



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

María L. Raymond Eder, Francisco Conti and Alberto L. Rosa\*

Laboratorio de Genética y Biología Molecular, IRNASUS-CONICET, Facultad de Ciencias Químicas, Universidad Católica de Córdoba, Córdoba, Argentina

## OPEN ACCESS

### Edited by:

Aline Lonvaud,  
Université de Bordeaux, France

### Reviewed by:

Ricardo Franco-Duarte,  
University of Minho, Portugal  
Rosanna Tofalo,  
Università di Teramo, Italy

### \*Correspondence:

Alberto L. Rosa  
alrosa@ucc.edu.ar;  
alberto\_l\_rosa@yahoo.com.ar

### Specialty section:

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

**Received:** 27 March 2018

**Accepted:** 30 May 2018

**Published:** 19 June 2018

### Citation:

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

Yeast communities associated with *Vitis vinifera* L. ecosystems have been widely characterized. Less is known, however, about yeast communities present in grapes and fermenting musts from *Vitis non-vinifera* ecosystems. Moreover, there are no comparative studies concerning yeast communities in grapes from *V. vinifera* L. and non-*vinifera* *Vitis* species in vineyards from a shared *terroir*. In this work, we have used a culture-dependent strategy, phenotypic analyses, and molecular genotyping, to study the most representative yeast species present in spontaneously fermenting musts of grapes harvested from neighboring *V. vinifera* L. (cv. Malbec) and *V. labrusca* L. (cv. Isabella) vineyards. Phenotypic analyses of H<sub>2</sub>S production, ethanol tolerance and carbon utilization, on randomly selected strains of each *Hanseniaspora uvarum*, *Starmerella bacillaris* and *Saccharomyces cerevisiae* strains, as well as microsatellite genotyping of *S. cerevisiae* isolates from each the Malbec and Isabella grape musts, suggest that *V. vinifera* L. and *V. labrusca* L. ecosystems could harbor different yeast strain populations. Thus, microbial communities in exotic *Vitis* species may offer opportunities to look for unique yeast strains that could not be present in conventional *V. vinifera* L. ecosystems.

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

## INTRODUCTION

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

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

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

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

## MATERIALS AND METHODS

### Spontaneous Fermentation of Malbec and Isabella Grape Musts

Malbec (*V. vinifera* L.) and Isabella (*V. labrusca* L.) grapes were harvested at their optimal ripeness stages from vineyards in Colonia Caroya (vintage of March, 2017), located at 31°02'00''S / 64°05'36''O and 491 meters above sea level, in the province of Córdoba, Argentina. The region has an annual rainfall of 765 mm and a mean temperature of 15.8°C. Separate spontaneous

fermentations of a pool of destemmed and partially crushed Malbec and Isabella grapes were performed in a local cooperative cellar. Grapes from ~80% of the Colonia Caroya's Malbec and Isabella vineyards (i.e., 18–20 Ha each) are processed at this cellar. About 20% of these closely located, small vineyards (i.e., ~1.5 Ha each), have intermixed rows of Malbec and Isabella plants. Must samples (70 liters) were fermented at 25–28°C in stainless steel tanks located in a room of the winery not previously used for winemaking. Musts were punched down twice a day and aliquots were taken daily for ten (i.e., 0–240 h) or five (i.e., 0–120 h) days from Malbec and Isabella musts, respectively, and stored in 30% (v/v) glycerol at –70°C.

### Isolation of Yeast Strains From Malbec and Isabella Ecosystems

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

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

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

## Molecular Identification of Yeast Species

Isolated yeasts were identified by PCR-RFLP and/or DNA sequencing of their 5.8-ITS (*Internal Transcribed Spacer*) rDNA regions (Esteve-Zarzoso et al., 1999). Total genomic DNA was extracted according to Raymond Eder et al. (2017). PCR was carried out using ITS1 and ITS4 primers (White et al., 1990). For PCR-RFLP, 10  $\mu$ l of each of the PCR products were digested for 3 h at 37°C with the restriction enzymes *Hinf* I (New England BioLabs, United States) and/or *Cfo* I (Promega, United States) and the resulting DNA fragments were characterized by agarose [3.0% (w/v)] gel electrophoresis and analyzed using data from www.yeast-id.org. In most of the cases, yeast species identification was confirmed by Sanger sequencing of their 5.8-ITS rDNA regions and analysis using the BLASTN software NCBI<sup>1</sup>. Species identification was considered valid when

<sup>1</sup><https://blast.ncbi.nlm.nih.gov/Blast.cgi>

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

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

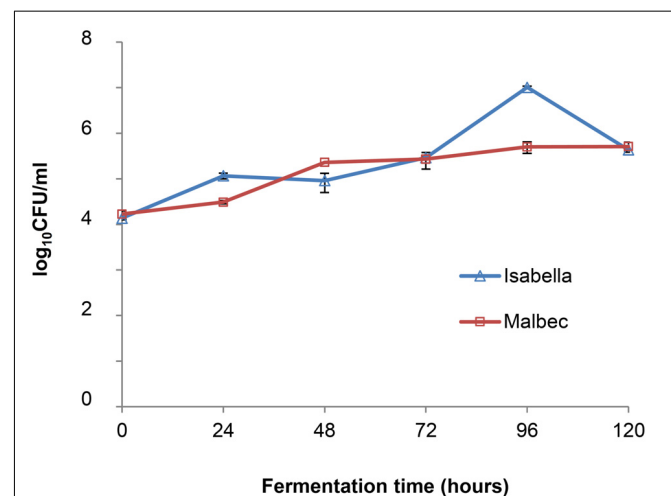
<sup>1</sup>*M* (Malbec) and *I* (Isabella) strains; <sup>2</sup>H<sub>2</sub>S production was evaluated in Biggy medium and scored as indicated in the Material and Methods section; <sup>3</sup>Tolerance to ethanol is indicated as the maximal ethanol concentration [i.e., % (v/v)] in solid media where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

the identity of a 5.8-ITS sequence and a reference sequence was 99–100%. Sequences from representative *H. uvarum*, *S. bacillaris*, and *S. cerevisiae* isolates were deposited in the NCBI GeneBank database (Table 2).

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

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

A simple weight loss microassay, dependent on CO<sub>2</sub> release (Quirós et al., 2010), was designed to characterize glucose and fructose fermentation profiles of the *H. uvarum*, *S. bacillaris*, and *S. cerevisiae* isolates. Similar small scale fermentation assays have been recently published (Liccioli et al., 2011; Peltier et al., 2018). In our studies, strains were grown during ~15 h at 25°C without agitation in 15 ml Falcon tubes containing 5 ml of YP medium supplemented with either glucose 10.0% (w/v) (YPD-10) or fructose 10.0% (w/v) (YPF-10). Duplicated 1.5 ml



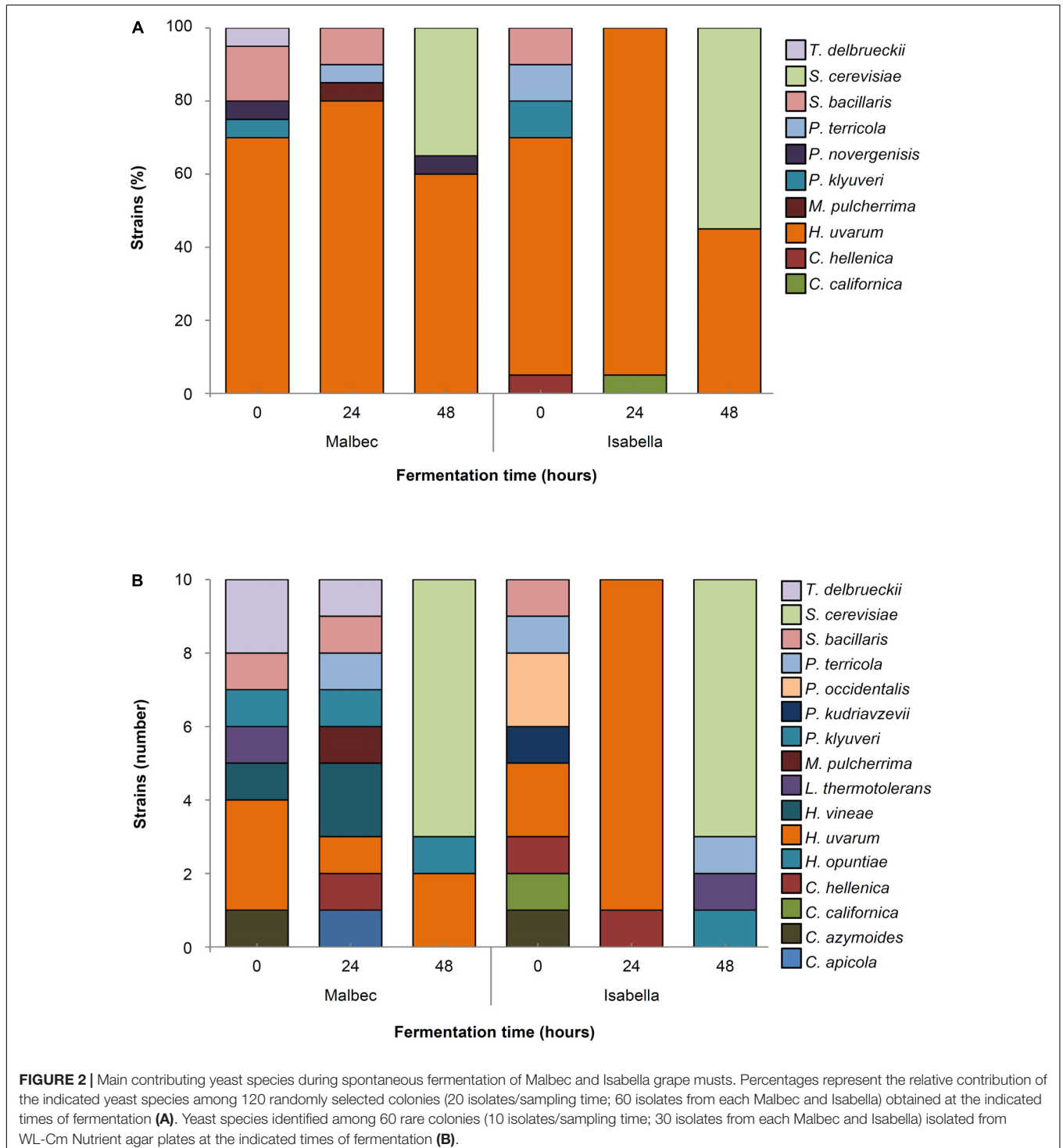
**FIGURE 1** | Population dynamics of total yeasts at initial times of spontaneous fermentation of Malbec and Isabella grape musts.

Eppendorf tubes, containing 1.0 ml liquid of YPD-10 or YPF-10, were inoculated with cells ( $10^7$ /ml) from the YPD-10 and YPF-10 cultures, respectively, and maintained at 25°C without agitation. Microtubes contained a 0.8 mm perforation on its cap, covered with a small piece of cotton, to allow CO<sub>2</sub> efflux. Tubes were weighed immediately after inoculation and every 24 h for 4 days, using non-inoculated tubes as control of weight loss via

evaporation. Fermentation rates were expressed as weight loss (i.e., CO<sub>2</sub> release) in function of time (i.e.,  $\text{g.l}^{-1}.\text{h}^{-1}$ ).

### *S. cerevisiae* Microsatellite Genotyping

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





(Legras et al., 2005). PCR reactions contained 100 ng of genomic DNA, 1.5 mM MgCl<sub>2</sub>, *Taq* polymerase buffer 1X (Invitrogen, United States), 200 μM dNTPs, 10 pmol of each primer and 1.25 units of *Taq* polymerase (Invitrogen, United States). Amplification reactions were performed in a MJ Mini Bio-Rad thermocycler (Bio-Rad, United States) using an initial denaturation step at 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, annealing at 57°C for 45 s, extension at 72°C for 1 min followed by a final extension at 72°C for 10 min. PCR products were separated in 8.0% polyacrylamide gels using TBE as the running buffer. Gels were stained with ethidium bromide, photographed under UV light and allele sizes were determined using the 100-bp-DNA-ladder (Inbio Highway, Argentina) as a reference molecular size standard.

## RESULTS

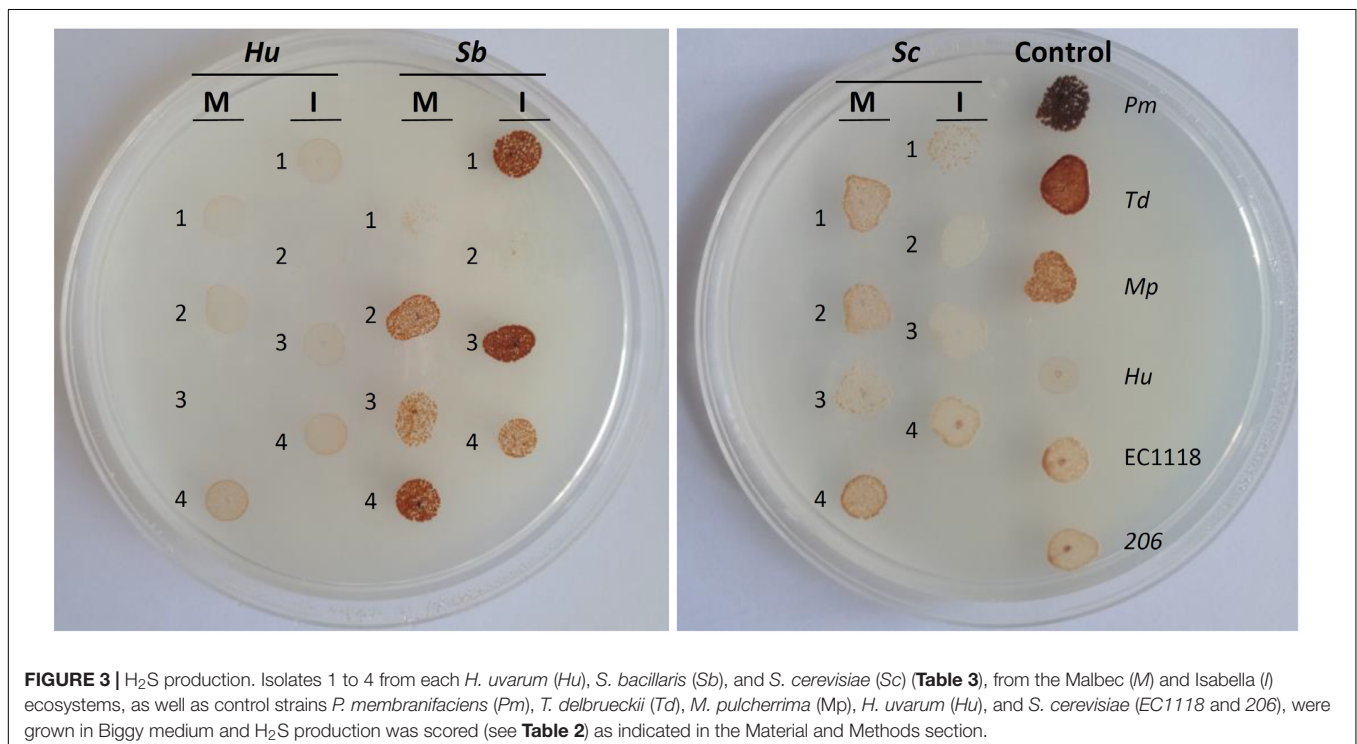
### Malbec (*V. vinifera* L.) and Isabella (*V. labrusca* L.) Spontaneously Fermenting Grape Musts

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

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

The population dynamics of cultivable yeast species in the Malbec and Isabella ecosystems were analyzed from time  $t_0$  to  $t_{120}$ , corresponding to the initial stages of fermentation of Malbec and the entire fermentation period of Isabella (Figure 1). The total yeast populations in both ecosystems started with similar counts, and increased similarly as fermentation progressed (Figure 1). The highest total yeast count in fermenting Isabella grape must was observed at  $t_{96}$  while fermenting Malbec must reached its highest yeast count at  $t_{120}$  (Figure 1). As expected, *S. cerevisiae* was the most predominant yeast species recognized among 75 isolates obtained at the middle/advanced stages of fermenting Malbec and Isabella musts (i.e.,  $t_{72}$ – $t_{120}$ ) (not shown). Based on this observation, our analyses of the predominant non-*Saccharomyces* species in the Malbec and Isabella ecosystems were limited to the early stages of spontaneous fermentation (i.e.,  $t_0$ ,  $t_{24}$ , and  $t_{48}$ ), at which a total of 180 isolates were identified by PCR-RFLP and/or DNA sequencing of their 5.8-ITS (*Internal Transcribed Spacer*) rDNA regions (Figure 2).

A great diversity of non-*Saccharomyces* species was evidenced among the yeast isolated from both Malbec and Isabella ecosystems (Figure 2). *H. uvarum* was the most common species isolated at early stages of fermentation of Malbec ( $t_0$ ,  $t_{24}$ , and  $t_{48}$ ) and Isabella ( $t_0$  and  $t_{24}$ ) grape musts (Figure 2A). Other non-*Saccharomyces* yeast species identified in both Malbec and Isabella musts were *Candida azymoides*, *Candida hellenica*, *Lachancea thermotolerans*, *Pichia klyuveri*, *Pichia terricola*, and



*Starmerella bacillaris*. Interestingly, *Torulaspora delbrueckii*, *Hanseniaspora vineae*, and *Metschnikowia pulcherrima*, were not among the yeast species identified in Isabella (Figure 2). *Candida californica*, previously recognized in Isabella fermenting must (Raymond Eder et al., 2017), was also isolated from Isabella in this work (i.e., at  $t_0$  and  $t_{24}$ ). *P. occidentalis* and *P. kudriavzevii* were isolated only from fermenting Isabella must (Figure 2B) while *P. norvegensis* was isolated only from fermenting Malbec must. *S. cerevisiae*, not isolated at initial stages of spontaneous fermentation, was the predominant yeast species in Isabella must at  $t_{48}$ , and started to become dominant at the same fermentation time in Malbec must.

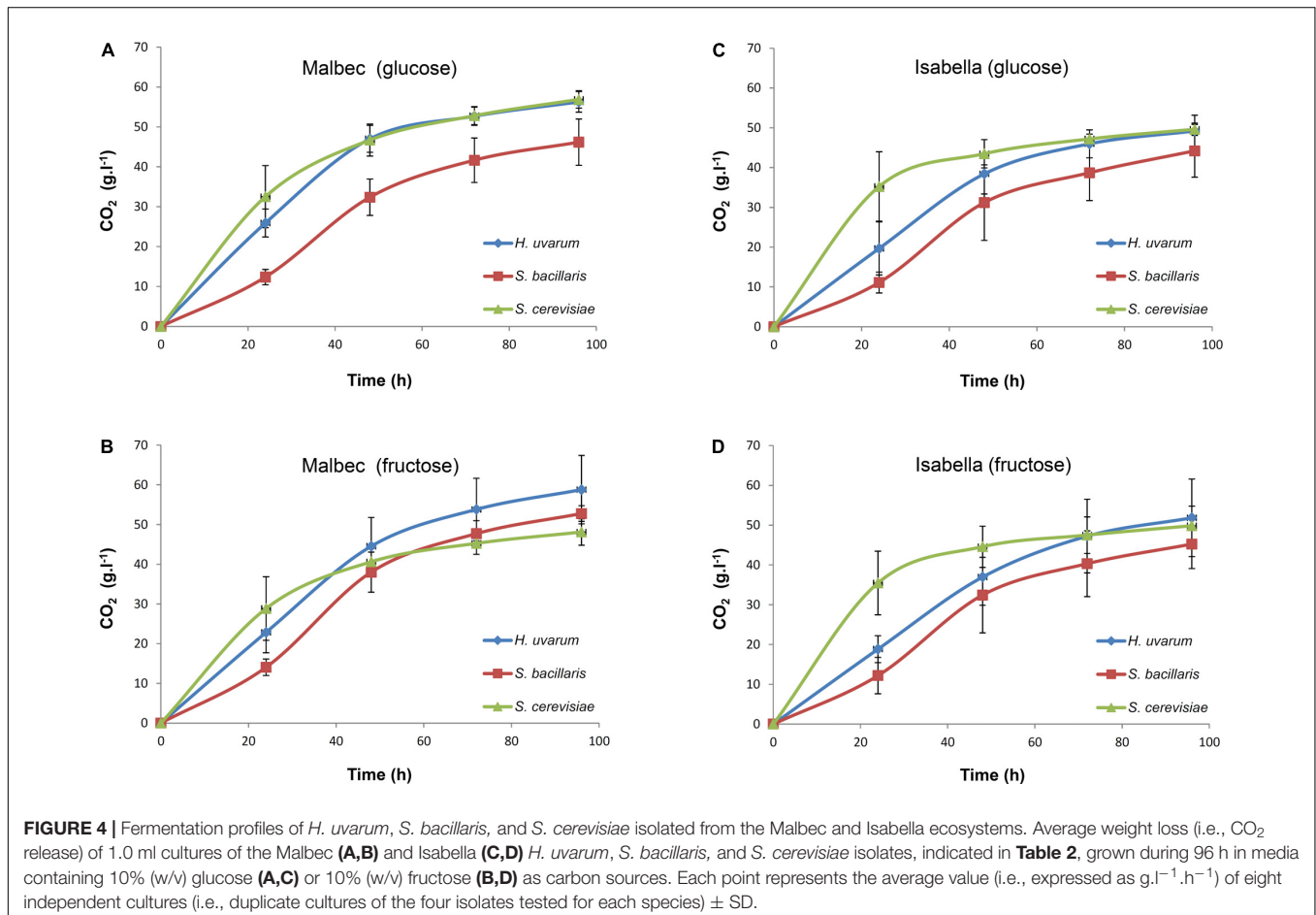
## H<sub>2</sub>S Production by *H. uvarum*, *S. bacillaris*, and *S. cerevisiae* Isolates From the Malbec and Isabella Ecosystems

In order to explore possible phenotypic differences among *S. bacillaris*, *H. uvarum*, and *S. cerevisiae* isolates from the Malbec and Isabella ecosystems, we analyzed the production of H<sub>2</sub>S in four randomly selected isolates from each of these species. H<sub>2</sub>S production varied greatly among yeast species, as well as between isolates of the same species from the same ecosystem (Figure 3

and Table 2). Interestingly, however, most of the *S. cerevisiae* isolates from Malbec (3 out of 4) showed higher production of H<sub>2</sub>S than their counterparts isolated from Isabella grape must (Figure 3). *S. bacillaris*, on the other hand, was the species with the most consistent production of relatively high levels of H<sub>2</sub>S, compared to *H. uvarum* and *S. cerevisiae* (Figure 3 and Table 2).

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

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

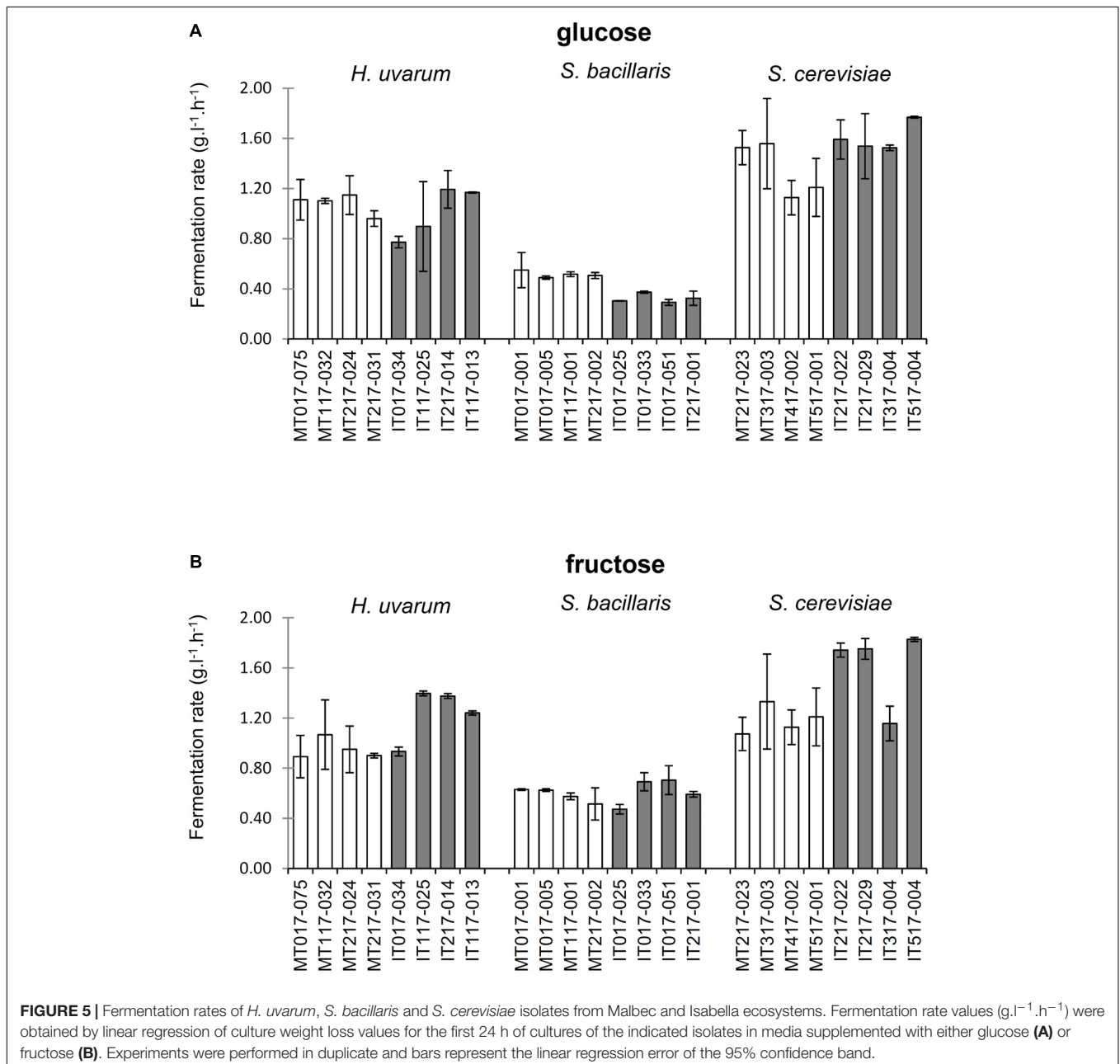


stages of fermentation of the Malbec must (not shown). Similar results were obtained when ethanol tolerance of the *H. uvarum*, *S. bacillaris*, and *S. cerevisiae* isolates was assayed in liquid media (not shown).

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

A simple microtube assay was designed to explore possible phenotypic differences in glucose versus fructose utilization among the *H. uvarum*, *S. bacillaris*, and *S. cerevisiae* isolates from

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



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

Isolate <sup>1</sup>	<i>S. cerevisiae</i> microsatellite <sup>2</sup>						
	AAT1	AAT2	AAT3	C3	C6	YPL009c	YOR267c
M1	1	1	2	1	1	3	2
M2	1	1	1	1	1	1	1
M3	1	1	2	1	1	2	2
M4	1	1	2	1	1	2	2
I1	4	1	2	1	1	3	2
I2	6	2	ND	1	1	2	ND
I3	5	1	2	1	1	3	3
I4	4	1	4	1	1	2	1

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

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

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

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

## DISCUSSION

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

We have recently proposed that some yeast species may be specifically associated with some *Vitis* species (Raymond Eder et al., 2017). Eventually, different *Vitis* species may harbor specific yeast communities (i.e., yeast species and/or strains of a given yeast species) even in neighboring *Vitis* ecosystems. In this work we explored this hypothesis by studying yeast isolates, from neighboring Malbec (*V. vinifera* L.) and Isabella (*V. labrusca* L.) vineyards, representative of the three major species recognized in Isabella (i.e., *H. uvarum*, *S. bacillaris*, and *S. cerevisiae*) (Raymond Eder et al., 2017). *H. uvarum* was the predominant non-*Saccharomyces* species in the Malbec and Isabella ecosystems,

both at early and middle stages of fermentation. In a previous work, we identified *S. bacillaris* as the main yeast species present at early stages of fermentation of Isabella grapes harvested in the same geographic region (i.e., vintage 2015) (Raymond Eder et al., 2017). A similar predominance of either *H. uvarum* or *C. stellata* (reclassified to *S. bacillaris*; Csoma and Sipiczki, 2008; Duarte et al., 2012), in consecutive vintages in the same geographic region, has been reported (Beltran et al., 2002). In addition to *H. uvarum*, a variety of non-*Saccharomyces* species were isolated at early stages of Isabella must fermentation. This diversity quickly decreased between *t*<sub>0</sub> and *t*<sub>24</sub> and three main yeast species (i.e., *H. uvarum*, *C. californica*, and *C. hellenica*) were recognized following 1 day of fermentation. In fermenting Malbec grape must, on the other hand, the great diversity of yeast species found at the beginning of fermentation continued at *t*<sub>24</sub> and *t*<sub>48</sub>, when *S. cerevisiae* species started to develop.

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

Phenotypic analyses of H<sub>2</sub>S production showed a remarkable diversity among the analyzed *S. cerevisiae* isolates from Malbec



and Isabella. Microsatellite genotyping of these *S. cerevisiae* isolates showed that, with the exception of isolates M3 and M4, they correspond to genetically different strains. Interestingly, Isabella's *S. cerevisiae* isolates I2 and I3, which are genetically different, were the lowest producers of H<sub>2</sub>S, even when compared with the industrial strain EC1118. Additional characterization of a larger number of *S. cerevisiae* isolates could indicate if medium and low H<sub>2</sub>S producer strains are preferentially associated with the Malbec and Isabella ecosystems, respectively. *H. uvarum* and *S. bacillaris* isolates were low and high producers of H<sub>2</sub>S, respectively. Although the observed phenotypes suggested genetic heterogeneity among the analyzed *H. uvarum* and *S. bacillaris* isolates, no specific association of the isolates with their Malbec or Isabella ecosystems was observed.

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

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

## REFERENCES

- Aponte, M., and Blaiotta, G. (2016). Potential role of yeast strains isolated from grapes in the production of Taurasi DOCG. *Front. Microbiol.* 7:809. doi: 10.3389/fmicb.2016.00809
- Barata, A., Malfeito-Ferreira, M., and Loureiro, V. (2012). The microbial ecology of wine grape berries. *Int. J. Food Microbiol.* 153, 243–259. doi: 10.1016/j.ijfoodmicro.2011.11.025
- Belloch, C., Orlic, S., Barrio, E., and Querol, A. (2008). Fermentative stress adaptation of hybrids within the *Saccharomyces sensu stricto* complex. *Int. J. Food Microbiol.* 29, 188–195. doi: 10.1016/j.ijfoodmicro.2007.11.083

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

## AUTHOR CONTRIBUTIONS

MR, FC, and AR made fundamental contributions to the conception and design of the work, contributed to the acquisition, analysis, and interpretation of data, and drafted the work and revised it critically for intellectual content. All authors approved the final version of the manuscript to be submitted for publication and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy and integrity of any part of the work are appropriately investigated and resolved.

## FUNDING

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

## ACKNOWLEDGMENTS

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

- Beltran, G., Torija, M. J., Novo, M., Ferrer, N., Poblet, M., Guillamón, J. M., et al. (2002). Analysis of yeast populations during alcoholic fermentation: a six year follow-up study. *Syst. Appl. Microbiol.* 25, 287–293. doi: 10.1078/0723-2020-00097
- Bezerra-Bussoli, C., Baffi, M. A., Gomes, E., and Da-Silva, R. (2013). Yeast diversity isolated from grape musts during spontaneous fermentation from a Brazilian winery. *Curr. Microbiol.* 67, 356–361. doi: 10.1007/s00284-013-0375-9
- Bokulich, N. A., Thorngate, J. H., Richardson, P. M., and Mills, D. A. (2014). Microbial biogeography of wine grapes is conditioned by cultivar, vintage, and climate. *Proc. Natl. Acad. Sci. U.S.A.* 111, 139–148. doi: 10.1073/pnas.1317377110

- Capece, A., Granchi, L., Guerrini, S., Mangani, S., Romaniello, R., Vicenzini, M., et al. (2016). Diversity of *Saccharomyces cerevisiae* strains isolated from Two Italian wine-producing regions. *Front. Microbiol.* 7:1018. doi: 10.3389/fmicb.2016.01018
- Combina, M., Elia, A., Mercado, L., Catania, C., Ganga, A., and Martinez, C. (2005). Dynamics of indigenous yeast populations during spontaneous fermentation of wines from Mendoza, Argentina. *Int. J. Food Microbiol.* 99, 237–243. doi: 10.1016/j.ijfoodmicro.2004.08.017
- Csoma, H., and Sipiczki, M. (2008). Taxonomic reclassification of *Candida stellata* strains reveals frequent occurrence of *Candida zemplinina* in wine fermentation. *FEMS Yeast Res.* 8, 328–336. doi: 10.1111/j.1567-1364.2007.00339.x
- Drumonde-Neves, J., Frando-Duarte, R., Lima, T., Schuller, D., and Pais, C. (2016). Yeast biodiversity in *Vineyard* environment is increased by human intervention. *PLoS One* 11:e0160579. doi: 10.1371/journal.pone.0160579
- Drumonde-Neves, J., Frando-Duarte, R., Lima, T., Schuller, D., and Pais, C. (2017). Association between grape yeast communities and the *Vineyard* Ecosystems. *PLoS One* 12:e0169883. doi: 10.1371/journal.pone.0169883
- Duarte, F. L., Pimentel, N. H., Teixeira, A., and Fonseca, A. (2012). *Saccharomyces bacillaris* is not a synonym of *Candida stellata*: reinstatement as *Starmerella bacillaris* comb. nov. *Antonie Van Leeuwenhoek* 102, 653–658. doi: 10.1007/s10482-012-9762-7
- Esteve-Zarzoso, B., Belloch, C., Uruburu, F., and Querol, A. (1999). Identification of yeasts by RFLP analysis of the 5.8S *rRNA* gene and the two ribosomal internal transcribed spacers. *Int. J. Syst. Bacteriol.* 1, 329–337. doi: 10.1099/00207713-49-1-329
- Filho, M. C., Bertéli, M. B., Valle, J. S., Paccola-Meirelles, L. D., Linde, G. A., Barcellos, F. G., et al. (2017). Genetic diversity and pectinolytic activity of epiphytic yeasts from grape carposphere. *Genet. Mol. Res.* 20:16. doi: 10.4238/gmr16029698
- Fleet, G. H. (2003). Yeast interactions and wine flavour. *Int. J. Food Microbiol.* 86, 11–22. doi: 10.1016/S0168-1605(03)00245-9
- Fleet, G. H. (2008). Wine yeasts for the future. *FEMS Yeast Res.* 8, 979–995. doi: 10.1111/j.1567-1364.2008.00427.x
- Jara, C., Laurie, V. F., Mas, A., and Romero, J. (2016). Microbial terroir in Chilean Valleys: diversity of non-conventional yeast. *Front. Microbiol.* 7:663. doi: 10.3389/fmicb.2016.00663
- Jolly, N. P., Augustyn, O. P. H., and Pretorius, I. S. (2006). The role and use of non-*Saccharomyces* yeasts in wine production. *S. Afr. J. Enol. Vitic.* 27, 15–35. doi: 10.21548/27-1-1475
- Jolly, N. P., Varela, C., and Pretorius, I. S. (2014). Not your ordinary yeast: non-*Saccharomyces* yeasts in wine production uncovered. *FEMS Yeast Res.* 14, 215–237. doi: 10.1111/1567-1364.12111
- Knight, S., Klaere, S., Fedrizzi, B., and Goddard, M. R. (2015). Regional microbial signatures positively correlate with differential wine phenotypes: evidence for a microbial aspect to terroir. *Sci. Rep.* 5:14233. doi: 10.1038/srep14233
- Legras, J. L., Ruh, O., Merdinoglu, D., and Karst, F. (2005). Selection of hypervariable microsatellite loci for the characterization of *Saccharomyces cerevisiae* strains. *Int. J. Food Microbiol.* 25, 73–83. doi: 10.1016/j.ijfoodmicro.2004.12.007
- Liccioli, T., Tran, T. M., Cozzolino, D., Jiranek, V., Chambers, P. J., and Schmidt, S. A. (2011). Microvinification—how small can we go? *Appl. Microbiol. Biotechnol.* 89, 1621–1628. doi: 10.1007/s00253-010-2992-6
- Masneuf-Pomarede, I., Bely, M., Marullo, P., and Albertin, W. (2016). The genetics of non-*Saccharomyces* wine yeasts: current knowledge and future challenges. *Front. Microbiol.* 6:1563. doi: 10.3389/fmicb.2015.01563
- Medina, K., Boido, E., Fariña, L., Gioia, O., Gomez, M. E., Barquet, M., et al. (2013). Increased flavour diversity of chardonnay wines by spontaneous fermentation and co-fermentation with *Hanseniaspora vineae*. *Food Chem.* 141, 2513–2521. doi: 10.1016/j.foodchem.2013.04.056
- Morgan, H. H., du Toit, M., and Setati, M. E. (2017). The grapevine and wine microbiome: insights from high-throughput amplicon sequencing. *Front. Microbiol.* 11:820. doi: 10.3389/fmicb.2017.00820
- Osorio-Cadavid, E., Chaves-López, C., Tofalo, R., Paparella, A., and Suzzi, G. (2008). Detection and identification of wild yeasts in champús, a fermented Colombian maize beverage. *Food Microbiol.* 25, 771–777. doi: 10.1016/j.fm.2008.04.014
- Padilla, B., Gil, J. V., and Manzanares, P. (2016). Past and future of non-*Saccharomyces* yeasts: from spoilage microorganisms to biotechnological tools for improving wine aroma complexity. *Front. Microbiol.* 7:411. doi: 10.3389/fmicb.2016.00411 doi: 10.3389/fmicb.2016.00411
- Peltier, E., Bernard, M., Trujillo, M., Prodhomme, D., Barbe, J. C., Gibon, Y., et al. (2018). Wine yeast phenomics: a standardized fermentation method for assessing quantitative traits of *Saccharomyces cerevisiae* strains in enological conditions. *PLoS One* 13:e0190094. doi: 10.1371/journal.pone.0190094
- Quirós, M., Gonzalez-Ramos, D., Tabera, L., and Gonzalez, R. (2010). A new methodology to obtain wine yeast strains overproducing mannoproteins. *Int. J. Food Microbiol.* 139, 9–14. doi: 10.1016/j.ijfoodmicro.2010.02.014
- Raymond Eder, M. L., Reynoso, C., Lauret, S. C., and Rosa, A. L. (2017). Isolation and identification of the indigenous yeast population during spontaneous fermentation of Isabella (*Vitis labrusca* L.) grape must. *Front. Microbiol.* 8:532. doi: 10.3389/fmicb.2017.00532
- Sipiczki, M., Romano, P., Lipani, G., Miklos, I., and Antunovic, Z. (2001). Analysis of yeasts derived from natural fermentation in a Tokaj winery. *Antonie Van Leeuwenhoek* 79, 97–105. doi: 10.1023/A:1010249408975
- Tofalo, R., Schirone, M., Torriani, S., Rantsiou, K., Cocolin, L., Perpetuini, G., et al. (2011). Diversity of *Candida zemplinina* strains from grapes and Italian wines. *Food Microbiol.* 29, 18–26. doi: 10.1016/j.fm.2011.08.014
- Tristezza, M., Tufariello, M., Capozzi, V., Spano, G., Mita, G., and Grieco, F. (2016). The enological potential of *Hanseniaspora uvarum* in simultaneous and sequential co-fermentation with *Saccharomyces cerevisiae* for industrial wine production. *Front. Microbiol.* 7:670. doi: 10.3389/fmicb.2016.00670
- Varela, C. (2016). The impact of non-*Saccharomyces* yeasts in the production of alcoholic beverages. *Appl. Microbiol. Biotechnol.* 100, 9861–9874. doi: 10.1007/s00253-016-7941-6
- Varela, C., and Borneman, A. R. (2017). Yeast found in vineyards and wineries. *Yeast* 34, 111–128. doi: 10.1002/yea.3219
- White, T., Bruns, T., Lee, S., and Taylor, J. (1990). “Amplification and direct sequencing of fungi ribosomal RNA genes for phylogenetics,” in *PCR Protocols. A Guide to Methods and Applications*, eds M. Innis, D. Gelfand, J. Sninsky, and T. White (San Diego, CA: Academic Press), 315–322.

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

Copyright © 2018 Raymond Eder, Conti and Rosa. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.