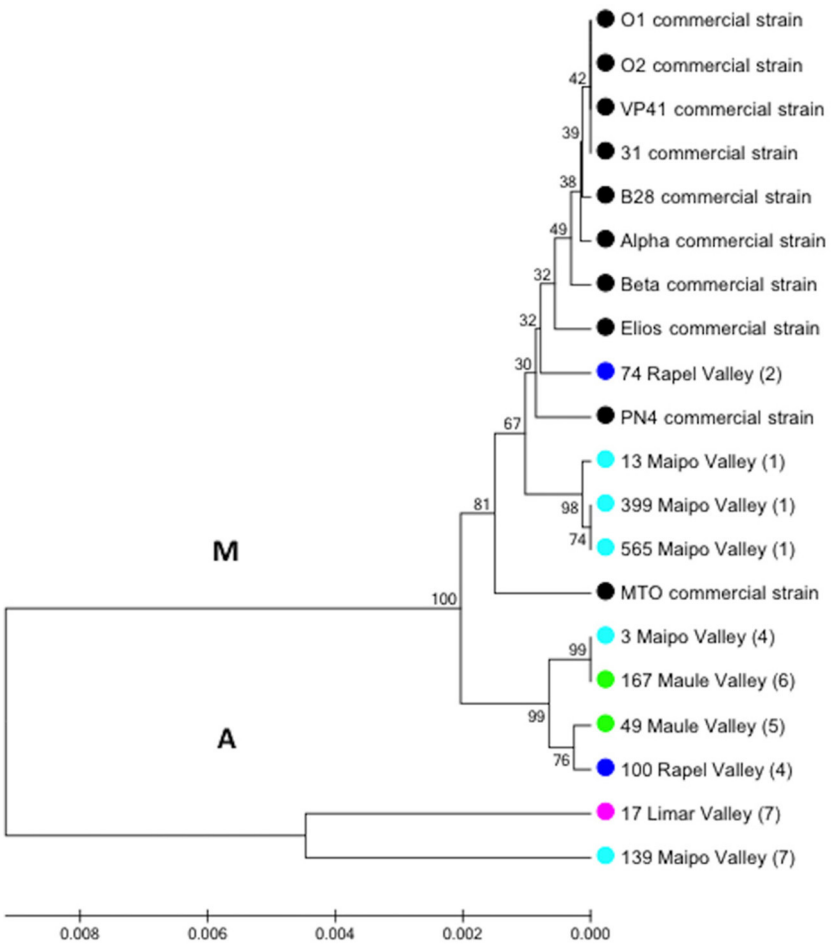

FIGURE 2 | Phylogenetic reconstruction based on the seven gene markers MLST scheme. Analysis included *O. oeni* commercial strains and Chilean autochthonous *O. oeni* isolates, which were distributed in two genetic groups indicated as M and A. Colored circles indicate the origin of each isolate (Chilean valley or commercial). Numbers correspond to signature group described in **Table 2**.

three methods showed that strain 139 had the lowest similarity to VP41 and PSU-1.

Genes that Contribute to Organoleptic Properties

To study the potential contributions to the organoleptic properties of the final product, genes related to the improvement of the wine's aromatic profile were analyzed. The autochthonous *O. oeni* genomes were compared to reference strains in terms of families and domains of interest for the enzymes that contributed to the organoleptic properties, such as esterases, glycosidases, enzymes involved in citrate metabolism, peptidases and proteases (**Table 4**). Our results showed that strain 139 presented a higher number of enzymes than strains 399 and 565.

The genome analysis screened for the presence and identity of 21 genes encoding enzymes potentially related to the aromatic profile of the wine as previously described (Mtshali et al., 2010; Cappello et al., 2014). **Table 5** showed that strain 139 harbored 14 of the 21 screened genes, which was higher than the numbers found for strains 565 and 399. Furthermore, the phenolic acid carboxylase gene (*pad*), carbamate kinase gene (*arcC*) and protease *ptrP* gene were absent in the autochthonous *O. oeni* genomes.

Genes linked to increased esters and ethyl esters that contributed to the wine's fruity aromas (*estA, estB, estC,* and *metB*) were present in the autochthonous *O. oeni* genomes. Genes linked to dyacetil, acetoin, butanediol, and acetate via citrate metabolism *(citE, citF, citD maeP,* and *alsA*) were present in isolate 139, but some of these genes were absent in the other autochthonous *O. oeni* genomes (565 and 399). Genes related to odorless non-volatile glycosides and glycosidase activities that contributed to the wine aroma (*bgl* and *abf*) were present in strain 139.

DISCUSSION

One of the main objectives of this study was to analyze the load and diversity of LAB in wine produced with spontaneous MLF in Chilean valleys and to pre-select future starter cultures.

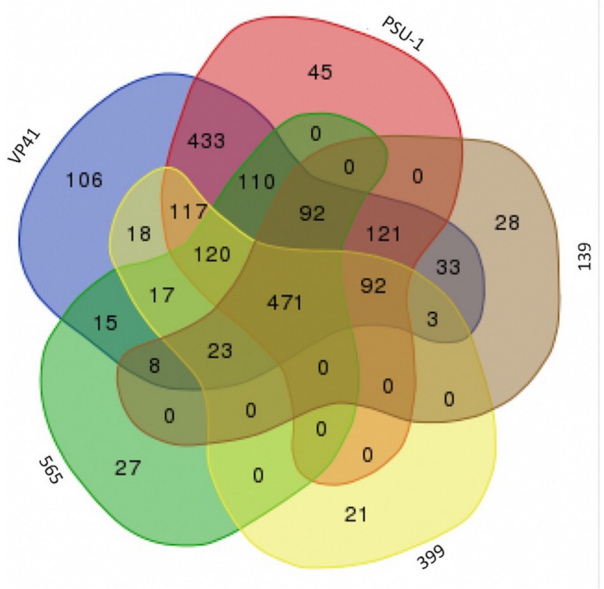

FIGURE 3 | Venn diagram between genome of Chilean autochthonous *O. oeni* isolates compared with two reference strains VP41 and PSU-1.

TABLE 3 | Results of DDH, ANIb, and OrthoANI algorithms of autochthonous *O. oeni* genomes compared to reference strain VP41.

	DDH	ANIb	OrthoANI
O. oeni	**AWRI429 or VP41 (reference)**	**AWRI429 or VP41 (reference)**	**AWRI429 or VP41 (reference)**
139	88,5	98,29	98,5702
399	96,7	99,38	99,5249
PSU-1	96,9	99,41	99,5289
565	96,5	99,3	99,5152

TABLE 4 | Occurrence of esterases, glycosidases, citrate metabolism, peptidases, and proteases enzymes of autochthonous *O. oeni* genomes compared with reference strains (VP41 and PSU-1).

	Reference strains		Autochthonous *O. oeni* isolates		
Enzymes	**VP41**	**PSU-1**	**139**	**399**	**565**
Glycosidases	28	18	10	6	10
Esterases	2	2	1	1	1
Citrate met	4	4	4	4	4
Peptidases	47	44	22	17	21
Proteases	3	1	1	1	1

This study covered an extensive Chilean enological region from 30°39′S to 35°50′S latitude and analyzed the total and lactic acid bacterial loads using culture-independent methodology. Our LAB screening results showed the highest prevalence *O. oeni*, which was coincident with reports from other countries (González-Arenzana et al., 2012; Pramateftaki et al., 2012; Cappello et al., 2017). Additionally, combinations of LAB, such as *O. oeni/Leuconostoc* and *O. oeni/Pediococcus* were found, which were coincident with the findings of Renouf et al. (2007), Pérez-Martín et al. (2015) and Miranda-Castilleja et al. (2016). However, the existence of *Pediococcus* in wine samples needs consideration, because these bacteria have been associated with ropiness of wine (Dols-Lafargue et al., 2008).

Furthermore, we found a predominance of the *O. oeni* load compared to the total bacterial load in all samples by qPCR analysis of the *rpo*B gen. This gene has been used for the description of LAB in fermentative environments (i.e., Renouf et al., 2006; Miranda-Castilleja et al., 2016 used the *rpo*B gene to study the dynamics and diversity of LAB in different cellars). Therefore, quantification of the bacterial DNA using the *rpo*B gen showed that this gene could be used as a marker of the *O. oeni* load and thus might be useful for monitoring MLF. However, the total bacterial and *O. oeni* loads were similar among the Limarí and Maule Valleys, which were located ∼760 km apart. The influence of the local bacterial diversity on wine elaboration and the peculiar characteristics provide a local product fingerprint, as reported by Bokulich et al. (2014). Furthermore, rpoB was useful to obtain a prior genetic screening of the strains, since the sequencing of this gene allow us to distinguish between autochthonous and commercial *O. oeni* strains. This approach has been reported previously in other Gram positive bacteria such as *Staphylococcus* (Drancourt and Raoult, 2002).

Genetic typing examined using MLST evidenced the existence of two major phylogenetic clusters. Interestingly, half of the autochthonous isolates could be distinguishable from the commercial isolates, whereas the other half grouped together with the commercial strains. Within group M, the commercial strain MTO presented insertion of a transposon element in *purK*, which is one of the most interesting loci to analyze the genetic variability of the O. oeni strains (González-Arenzana et al., 2013). This insertion was reported in 7 *O. oeni* strains from Champagne, Burgundy, and Jura, France, 1 strain from Italy (Bridier et al., 2010) and 2 strains from Pineau and Jura, France (Bilhère et al., 2009). According to the MLST results, low genetic diversity among the autochthonous *O. oeni* isolates were found, which might be related to the use of housekeeping genes that could be under restricted variation. Other explanations are the exchange of DNA among *O. oeni* strains, as proposed by de las Rivas et al. (2004), where a favorable environment for horizontal gene transfer could be created in the fermentation tank/barrel. Dicks (1994) and Zúñiga et al. (1996) showed that *O. oeni* was able to receive foreign DNA by transformation *in vitro* and by conjugation. Interestingly, Campbell-Sills et al. (2015) reported that *O. oeni* isolated from MLF grouped together in a phylogenomic analysis and that strains outside this genetic group were absent during MLF.

Spontaneous MLF has drawbacks, including stuck fermentation and contamination by microorganisms, which risk altering the wine quality. To solve this problem, the use of microbial starters has been introduced with commercial strains isolated from other wine-producing countries. However, starter strains selected from the wine native microbiota of each region

TABLE 5 | Identities of aromatic genes found between autochthonous *O. oeni* genomes compared with reference strains (VP41 and PSU-1).

Aromatic Genes	Specie	NCBI/UNIPROT ACC. NUM.	Reference strain		*O. oeni* a isolates			Coded protein
			PSU-1	VP41	139	399	565	
mleA	*Oenococcus oeni*	AAV65766.1	99,82	99,82	99,82	99,82	99,82	*mleA* Malolactic enzyme
alsS	*Oenococcus oeni*	AEW09411.1	99,64	99,64	99,64	99,82	99,82	*alsS* alfa-Acetolactate synthase
alsD	*Oenococcus oeni*	AEW09410.1	100	100	0	99,58	0	*alsD* alfa-Acetolactate descarboxylase
citD	*Oenococcus oeni*	CITD_OENOB	100	100	100	0	100	*citD* Citrate lyase g-subunit
citE	*Oenococcus oeni*	W5XLJ3_OENOE	99,01	99,01	100	0	0	*citE* Citrate lyase β-subunit
citF	*Oenococcus oeni*	A0NL52_OENOE	99,61	99,61	99,61	99,61	99,61	*citF* Citrate lyase a-subunit
maeP	*Oenococcus oeni*	AEW09418.1	100	100	98,78	99,69	99,69	*maeP* Putative citrate transporter
bgl	*Oenococcus oeni*	AIZ50378.1	99,32	99,46	99,05	0	0	*bgl* ß Glucosidase - related glycosidase
abf	*Oenococcus oeni*	ADJ95768.1	0	99,67	100	0	0	*abf*, a-L-arabinofuranosidase
estA	*Oenococcus oeni*	AFV75079.1	100	100	99,24	99,24	99,24	*estA* Predicted esterase
estB	*Oenococcus oeni*	AFV75078.1	99,01	99,01	99,67	98,64	98,64	*estB* Predicted esterase
estC	*Oenococcus oeni*	AFV75077.1	26,79	95,91	23,74	95,7	95,7	*estC* Predicted esterase
metB	*Oenococcus oeni*	R4HZQ9_OENOE	99,21	99,21	99,21	0	41,48	*metB* Cystathionine g-lyase
metC	*Oenococcus oeni*	AEW09413.1	100	100	47,31	0	39,89	*metC* Cystathionine β-lyase
metK	*Oenococcus oeni*	AEW09412.1	100	100	100	0	0	*metK* S-adenosylmethionine synthase
gshR	*Oenococcus oeni*	AEW09415.1	100	100	99,78	99,78	99,78	*gshR* Glutathione reductase
arcA	*Oenococcus oeni*	ARCA_OENOB	100	99,38	98,55	0	0	*arcA* Arginine deiminase
arcB	*Oenococcus oeni*	OTCC_OENOE	29,57	100	29,79	29,03	29,03	*arcB* Ornithine Transcarbamylase
arcC	*Oenococcus oeni*	ARCC_OENOE	0	100	0	0	0	*arcC* Carbamate kinase
pad	*Lactobacillus plantarum*	AAC45282.1	0	0	0	0	0	*pad p*henolic acid decarboxylases
prtP	*Lactobacillus plantarum*	CAT14096.1	0	0	0	0	0	*prtP* Proteinase

have better natural adaptation to the wine and maintain regional typicity (Zapparoli et al., 2003; Izquierdo et al., 2004). Ethanol and acidic environments are determinant factors for the growth of *O. oeni* in wine (Liu et al., 2014). In this context, strains able to tolerate 15% v/v ethanol and a pH of 3.1 were obtained. These results were different from the reports of Capozzi et al. (2010); Solieri et al. (2010) and Lerm et al. (2011), which showed that *O. oeni* strains were unable to survive in high ethanol concentrations (13% v/v). Strain 139 had high growth in 15% v/v ethanol and at a pH of 3.1 and exhibited high malolactic activity; these results suggested that this strain (139) adapted better to the wine environment than the other two strains (565 and 139). Hence, strain 139 may be proposed as the best candidate for use as a starter in MLF.

O. oeni strains have a compact genome of 1.8 Mb and several metabolic pathways related to growth in enological environments, including MLF and aroma production (Mills et al., 2005; Makarova et al., 2006; Makarova and Koonin, 2007). Furthermore, its compact genome most likely reflects a high level of organization and simplicity (Jara and Romero, 2015; Sternes and Borneman, 2016). This genomic organization may be the basis for its adaptation to the wine environment (Zé-Zé et al., 1998, 2000; Mills et al., 2005). Interestingly, the analyses of these genomes using ANIb, DDH and OrthoANI revealed 139 consistent differences from the autochthonous strains when the distance between genomes was calculated by aligning the whole sequences. According to Thompson et al. (2013), these isolates and reference strains (VP41 and PSU-1) shared more than 95% ANIb and hence could be considered the same species. A similar observation including more than 30 *O. oeni* genomes was also reported by Campbell-Sills et al. (2015).

A report by Borneman et al. (2010) compared the genomes of three *O. oeni* strains (PSU-1, BAA1163, and AWRIB429). These strains shared conserved genes corresponding to 52% of the observed ORFs. These authors claimed that this conserved region could be considered the core genome. A similar result was reported by Campbell-Sills et al. (2015) and Sternes and Borneman (2016), but these studies included more CDSs due to differences in the orthologous calculation. Borneman et al. (2010) posited that unique ORFs associated with bacteriophage-derived sequences or glycosyl hydrolases might be key from a winemaking perspective, because these ORFs might contribute to aromatic compound formation through the cleavage of the sugar moiety from the non-volatile (and therefore aroma-less) glycosidic precursors present in grape juice. These analyses suggest that genomic variation may be the key to ascertaining the phenotypic differences between *O. oeni* strains. In this context, our data showed that the Chilean strain contained 21–28 unique genes per strain related to metabolism and transport, some of which possibly explained some of the technological properties of the bacteria. Similarly, Campbell-Sills et al. (2015) reported that *O. oeni* isolates from Champagne showed 27 unique genes that might be related to technical properties. Taken together, the technical properties, unique characteristics and capacity for local adaptation of some LAB could provide the basis for obtaining suitable strains to serve as inocula

in future products and contribute to the typicity of Chilean wines.

Several enzymes from *O. oeni* may contribute to the aromatic profile of the wine during MLF, including β-glucosidase, citrate lyase, esterases, proteases, and peptidases (Mtshali et al., 2010; Michlmayr et al., 2012; Cappello et al., 2017). The activity of some of these enzymes may be modulated by enological parameters, such as the pH, temperature, ethanol, or glucose and fructose concentrations (Grimaldi et al., 2005). Furthermore, Olguín et al. (2011) demonstrated that the expression of the β-glucosidase gene (*bgl*) in *O. oeni* might be induced by a moderate ethanol concentration.

Citrate metabolism is involved in the production of compounds, such as diacetyl, acetoin, butanediol and acetate, which are important for the wine aroma (Olguín et al., 2009). Diacetyl is the most important aroma compound during MLF (Cappello et al., 2017). Additionally, some genes involved in citrate metabolism have been shown to provide metabolic traits to different strains (Olguín et al., 2009). The inter-strain comparison of the transcriptional levels of genes involved in citrate metabolism (*ack*A and *als*D) revealed that the strains had different metabolic features.

Esters are a key group of volatile compounds that can contribute to the wine aromatic profile (Swiegers et al., 2005). These compounds depend on the activity of esterases (Cappello et al., 2017). Sumby et al. (2013) showed that two purified *O. oeni* esterases (EstA2 and EstB28) had two activities (synthesis and hydrolysis) that suggested the contribution of *O. oeni* to the wine aroma profile.

The contribution of specific *O. oeni* strain to the organoleptic properties of wine may affect flavor formation depending on the wine parameters (Cappello et al., 2017). The selection of bacterial strains for MLF should consider the potential to improve the wine typicity. In this context, Chilean isolates showed different contents of genes encoding enzymes contributing to the aromatic profile; among them, strain 139 presented a higher number of glucosidases and promising enological properties and thus might be proposed as the best candidate for use as a starter in MLF.

To the best of our knowledge, this study is the first report to focus on both the genetic and technological characterization of *O. oeni* strains in Chile. This study reported that genetic (MLST) and genomic tools (ANI) might reveal the differences between commercial and autochthonous *O. oeni* strains. Similarly, autochthonous *O. oeni* strains showed some advantages in terms of technological properties. Thus, future studies should focus on determining the potential relationships between the phylogenetic and phenotypic characteristics of *O. oeni* strains; these results could help identify the effect of environmental conditions on the genetic content and evolution of the species. Furthermore, these analyses may provide useful information for the selection of strains with better industrial performances.

AUTHOR CONTRIBUTIONS

JR and CJ: designed of the work; CI: data collection, data analysis, and interpretation; MR: Genetic and genomic analyses; CJ: drafting the article; JR: critical revision and edition of the article; CJ, CI, JR: Final approval of the version to be published.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.00090/full#supplementary-material

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