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Culture-dependent and independent techniques to monitor yeast species during cold soak carried out at different temperatures in winemaking

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ABSTRACT

Transformation of grape must into wine is a process that may vary according to the consumers' requirements. Application of cold soak prior to alcoholic fermentation is a common practice in cellars in order to enhance flavor complexity and extraction of phenolic compounds. However, the effect of this step on wine yeast microbiota is not well-known. The current study simultaneously analyzed the effect of different cold soak temperatures on the microbiological population throughout the process and the use of culture-dependent and independent techniques to study this yeast ecology. The temperatures assayed were those normally applied in wineries: 2.5, 8 and 12 °C. PCR-DGGE allowed detection of the most representative species such as *Hanseniaspora uvarum*, *Starmerella bacillaris* and *Saccharomyces cerevisiae*. As could be expected, highest diversity indices were obtained at the beginning of each process, and survival of *H. uvarum* or *S. bacillaris* depended on the temperature. Our results are in agreement with those obtained with culture independent methods, but qPCR showed higher precision and a different behavior was observed for each yeast species and at each temperature assayed. Comparison of both culture-independent techniques can provide a general overview of the whole process, although DGGE does not reveal the diversity expected due to the reported problems with the sensitivity of this technique.

1. Introduction

Alcoholic fermentation of grape juice, conducted in a spontaneous way, is a complex ecological and biochemical process that mainly depends on the sequential development of various yeast species and strains (Sun and Liu, 2014). Some species of the genera *Candida*, *Debaryomyces*, *Hanseniaspora*, *Pichia*, *Kloeckera*, *Metschnikowia*, *Pichia*, *Schizosaccharomyces*, *Torulaspora* and *Zygosaccharomyces,* commonly known as non-*Saccharomyces* yeasts, are present on the grape surface. These yeast species are predominant in grape musts and during the early fermentation stages. *Saccharomyces* is sometimes present on the grapes and winery equipment and, subsequently, the strongest fermenting and most ethanol tolerant species of this genus take control of the fermentation (Clavijo et al., 2010).

Wine yeasts are influenced by multiple factors that can be grouped into viticultural and oenological practices (Andorrà et al., 2010a; Hierro et al., 2006; Tello et al., 2012). Pre-fermentative cold maceration or cold soak (CS), one of the most commonly used oenological practices, is widely applied in winemaking to produce red wine. It consists of keeping the must at a low temperature (0 to 15 °C) for a certain time (3 to 10 days) before alcoholic fermentation takes place (Zott et al., 2008).

The wine industry has become increasingly interested in the use of cold maceration prior to fermentation because it enhances flavor complexity and extraction of phenolic compounds (Casassa et al., 2015; Parenti et al., 2004). However, low temperatures can affect the survival rate of non-*Saccharomyces* yeast populations present in must. Growth of certain non-*Saccharomyces* yeasts and their metabolism may be involved in the improvement of organoleptic characteristic of wines (Andorrà et al., 2010a), but some wild spoilage yeasts can produce off-flavors that adversely affect the final product (Malfeito-Ferreira, 2011). Thus it is important to study the diversity and dynamics of non-*Saccharomyces* yeasts and their role during pre-fermentative CS as well as the consequences for the vinification process and quality of the final product.

Effects of CS on the wine yeast populations have previously been studied with culture-dependent analyses in different culture media (Hierro et al., 2006; Maturano et al., 2015; Zott et al., 2008). These

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methods are very laborious and time-consuming and include isolation, cultivation and characterization of each species. In addition, the minor populations present are difficult to detect using cultural methods because they may be masked on the plates (Cocolin et al., 2013). Selective cultivation and isolation of microorganisms from natural samples should be avoided, because traditional culture-dependent methods are easily biased (Rantsiou et al., 2005). Fortunately, molecular biology has progressed considerably over the years. Development and employment of many new techniques have allowed identification and enumeration of microorganisms using culture-independent methods. An extra advantage of culture-independent techniques is the potential to detect and quantify the non-*Saccharomyces* populations even at the end of the fermentation (Andorrà et al., 2008, 2010a; Wang et al., 2014) relatively quickly and without the need of previous steps of enrichment in culture media. Previously, non-*Saccharomyces* populations were generally only detected during the first step of fermentation (Ribéreau-Gayon et al., 2006), although detection could be extended through the use of selective media, such as Lysine medium. This medium only allows growth of yeast species able to assimilate lysine as sole nitrogen source (Angelo and Siebert, 1987).

Molecular techniques have demonstrated their usefulness to determine yeast diversity in ecological studies during winemaking. Culture-dependent techniques are efficient for typifying and monitoring of yeast strains throughout the fermentation process, whereas culture-independent techniques enable analysis of total yeast diversity during wine production. Moreover, some studies have reported a good correlation between both techniques (Alessandria et al., 2015; Andorrà et al., 2010a). Therefore, a combined approach could be a good strategy to study microbial ecology, using different molecular tools to infer microbial patterns and pair these with quantifiable functional attributes that affect the fermentation. The current study was conducted during the pre-fermentative CS and fermentative process to enhance the results of previous ecological research carried out using culture-dependent methods (Maturano et al., 2015). In these previous studies, high populations of *H. uvarum* and *C. zemplinina* were detected during CS. However, only *S. cerevisiae* was isolated after inoculation with the commercial yeast. In the present study, two of the most common and accessible culture-independent techniques were used to assess the presence or absence of yeast species. Denaturing Gradient Gel Electrophoresis (DGGE) was used to obtain an overview of the total yeast populations in the sample and Quantitative PCR (qPCR) to monitor the population of the main yeast species, *Saccharomyces*, *Hanseniaspora* and *Starmerella* during the cold soak at different temperatures. Their behavior after inoculation with a commercial *S. cerevisiae* strain was also studied. In order to better understand the dynamics and biodiversity of yeast species during the pre-fermentative stage, classical ecological indices were calculated.

2. Materials and methods

2.1. Reference strains

The yeast species used in this study are commonly isolated from different viticulture regions worldwide (Fleet, 2008; Jolly et al., 2014). Reference strains of yeasts were used as mobility patron in PCR-DGGE and to build the standard curve for qPCR. These yeasts were obtained from CECT (Spanish Type Culture Collection): *Dekkera anomala* CECT1008, *Dekkera bruxellensis* CECT11045, *Hanseniaspora guilliermondii* CECT11027, *Hanseniaspora uvarum* CECT11105, *Issatchenkia terricola* CECT11176, *Saccharomyces cerevisiae* CECT1942, *Schizosaccharomyces pombe* CECT1379, *Torulaspora delbrueckii* CECT1880, *Zygosaccharomyces rouxii* CECT1232, *Zygosaccharomyces bailii* CECT11042. Strains belonging to *Candida zemplinina*, now reclassified as *Starmerella bacillaris* (Duarte et al., 2012), were obtained from the collection of the Biotechnological Research group of the URV and isolated from enological environments. All yeast strains were grown in YEPD (2% glucose, 2% peptone, 1% yeast extract).

2.2. Wine fermentation and sampling

Malbec grapes were harvested in 2012 from vineyards located in Drummond, Lujan de Cuyo (Latitude 33° S, Longitude 68° 51′ W, at an altitude of 912 m), Mendoza, Argentina. Winemaking was conducted at the experimental winery of the Wine Research Centre at the National Institute of Agricultural Technology (INTA) in Mendoza. Mature and healthy grapes were crushed and destemmed, followed by the addition of 50 mg/L SO_2. Fresh grape juice contained 220 g/L reducing sugars, a density of 1100 g/L, and displayed a titratable tartaric acid acidity of 5.25 g/L and a pH of 3.6. Grape must was distributed into 100-L stainless steel tanks.

Pre-fermentative cold soaks were carried out at three different assay temperatures for 7 consecutive days: at 12 ± 1 °C, 8 ± 1 °C and 2.5 ± 1 °C (treatment 2, 3 and 4, respectively). Temperature was controlled by placing the tanks in refrigerated chambers. Daily addition of solid CO_2 was necessary to keep the temperature below 4 °C. At the end of each cold soak and prior to inoculation with active dry yeast, musts were warmed up to room temperature (24 °C). Tanks were inoculated with 25 g/hL of commercial active dry Lalvin ICV D254 yeast (Lallemand Inc., Montreal, Canada). This dosage rate was assumed to provide an initial cell population of approximately 5×10^6 viable cells/mL. Alcoholic fermentation was performed at controlled temperature (24 ± 2 °C). A control (treatment 1) was included and consisted of inoculation of fresh must with the same commercial *S. cerevisiae* strain, and with simultaneous maceration and alcoholic fermentation at 24 ± 2 °C. The temperature was monitored during pre-fermentative and fermentative phases with an iButton® temperature data logger (Maxim Integrated, San Jose CA, United States) placed inside each tank.

Must and wine samples were taken from each treatment during the following stages: fresh must upon crushing (GJ), pre-fermentative cold soak after 2 (D2), 5 (D5) and 7 days (D7), and at the beginning (BF), in the middle (MF: density 1050–1040 g/L) and at the end of the alcoholic fermentation (FF: density 995–990 g/L). All samples were immediately submitted to microbial analysis using culture-dependent techniques; 1 mL samples for culture-independent analysis were washed with distilled sterile water to remove PCR inhibitors, and the cell pellet was kept at −20 °C until DNA extraction and analysis.

2.3. Culture-dependent techniques

2.3.1. Yeast count and isolation

Decimal dilutions (0.1 mL) were plated onto Wallerstein Laboratory (WL) Nutrient Agar medium (Oxoid, Hampshire, UK), supplemented with 0.2 g/L dichloran (Fluka A.G., St. Gallen, Switzerland) and 0.5 g/L chloramphenicol (Sigma Aldrich, Saint Louis MO, United States) to inhibit molds and bacteria, respectively. Petri dishes were incubated at 28 °C for 48–72 h. Colonies were counted (total viable yeasts) and examined daily until they were large enough to allow discrimination between the different colony types according to Pallmann et al. (2001). An average of 30 colonies was isolated from each sample. Isolates were purified by streak plating, sub-cultured on Malt Extract Agar (MEA) and incubated at 28 °C for 48–72 h for subsequent identification.

2.3.2. Molecular identification of yeasts

Yeast colonies were identified after DNA extraction and D1-D2 sequencing analysis according to Maturano et al. (2015). The BLAST search (Basic Local Alignment Search Tool, http://blast.ncbi.nlm.nih.gov) was used to compare the sequences obtained with databases of the National Center for Biotechnology Information (NCBI). Identification was considered correct when gene sequences showed ≥99% identity. Implantation of strain *S. cerevisiae* Lalvin D254 was assayed at the end of the alcoholic fermentation. Yeast isolates previously identified as *S. cerevisiae* in samples taken at the end of the alcoholic fermentation were submitted to interdelta PCR analysis for intraspecific differentiation (Legras and Karst, 2003).

2.4. Culture-independent techniques

2.4.1. DNA extraction

DNA from all pre-fermentation and fermentation samples (1 mL) and fresh cultures of the reference strains was isolated as described by Hierro et al. (2006) using the DNeasy Plant mini kit (Qiagen, Valencia, California). The same DNA extraction was used for both PCR techniques.

2.4.2. PCR-DGGE analysis

Amplification of the specific ribosomal region of yeasts using the primer pair U1GC-U2 and DGGE electrophoresis were carried out according to Andorrà et al. (2008). All PCR amplifications were performed in a GeneAmp PCR System 2720 (Applied Biosystems, Fosters City, USA), using EcoTaq DNA Polymerase (Ecogen, Spain) and DGGE electrophoresis was carried out using a Dcode universal mutation detection system (Bio-Rad, Hercules, California). The DNA fragments from the PCR-DGGE gels were excised from the acrylamide electrophoresis gel and transferred into 50 μL of sterile water to allow diffusion during 12 h at 4 °C. One microlitre of this solution was used for re-amplification with primers without the GC clamp. PCR products were purified and sequenced by Macrogen Inc. facilities (Seoul, South Korea) using an ABI3730 XL automatic DNA sequencer. The BLAST search (Basic Alignment Search Tool, Internet address: http://www.ebi.ac.uk/Tools/sss/ncbiblast/nucleotide.html) was used to compare the sequences obtained in the present study with those published in databases of the European Molecular Biology Laboratory (EMBL). Identification was considered correct when gene sequences showed ≥99% identity.

2.4.3. Quantitative PCR analysis

In all cases qPCR was performed on an Applied Biosystems 7300 Fast Real-Time PCR System (Applied Biosystems) using 5 μL of DNA solution and Power Syber Green master mix according to the manufacturer's instructions (Applied Biosystems, California). The instrument automatically determined Ct (Cycle threshold) values. Samples and cultures for standard curves were analyzed in triplicate.

Quantification of *Saccharomyces cerevisiae, Starmerella bacillaris* species and *Hanseniaspora* genus was obtained using the primers CESP-F/SCER-R, AF/200R and CESP-F/HUV-R, respectively, under conditions described by Andorrà et al. (2010a).

To determine the sensitivity and detection limits of the qPCR, yeast cultures at a concentration of 10^7 CFU/mL were serially diluted 10-fold. For each yeast concentration DNA extraction was done, and used to construct a standard curve. The assay was linear over 5 orders of magnitude, and the detection limit was approximately 10^2 CFU/mL. Standard curves were created by plotting the cycle threshold (CT) values of the qPCR performed against the log input cells per mL. The yeast strains, cited previously, *H. uvarum*, *Starm. bacillaris* and *S. cerevisiae* were used to achieve their specific standard curves. The following values for R^2 (0.995 ± 0.01, 0.985 ± 0.04 and 0.98 ± 0.03), Slope (−3.73 ± 0.02, −3.16 ± 0.04 and −2.61 ± 0.06) and Intercept (38.85 ± 0.25, 38.35 ± 0.12 and 33.67 ± 0.09) were obtained for the three yeast strains used (*H. uvarum*, *Starm. bacillaris* and *S. cerevisiae*) respectively.

2.5. Biodiversity analysis

The following two ecology indices were used to evaluate the biodiversity (H') and the dominance (D) of the yeast species found during the pre-fermentative cold soak treatments assayed (Cordero-Bueso et al., 2011):

1) Shannon–Wiener index (H') to obtain the general biodiversity:

$$H' = -\sum S \; pi \log_n (pi).$$

where S is the number of species and pi is the proportion of colonies of the sample belonging to the species.

2) Simpson's index (D), which gives more weight to dominant species:

$$D = \sum S \; (pi)^2.$$

where S is the number of species and pi is the proportion of colonies of the sample belonging to the species.

2.6. Statistical analyses

Because of the large volume of the fermentation tanks and the limitation of the installations of the Wine Research Centre to conduct CS and wine fermentations (100 L), single fermentations were carried out. Each analysis was performed independently and the results represent the mean of three determinations with the corresponding standard deviation (±SD). Experimental data obtained during fermentations were analyzed by repeated measures analysis of variance (ANOVA), using IBM SPSS software (version 19.0, Chicago, United States).

3. Results

Samples obtained from the control treatment (T1), the three pre-fermentative cold soak (CS) treatments (T2: 12 °C, T3: 8 °C and T4: 2.5 °C) and alcoholic fermentation were analyzed in the present study. Microbial analysis was carried out using culture-dependent and two culture-independent techniques: DGGE (to detect yeast species) and qPCR (to quantify and monitor the main yeast species during winemaking).

3.1. Culture-dependent techniques

3.1.1. Yeast species identification

Results of percentages of total population and counting of yeasts isolated on plates are detailed on Table 1. The total number of yeast colonies isolated was 570; 210 colonies were isolated from grape juice (GJ) and during pre-fermentative treatments (CS) and 360 were isolated during fermentation (120 for each stage of the fermentation). GJ samples were taken from each tank before must distribution; the total yeast population at this moment was about 3.3 log CFU/mL, with non-*Saccharomyces* species such as *Starm. bacillaris, P. occidentalis* and *H. uvarum* found at high numbers (25%, 23.5% and 9.5%, respectively), while *Pichia kudriavzevii* was found at a low proportion (3%). Unexpectedly, *S. cerevisiae* was detected at a high proportion in GJ (39%). *H. uvarum*, *Starm. bacillaris*, *P. occidentalis* and *S. cerevisiae* were the main species found among the yeasts isolated during pre-fermentative cold soak (Table 1). *S. cerevisiae* was detected in all samples and at all temperatures assayed, even during CS and alcoholic fermentation. Other yeast species including *H. guilliermondii*, *M. pulcherrima*, *P. kluyveri*, *P. kudriavzevii* and *Wickerhamomyces anomalus* were also detected in some of the samples analyzed (Table 1). However, none of these non-*Saccharomyces* yeast species were isolated after inoculation of the *S. cerevisiae* starter culture at the end of the pre-fermentative CS. *S. cerevisiae* was the only species detected at the beginning, middle and at the end of the alcoholic fermentation (data not shown).

3.1.2. Evaluation of starter implantation

Because of the high proportion of native *S. cerevisiae* recorded at the end of cold soak treatments, intraspecific analysis of *S. cerevisiae* was carried out in order to assess implantation of the commercial yeast at the end of the alcoholic fermentation. Our results showed an implantation percentage for commercial strain ICV D254 of 82.5% at T2 and 100% at T3 and T4. At the end of the alcoholic fermentation of the control treatment (without CS), only the unique molecular pattern of the commercial yeast strain was observed.

3.1.3. Biodiversity analysis

Fig. 1 shows a Venn diagram representing 9 yeast species belonging to 6 genera, which were isolated from grape juice and during the

Table 1
Yeast species analyzed using culture-dependent (plating) and culture-independent technique (PCR-DGGE) in the different samples obtained during pre-fermentative cold soak treatments.

YEAST SPECIES	Grape juice		T2 (12 °C)			T3 (8 °C)			T4 (2.5 °C)		
			D2	D5	D7	D2	D5	D7	D2	D5	D7
Starmerella bacillaris	%	25	46.7		10	56.2	50	23.5	28	27	27
	Count	0.83	1.76		0.63	1.58	1.6	0.64	0.84	0.75	0.76
	DGGE					X	X	X			
Hanseniaspora uvarum	%	9.5	9.6	86.3	50	9.4		3.9	14.3	9.9	12.5
	Count	0.9	0.25	4.03	3.15	0.26		0.1	0.43	0.28	0.35
	DGGE	X	X	X	X	X	X			X	X
Hanseniaspora guilliermondii	%			7.7					20.4		
	Count			0.35					0.61		
	DGGE										
Wickerhamomyces anomalus	%			1.5		7.8					
	Count			0.07		0.22					
	DGGE										
Pichia kluyveri	%		3.3	1.1					9.2		
	Count		0.13	0.05					0.28		
	DGGE										
Pichia kudriavzevii	%	3					3.9			13.1	
	Count	0.31					0.12			0.36	
	DGGE										
Pichia occidentalis	%	23.5	13.4		5	12.5	3.8	17.6	4.1	9.8	9.5
	Count	0.78	0.50		0.32	0.35	0.13	0.78	0.12	0.27	0.26
	DGGE										
Metschnikowia pulcherrima	%							4			
	Count							0.11			
	DGGE										
Saccharomyces cerevisiae	%	39	30	3.5	35	14.1	42.3	31	24.1	42.3	51
	Count	0.48	1.13	0.16	2.2	0.4	1.35	1.38	0.72	1.17	1.44
	DGGE	X	X	X	X	X	X	X	X	X	X

T2: cold soak at 12 ± 1 °C; T3: cold soak at 8 ± 1 °C; T4: cold soak at 2.5 ± 1 °C. D2: Day 2, D5: Day 5 and D7: Day 7 of pre-fermentative cold soak. %: species percentage of total population; Count: (Log CFU/mL).

different pre-fermentative CS treatments assayed. All species found in grape must were also detected during CS carried out at different temperatures (*S. cerevisiae*, *H. uvarum*, *Starm. bacillaris* and *P. occidentalis*).

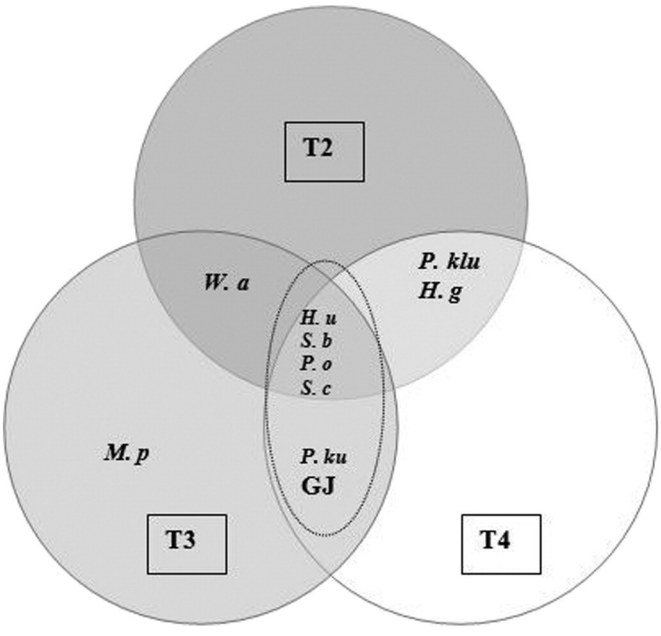

Fig. 1. Yeast species identified using culture-dependent methods during pre-fermentative cold soak treatments. Data of each treatment are represented by Venn diagrams. Species found in Grape Juice are surrounded by a vertical ellipse with dotted line. GJ: Grape Juice, T2: cold soak at 12 ± 1 °C, T3: cold soak at 8 ± 1 °C, T4: cold soak at 2.5 ± 1 °C. *S.b.: Starmerella bacillaris, H.u.: Hanseniaspora uvarum, H.g.: Hanseniaspora guilliermondii, M.p.: Metschnikowia pulcherrima, W.a.: Wickerhamomyces anomalus, P.klu.: Pichia kluyveri, P.ku.: Pichia kudriavzevii, P.o.: Pichia occidentalis, S.c.: Saccharomyces cerevisiae.*

An exception was *P. kudriavzevii*, which was not detected at 12 °C. Two other species, *P. kluyveri* and *H. guilliermondii*, were only isolated at 12 °C and 2.5 °C, but not at 8 °C. Similarly, *W. anomalus* was only isolated at 12 °C and 8 °C CS treatments, while *M. pulcherrima* was only isolated at 8 °C (Fig. 1).

The Shannon diversity (H´) and Simpson dominance (D) indices during the 3 CS conditions assayed are shown in Fig. 2a and b, respectively. At the beginning of the pre-fermentative CS, all treatments registered the highest microbial diversity values (H′), which were 1.7 for T4 and 1.3 for T2 and T3, but the lowest dominance values (D): 0.21 for T4, 0.33 for T2 and 0.37 for T3. Remarkably, cold soak treatments carried out at 2.5 °C showed the highest Shannon indices, but this value decreased during the CS treatment, reaching values similar to the other two temperatures assayed. All treatments presented a time effect for the diversity index (Shannon-H´) between the initial and final maceration period (Fig. 2a). Percentages of the dominant species are given in fig. 2b. Although differences were observed among the maceration temperatures assayed, *H. uvarum, Starm. bacillaris* and *S. cerevisiae* were the main species isolated in all samples assayed.

In general, the dominance indices registered values between 0.21 and 0.43, with the exception of day 5 during treatment T2, with a D index of 0.75 (Fig. 2b). On day 5, T2 demonstrated a strong presence of *H. uvarum*, which was also detected at the end of the CS, representing half of the total number of species identified. The situation for T3 was different; at this temperature (8 °C) the main yeast species isolated on day 2 (*Starm. bacillaris*) was also recovered on day 5, but at concentrations similar to *S. cerevisiae*, the dominant species at the end of the CS. T4, the lowest temperature assayed (2.5 °C), showed an increasing D value vs time. On day 2, all three yeast species were isolated at similar levels, but after three days of CS, *H. uvarum* was not detected whereas the *S. cerevisiae* population increased at the end of the CS. At this temperature, the *Starm. bacillaris* population remained constant during the CS stage (Fig. 2b).

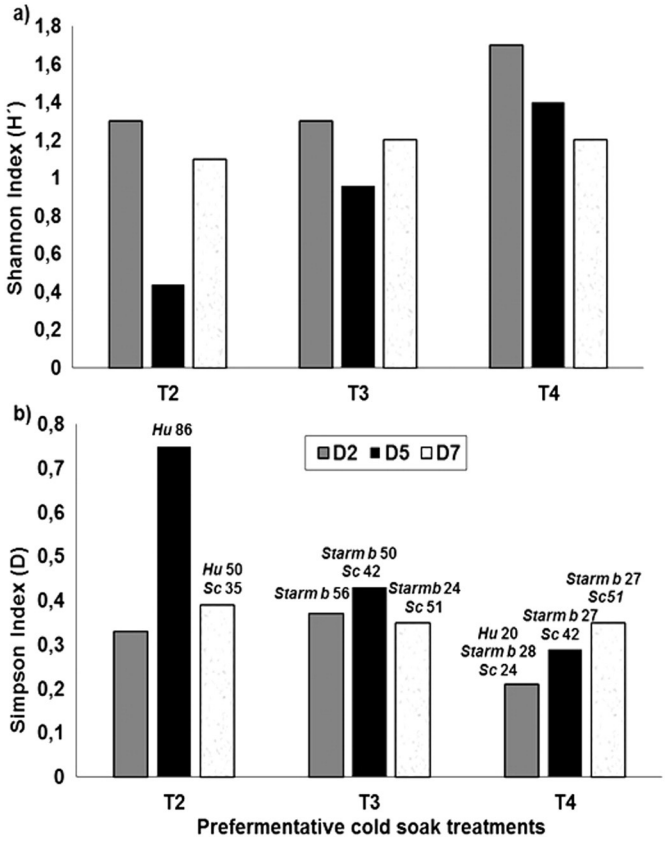

Fig. 2. Main biodiversity indices during pre-fermentative cold soak treatments (T2: 12 ± 1 °C, T3: 8 ± 1 °C and T4: 2.5 ± 1 °C). a) Shannon Index (H′) and b) Simpson index (D). Percentage of the main yeast species is included at the top of each bar. Sc: *Saccharomyces cerevisiae*, Hu: *Hanseniaspora uvarum*, Starm.b: *Starmerella bacillaris*. D2: Day 2, D5: Day 5 and D7: Day 7 of pre-fermentative cold soak.

3.2. Culture-independent techniques

3.2.1. DGGE analysis

Culture-independent technique PCR-DGGE allowed detection of yeast-like fungi, such as *Aureobasidium pullulans* and *Galactomyces geotrichum*, and the filamentous fungus *Aspergillus niger* in grape juice. Only three yeast species, *H. uvarum*, *Starm. bacillaris* and *S. cerevisiae*, were detected by PCR-DGGE (Table 1). *H. uvarum* and *S. cerevisiae* were detected in most samples during CS treatments and in grape juice (Table 1). *Starm. bacillaris* was only in evidence at 8 °C, and detected until the end of the CS treatment, displacing *H. uvarum*. At 12 °C and 2.5 °C, *H. uvarum* was the only non-*Saccharomyces* yeast found together with *S. cerevisiae* (Table 1). Surprisingly, after inoculation *S. cerevisiae* was the only species detected by PCR-DGGE during the alcoholic fermentation. This technique allowed detection of *S. cerevisiae* throughout the three cold soak processes assayed and during the alcoholic fermentation. *H. uvarum* was not detected on the second day of CS at 2.5 °C, and thus *S. cerevisiae* was the only yeast species detected (Table 1).

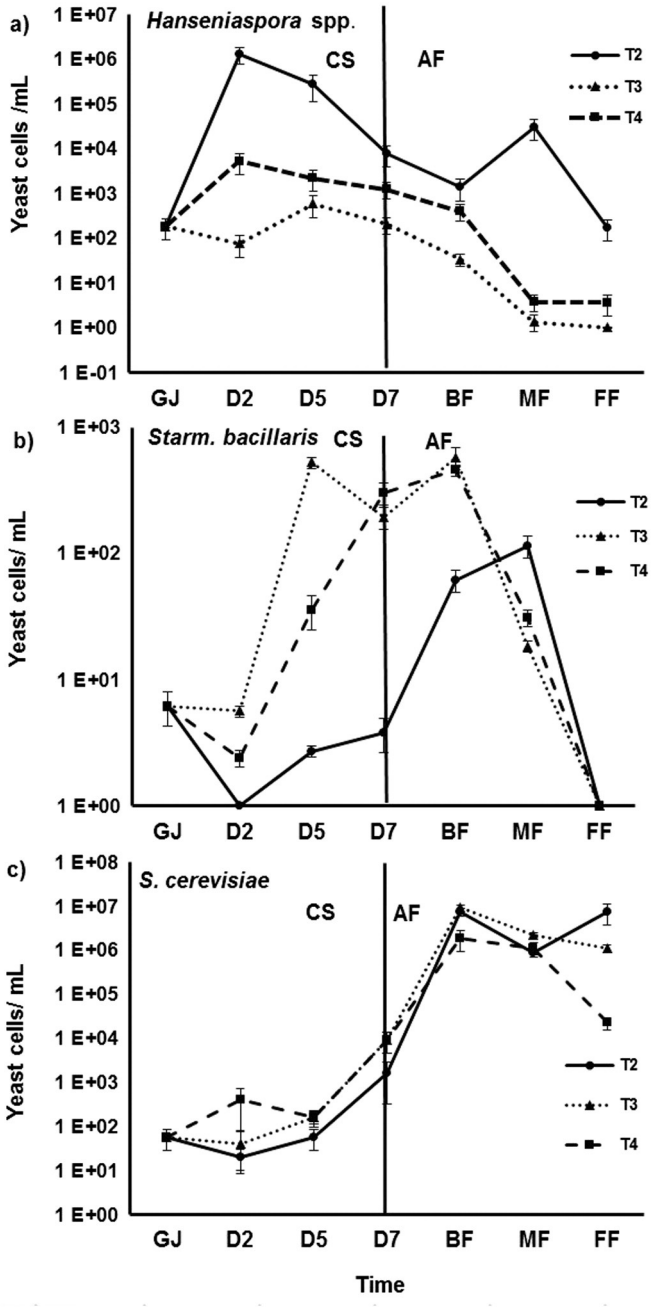

Fig. 3. Evolution of the yeast population measured with qPCR, using specific primers for each yeast species. a) *Hanseniaspora*, b) *Starmerella bacillaris*, and c) *Saccharomyces cerevisiae*. CS: cold soak, AF: Alcoholic Fermentation; T2: cold soak at 12 ± 1 °C; T3: cold soak at 8 ± 1 °C; T4: cold soak at 2.5 ± 1 °C. GJ: Grape Juice, D2: Day 2, D5: Day 5 and D7: Day 7 of pre-fermentative cold soak. BF: Beginning of Fermentation, MF: Middle or halfway Fermentation, FF: Final Fermentation.

3.2.2. Quantitative analysis of the main yeast species using qPCR

The second culture-independent technique, qPCR, was applied to quantify the most representative species found with PCR-DGGE and plate count methods (Table 1, Fig. 3). This technique revealed the evolution of the main yeast species detected: *Starm. bacillaris*, *Hanseniaspora* spp. and *S. cerevisiae*. In general, and at all temperatures during pre-fermentative CS, the same tendency was observed: an increase in non-*Saccharomyces* populations at the beginning of the CS, whereas *S. cerevisiae* levels remained similar. However, at the end of the CS at all temperatures the *S. cerevisiae* population increased, thus becoming the main yeast species detected during the alcoholic fermentation. A higher CS temperature (12 °C) allowed better growth of *Hanseniaspora* spp. than at other CS temperatures, reaching about 10^6 cells/mL. However, after the pre-fermentative CS period all tanks showed a similar *Hanseniaspora* spp. population between 10^2–10^3 cells/mL (an exception was MF in T2). This genus grew better at 2.5 °C than at 8 °C. After inoculation (start of the alcoholic fermentation), the reduction in the yeast population was more evident in treatments at lower CS temperature. In the case of CS conducted at 12 °C, *Hanseniaspora* spp. increased, resulting in a higher presence of this yeast genus at the end of the fermentation; nearly 10^2 cells/mL (Fig. 3a).

Monitoring of *Starm. bacillaris* revealed a different behavior. The highest population for this yeast was found at 8 °C; at 2.5 °C the population reached a similar number, but two days later. This yeast species seems to grow better at a lower CS temperature (Fig. 3b). In pre-fermentative CS at 12 °C the population of this yeast reached values near the detection limit throughout the pre-fermentative period. After inoculation of *S. cerevisiae*, the *Starm. bacillaris* population slightly increased in all treatments, but halfway the fermentation process, the populations decreased in treatments at lower CS temperature. This reduction was postponed in treatment 2. The species was not detected in any of the treatments at the end of the fermentation (Fig. 3b).

Saccharomyces cerevisiae was detected under all CS conditions at a concentration of 10^2 to 10^3 cells/mL and of course at much higher populations immediately after inoculation of the starter culture during the alcoholic fermentation. Although the differences in population of this yeast were not statistically significant ($p = 0.11$), lower temperatures noticeably presented higher populations during CS. After inoculation of the commercial *S. cerevisiae* strain, the *Saccharomyces* population increased during all treatments (Fig. 3c). These levels were maintained practically until the end of the fermentation; CS carried out at 2.5 °C showed a greater decrease than the two higher temperatures.

4. Discussion

Temperature is one of the main factors that exerts a selective pressure on the biodiversity and evolution of yeast species during fermentation (Fleet, 2003). Our results demonstrate that the pre-fermentative CS carried out at 2.5 °C favored a more uniform interspecific distribution compared with treatments at 8 °C and 12 °C. This is reflected by the ($H´$) biodiversity indices obtained from the results of the culture-dependent technique. Furthermore, it was observed that the populations of the most representative species remained relatively constant during CS. In contrast, pre-fermentative CS carried out at higher temperature favored dominance (D) of *H. uvarum* (12 °C) and *Starm. bacillaris* (8 °C) throughout the maceration period. Zott et al. (2008) identified non-*Saccharomyces* yeasts during pre-fermentative CS at 4, 10 and 15 °C in Merlot must from the Bordeaux region, France. In line with our results, the authors found that *H. uvarum* was the most abundant species during CS at 15 °C, whereas the other main non-*Saccharomyces* species, *Starm. bacillaris,* was favored by lower temperatures. Similar results were obtained by Andorrà et al. (2010a) during wine fermentation. Besides, the remarkable presence of *S. cerevisiae* in grape must and throughout the pre-fermentative CS should be highlighted. Presence of this "non-habitual" yeast species in must could mask the population of other non-*Saccharomyces* wine yeasts. *S. cerevisiae* was probably incorporated to the grape must during cellar manipulation of the grape juice, because it is rarely isolated from healthy grapes (Mercado et al., 2007). However, in recent years, several authors have reported significant population levels of *Saccharomyces* species, both commercial and indigenous, on grapes and during the first step of winemaking without any apparent relationship to machinery or cellar equipment (Bezerra-Bussoli et al., 2013; Capece et al., 2012; Ortiz et al., 2013).

Generally, with the use of culture-dependent techniques it was assumed that non-*Saccharomyces* yeasts were only present during the first vinification stages (Heard and Fleet, 1985). However, this assumption changed with the development of culture-independent techniques to monitor the wine yeast population (Andorra et al., 2008, 2010a; Zott et al., 2010).

Culture-independent techniques have been widely applied to wine research, using PCR-DGGE, qPCR and high-throughput sequencing technologies such as 454 pyrosequencing of amplicons, Ion Torrent and Illumina (Bokulich and Mills, 2013; David et al., 2014; Ercolini, 2004, 2013; Wang et al., 2015b). At present, all these techniques are well known, and the results obtained with each technique are well understood. For example, the most well-known biases of PCR-DGGE are its low detection limit; it is impossible to detect cells with at least two orders of magnitude lower than the principal yeast species (Andorrà et al., 2008); and the preferential amplification of the primers for some sequences (Wang et al., 2015a). In the present study, some quantitatively minor populations of the culturable community such as certain *Candida*, *Metschnikowia* and *Pichia* species did not appear on DGGE gels, whereas these species were detected by plating. Therefore, PCR-DGGE is ideal for detecting species diversity in a mixed population with similar relative proportions, but the massive presence of one species decreases the chances of detecting minor species (Andorrà et al., 2008). In the present study, presence of filamentous fungi and yeast-like fungi that can be amplified with the primers used can lower the yeast detection limit. This fact was previously observed by Andorrà et al. (2010a), but in both studies detection of filamentous fungi or yeast-like fungi only occurred in must or during the first fermentation step; the microorganisms were not detected with DGGE once the fermentation had started. As previously mentioned by Andorrà et al. (2010a), red wine maceration with grape skins is a complex matrix, and the presence of these "contaminant" microorganisms is higher than in white wine. On the other hand, not all species are amplified with PCR-DGGE primers equally efficiently. Mills et al. (2002) reported that *M. pulcherrima* exhibited poor PCR efficiency with the NL1-LS2 primer set. This may be a possible explanation for not detecting *M. pulcherrima* in DGGE gels although this microorganism was detected in the culturable yeast community. Two other species, *H. uvarum* and *H. guilliermondii*, displayed a similar situation. Although both species can be discriminated by PCR-DGGE, the main wine yeast *H. uvarum* was the only one that could be detected. The detection limit depends on the target yeast species. Wang et al. (2015a) showed that when 10^7 cells/mL of *S. cerevisiae* were mixed with 10^5 cells/mL of *Starm. bacillaris*, this latter yeast species could be detected by PCR-DGGE, but when *S. cerevisiae* was mixed with *H. uvarum*, the latter species could not be detected with this technique.

Findings with qPCR were more accurate than those obtained with PCR-DGGE. However, PCR-DGGE is a good and inexpensive technique to study the total population of a sample, although some modifications should be introduced to prevent the differential amplification of the yeast species present in samples as described by Wang et al. (2015a). Comparison of the two quantitative techniques, qPCR and plate count, showed that *H. uvarum*, *Starm. bacillaris* and *S. cerevisiae* populations were significantly higher with qPCR during the CS period in wines assayed at lower temperatures (8 °C and 2.5 °C). This could indicate that the stress effect of the lower temperature induced a viable but non-culturable (VBNC) cellular state of the microorganisms (Salma et al., 2013). Three different population levels among the yeast species studied were observed. *Hanseniaspora* showed the highest population of the non-*Saccharomyces* yeast species during all CS treatments. Highest growth was observed at 12 °C, whereas the lowest population was found at 8 °C. A different pattern was found for *Starmerella*. This yeast species showed a longer lag phase, and the increase in population was highest at 8 ± 1 °C, although it showed a lower concentration than *Hanseniaspora*. Andorrà et al. (2010a) compared two fermentations conducted at 13 and 25 °C and concluded that the fermentation temperature exerted limited influence on yeast populations during wine fermentation. However, from our results it can be inferred that each non-*Saccharomyces* yeast species showed a different behavior during the CS treatment, which was temperature-dependent. It has been suggested that non-*Saccharomyces* yeasts grow better at lower temperatures (Heard and Fleet, 1988; Sharf and Margalith, 1983; Sipiczki, 2003; Tofalo et al., 2012). The use of a culture-independent technique in the present study showed a good relationship between the temperature of the pre-fermentative CS period and survival of the yeast species during alcoholic fermentation. Indigenous non-*Saccharomyces* yeasts, already present in the grape must, and often at high proportions, become acclimatized to the specific environment (pre-fermentative CS stage), which gives them a competitive edge that allows their presence and permanence during alcoholic fermentation.

The three techniques employed in this study corroborate observations that *S. cerevisiae* was present in grape must, throughout the pre-fermentative CS and during alcoholic fermentation. Despite high populations of native *Saccharomyces*, the population of commercial *S. cerevisiae* was high. However, it is important to highlight that according to the qPCR results, non-*Saccharomyces* populations were detected halfway (*Starm. bacillaris*) and at the end (*Hanseniaspora*) of the fermentation period, although 2–4 orders of magnitude lower than *Saccharomyces*. It could be inferred that all treatments showed co-participation of both *Saccharomyces* and non-*Saccharomyces* yeasts throughout the winemaking process. Presence of non-*Saccharomyces* populations during the alcoholic fermentation was only detected with qPCR and in some cases, the population size was quite low, near the detection limit. The use of a very stable molecule such as DNA as template may overestimate cell counts through amplification of DNA from dead cells, but previous studies only detected 1% of dead cells (Hierro et al., 2006, Zott et al., 2010). DNA amplification of dead cells can be avoided by previous addition of some "vital dyes" to the DNA extract (Andorrà et al., 2010b).

As mentioned previously, yeast populations were elevated during alcoholic fermentation, and therefore it was necessary to carry out serial dilutions for plate counting. This may have caused loss of information about non-*Saccharomyces* populations present during this stage and, therefore, the non-*Saccharomyces* population can be underestimated using this technique.

Analysis of the data leads to the conclusion that the combined use of culture-dependent and independent techniques enabled a comprehensive study of microbial ecology present throughout the winemaking process. PCR-DGGE allowed detection of the most representative species such as *H. uvarum*, *Starm. bacillaris* and *S. cerevisiae*. These species were also the most important after plate culturing, but during the pre-fermentative CS other non-*Saccharomyces* species were detected, although at minor proportions. The use of qPCR, a culture-independent technique, showed a higher presence and permanence of non-*Saccharomyces* species and their contribution was not only limited to the pre-fermentative stage, as observed with PCR-DGGE and plate counts.

The optimal temperature for non-*Saccharomyces* yeasts was also important: *Hanseniaspora* performed and acclimatized best at 12 °C and *Starm. bacillaris* grew better at lower temperature (8 and 2.5 °C). The pre-fermentative step (cold soak) could be considered helpful to a better adaptation of non-*Saccharomyces* yeast populations to alcoholic fermentation.

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References

Alessandria, V., Marengo, F., Englezos, V., Gerbi, V., Rantsiou, K., Cocolin, L., 2015. Mycobiota of barbera grapes from the piedmont region from a single vintage year. Am. J. Enol. Vitic. 66, 244–250.

Andorrà, I., Landi, S., Mas, A., Guillamón, J.M., Esteve-Zarzoso, B., 2008. Effect of oenological practices on microbial populations using culture-independent techniques. Food Microbiol. 25, 849–856.

Andorrà, I., Landi, S., Mas, A., Esteve-Zarzoso, B., Guillamón, J.M., 2010a. Effect of fermentation temperature on microbial population evolution using culture-independent and dependent techniques. Food Res. Int. 43, 773–779.

Andorrà, I., Esteve-Zarzoso, B., Guillamón, J.M., Mas, A., 2010b. Determination of viable wine yeast using DNA binding dyes and quantitative PCR. Int. J. Food Microbiol. 144, 257–262.

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

Bezerra-Bussoli, C., Baffi, M.A., Gomes, E., Da-Silva, R., 2013. Yeast diversity isolated from grape musts during spontaneous fermentation from a Brazilian winery. Curr. Microbiol. 67 (3), 356–361.

Bokulich, N.A., Mills, D.A., 2013. Improved selection of internal transcribed spacer-specific primers enables quantitative, ultra-high-throughput profiling of fungal communities. Appl. Environ. Microbiol. 79 (8), 2519–2526.

Capece, A., Romaniello, R., Siesto, G., Romano, P., 2012. Diversity of *Saccharomyces cerevisiae* yeasts associated to spontaneously fermenting grapes from an Italian "heroic vine-growing area". Food Microbiol. 31 (2), 159–166.

Casassa, L.F., Bolcato, E.A., Sari, S.E., 2015. Chemical, chromatic, and sensory attributes of 6 red wines produced with prefermentative cold soak. Food Chem. 174, 110–118.

Clavijo, A., Calderon, I.L., Paneque, P., 2010. Diversity of *Saccharomyces* and non-*Saccharomyces* yeasts in three red grape varieties cultured in the Serrania de Ronda (Spain) vine-growing region. Int. J. Food Microbiol. 143, 241–245.

Cocolin, L., Alessandria, V., Dolci, P., Gorra, R., Rantsiou, K., 2013. Culture independent methods to assess the diversity and dynamics of microbiota during food fermentation. Int. J. Food Microbiol. 167 (1), 29–43.

Cordero-Bueso, G., Arroyo, T., Serrano, A., Tello, J., Aporta, I., Vélez, M., Valero, E., 2011. Influence of the farming system and vine variety on yeast communities associated with grape berries. Int. J. Food Microbiol. 145 (1), 132–139.

David, V., Terrat, S., Herzine, K., Claisse, O., Rousseaux, S., Tourdot-Maréchal, R., Alexandre, H., 2014. High-throughput sequencing of amplicons for monitoring yeast biodiversity in must and during alcoholic fermentation. J. Ind. Microbiol. Biotechnol. 41 (5), 811–821.

Duarte, F.L., Pimentel, N.H., Teixeira, A., Fonseca, Á., 2012. *Saccharomyces bacillaris* is not a synonym of *Candida stellata*: reinstatement as *Starmerella bacillaris* comb nov. Antonie Van Leeuwenhoek 102, 653–658.

Ercolini, D., 2004. PCR-DGGE fingerprinting: novel strategies for detection of microbes in food. J. Microbiol. Methods 56 (3), 297–314.

Ercolini, D., 2013. High-throughput sequencing and metagenomics: moving forward in the culture-independent analysis of food microbial ecology. Appl. Environ. Microbiol. 79 (10), 3148–3155.

Fleet, G.H., 2003. Yeast interactions and wine flavour. Int. J. Food Microbiol. 86 (1), 11–22.

Fleet, G.H., 2008. Wine yeast for the future. FEMS Yeast Res. 8, 979–995.

Heard, G.M., Fleet, G.H., 1985. Growth of natural yeast flora during the fermentation of inoculated wines. Appl. Environ. Microbiol. 50 (3), 727–728.

Heard, G.M., Fleet, G.H., 1988. The effects of temperature and pH on the growth of yeast species during the fermentation of grape juice. J. Appl. Bacteriol. 65, 23–28.

Hierro, N., Gonzalez, A., Mas, A., Guillamón, J.M., 2006. Diversity and evolution of non-*Saccharomyces* yeast populations during wine fermentations: effect of grape ripeness and cold maceration. FEMS Yeast Res. 6, 102–111.

Jolly, N.P., Varela, C., Pretorius, I.S., 2014. Not your ordinary yeast: non-Saccharomyces yeasts in wine production uncovered. FEMS Yeast Res. 14 (2), 215–237.

Legras, J.L., Karst, F., 2003. Optimisation of interdelta analysis for *Saccharomyces cerevisiae* strain characterisation. FEMS Microbiol. Lett. 221 (2), 249–255.

Malfeito-Ferreira, M., 2011. Yeasts and wine off-flavours: a technological perspective. Ann. Microbiol. 61 (1), 95–102.

Maturano, Y.P., Mestre, M.V., Esteve-Zarzoso, B., Nally, M.C., Lerena, M.C., Toro, M.E., Vazquez, F., Combina, M., 2015. Yeast population dynamics during prefermentative cold soak of Cabernet Sauvignon and Malbec wines. Int. J. Food Microbiol. 199, 23–32.

Mercado, L., Dalcero, A., Masuelli, R., Combina, M., 2007. Diversity of *Saccharomyces* strains on grapes and winery surfaces: analysis of their contribution to fermentative flora of Malbec wine from Mendoza (Argentina) during two consecutive years. Food Microbiol. 24 (4), 403–412.

Mills, D.A., Johannsen, E.A., Cocolin, L., 2002. Yeast diversity and persistence in botrytis-affected wine fermentations. Appl. Environ. Microbiol. 68 (10), 4884–4893.

Ortiz, M.J., Barrajón, N., Baffi, M.A., Arévalo-Villena, M., Briones, A., 2013. Spontaneous must fermentation: identification and biotechnological properties of wine yeasts. LWT- Food Sci. Technol. 50 (2), 371–377.

Pallmann, C.L., Brown, J.A., Olineka, T.L., Cocolin, L., Mills, D.A., Bisson, L.F., 2001. Use of WL medium to profile native flora fermentations. Am. J. Enol. Vitic. 52 (3), 198–203.

Parenti, A., Spugnoli, P., Calamai, L., Ferrari, S., Gori, C., 2004. Effects of cold maceration on red wine quality from Tuscan Sangiovese grape. Eur. Food Res. Technol. 218, 360–366.

Rantsiou, K., Urso, R., Iacumin, L., Cantoni, C., Cattaneo, P., Comi, G., Cocolin, L., 2005. Culture-dependent and-independent methods to investigate the microbial ecology of Italian fermented sausages. Appl. Environ. Microbiol. 71 (4), 1977–1986.

Ribéreau-Gayon, P., Dubordieu, D., Donèche, B., Lonvaud, A., 2006. Citology, Taxonomy and Ecology of Grape and Wine Yeasts. Handbook of Enology. The Microbiology of Wine and Vinifications. second ed. John Wiley and Soons, Chichester.

Salma, M., Rousseaux, S., Sequeira-Le Grand, A., Divol, B., Alexandre, H., 2013. Characterization of the viable but nonculturable (VBNC) state in *Saccharomyces cerevisiae*. PLoS One 8 (10), e77600.

Sharf, R., Margalith, P., 1983. The effect of temperature on spontaneous wine fermentation. Eur. J. Appl. Microbiol. Biotechnol. 17, 311–313.

Sipiczki, M., 2003. *Candida zemplinina* sp. nov., an osmotolerant and psychrotolerant yeast that ferments sweet botrytized wines. Int. J. Syst. Evol. Microbiol. 53 (6), 2079–2083.

Sun, Y., Liu, Y., 2014. Investigating of yeast species in wine fermentation using terminal restriction fragment length polymorphism method. Food Microbiol. 38, 201–207.

Tello, J., Cordero-Bueso, G., Aporta, I., Cabellos, J.M., Arroyo, T., 2012. Genetic diversity in commercial wineries: effects of the farming system and vinification management on wine yeast. J. Appl. Microbiol. 112, 302–315.

Tofalo, R., Schirone, M., Torriani, S., Rantsiou, K., Cocolin, L., Perpetuini, G., Suzzi, G., 2012. Diversity of *Candida zemplinina* strains from grapes and Italian wines. Food Microbiol. 29 (1), 18–26.

Wang, C., Esteve-Zarzoso, B., Mas, A., 2014. Monitoring of *Saccharomyces cerevisiae*, *Hanseniaspora uvarum*, and *Starmarella bacillaris* (synonym *Candida zemplinina*) populations during alcoholic fermentation by flurorescence in situ hybridisation. Int. J. Food Microbiol. 191, 1–9.

Wang, C., Esteve-Zarzoso, B., Cocolin, L., Mas, A., Rantsiou, K., 2015a. Viable and culturable populations of *Saccharomyces cerevisiae*, *Hanseniaspora uvarum* and *Starmerella bacillaris* (synonym *Candida zemplinina*) during Barbera must fermentation. Food Res. Int. 78, 195–200.

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

Zott, K., Miot-Sertier, C., Claisse, L.-F., Masneuf, A., Pomarede, I., 2008. Dynamics and diversity of non-Saccharomyces yeasts during the early stages in winemaking. Int. J. Food Microbiol. 125, 197–203.

Zott, K., Claisse, O., Lucas, P., Coulon, J., Lonvaud-Funel, A., Masneuf-Pomarede, I., 2010. Characterization of the yeast ecosystem in grape must and wine using real-time PCR. Food Microbiol. 27 (5), 559–567.