International Journal of Food Microbiology 199 (2015) 23–32

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Yeast population dynamics during prefermentative cold soak of Cabernet Sauvignon and Malbec wines

Y. Paola Maturano [a,b], M. Victoria Mestre [a], Braulio Esteve-Zarzoso [c], María Cristina Nally [a,b], María Cecilia Lerena [b,d], María Eugenia Toro [a], Fabio Vazquez [a], Mariana Combina [b,d,*]

[a] Instituto de Biotecnología, UNSJ, Av. San Martín 1109 (O), San Juan, Argentina
[b] Consejo Nacional de Investigaciones Científicas y Tecnológicas (CONICET), Argentina
[c] Biotecnologia Enològica, Departament de Bioquímica i Biotecnologia, Facultat d' Enologia, Universitat Rovira i Virgili, Marcel·lı' Domingo s/n, Tarragona 43007, Spain
[d] Estación Experimental Agropecuaria Mendoza, Instituto Nacional de Tecnología Agropecuaria (INTA), Argentina

A R T I C L E   I N F O

Article history:
Received 2 October 2014
Received in revised form 19 December 2014
Accepted 8 January 2015
Available online 13 January 2015

Keywords:
Red wine
Yeast
Low temperature
Prefermentative cold soak
Sensory analysis

A B S T R A C T

Prefermentative cold soak is a widely used technique in red wine production, but the impact on the development of native yeast species is hardly described. The aim of this work was to analyse the dynamics and diversity of yeast populations during prefermentative cold soak in red wines. Three different temperatures (14 ± 1 °C; 8 ± 1 °C and 2.5 ± 1 °C) were used for prefermentative cold soak in Cabernet Sauvignon and Malbec grape musts. *Saccharomyces* and non-*Saccharomyces* populations during cold soak and alcoholic fermentation were analysed. In addition, the impact on chemical and sensory properties of the wines was examined. Yeast dynamics during prefermentative cold soak were temperature dependent. At 14 ± 1 °C, the total yeast population progressively increased throughout the cold soak period. Conversely, at 2.5 ± 1 °C, the yeast populations maintained stable during the same period. Prefermentative cold soak conducted at 14 ± 1 °C favoured development of *Hanseniospora uvarum* and *Candida zemplinina*, whereas cold soak conducted at 8 ± 1 °C favoured growth of *Saccharomyces cerevisiae*. At 2.5 ± 1 °C, no changes in yeast species were recorded. Acidity and bitterness, two sensory descriptors, appear to be related to wines produced with prefermentative cold soak carried out at 14 ± 1 °C. This fact could be associated with the increase in non-*Saccharomyces* during the prefermentation stage. Our results emphasise the importance of the temperature as a determinant factor to allow an increase in non-*Saccharomyces* population during prefermentative cold soak and consequently to modify sensorial attributes of wines as well as their sensorial impact.

© 2015 Elsevier B.V. All rights reserved.

1. Introduction

Wine is the product of a complex process involving yeasts, grape must and winemaking practices. Yeasts are responsible for the alcoholic fermentation of grape juice into wine, producing ethanol, CO_2 and flavour compounds such as esters, higher alcohols, carbonyl compounds and organic acids (Fleet, 2003; Parish and Carroll, 1985). The yeast community present in fresh grape must and during alcoholic fermentation has been described for several wine growing regions in the world. Regardless of the place and grape cultivar, non-*Saccharomyces* yeasts belonging to the genera *Candida, Hanseniaspora, Torulaspora, Pichia, Issatchenkia* and *Metschnikowia* are often present in the grape juice and during the early stages of fermentation (Barata et al., 2012; Combina et al., 2005ab; Ribéreau-Gayon et al., 2006). Nowadays, an ever-increasing competition is forcing winemakers to introduce novel winemaking practices in order to enhance the quality of their products. Different winemaking practices have been widely applied to improve wine quality. One of the most used techniques for red wine production is prefermentative cold soak (Marais, 2003). This is a maceration technique based on the contact of skins, seeds and other fermentation solids with the must in a non-alcoholic setting, in order to favour the extraction of water-soluble compounds such as anthocyanins and aroma precursors (Sacchi et al., 2005). Prefermentative cold soak has been shown to positively contribute to wine colour, taste, and mouthfeel attributes of red wines (Casassa and Harbertson, 2014; Casassa et al., 2015; Marais, 2003).

The maceration technique, particularly when it involves the use of low temperatures, is critical to the quantitative and qualitative microbiological composition of the must during the prefermentative phase (Albertin et al., 2014; Hierro et al., 2006). In the first place, the natural bio-selective effect that ethanol exerts is absent; additionally, low temperatures favour the development of some cryotolerant yeasts that could affect the subsequent alcoholic fermentation performance (Hierro et al., 2006; Mendoza et al., 2009). Low temperatures have proved to modify the competition between *Saccharomyces* and non-

* Corresponding author at: Estación Experimental Agropecuaria Mendoza, INTA San Martín 3853 (5507) Luján de Cuyo, Mendoza, Argentina. Tel.: +54 261 4963020x295.
E-mail addresses: combina.mariana@inta.gob.ar, maricombina@yahoo.com (M. Combina).

http://dx.doi.org/10.1016/j.ijfoodmicro.2015.01.005
0168-1605/© 2015 Elsevier B.V. All rights reserved.

Saccharomyces yeasts, possibly causing starvation of some essential nutrients for *S. cerevisiae* in the medium (Fleet, 2003; Fugelsang and Edwards, 2007).This may be attributed to the glucophilic nature of some non-*Saccharomyces* yeasts (Granchi et al., 2002) or inhibition of *S. cerevisiae* by non-*Saccharomyces* species mediated by the release of acetaldehyde and acetic acid (Mendoza et al., 2007; Mortimer, 2000). All these factors or a combination of them could lead to stuck or sluggish fermentations (Fleet and Heard, 1993).

The microbial community present in grape juice is thought to originate from grapes and cellars (Mercado et al., 2007; Ribéreau-Gayon et al., 2006), but it may be modified and affected by factors such as the temperature and extension of cold soak. As prefermentative cold soak can modify yeast populations, an understanding of the changes in communities during this period is critical because any change can affect the alcoholic fermentation performance and the quality of the wines obtained (Hierro et al., 2006). Therefore, the objective of the present study was to assess the diversity and evolution of yeast populations during prefermentative cold soak conducted at three different temperatures. In addition, the yeast population during alcoholic fermentation was analysed. Furthermore, chemical parameters and sensory properties of wines obtained at different prefermentative cold soak temperatures were examined.

2. Materials and methods

2.1. Wine fermentations and sampling

Grapes from two red cultivars, Cabernet Sauvignon and Malbec, were harvested from vineyards located in Lujan de Cuyo (Latitude 33° 06′ South, Longitude 68° 51′ West), Mendoza, Argentina, during the 2011 season. Winemaking was conducted in 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/LSO_2. Fresh grape juice compositions were 24.5 and 24 Brix, with a titratable acidity of 5.85 g/L and 5.25 g/L of tartaric acid and pH 3.80 and 3.63 for Cabernet Sauvignon and Malbec, respectively.

Grape juices were distributed into 100-L stainless steel tanks. Prefermentative cold soaks were carried out with each grape cultivar at three different temperatures: 14 ± 1 °C; 8 ± 1 °C and 2.5 ± 1 °C, during 7 days. Temperatures were controlled by placing the tanks in refrigerated chambers. Daily addition of solid CO_2 was necessary to maintain 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 by addition of 25 g/hL of commercial active dry yeast, Lalvin D254 (Lallemand Inc., Montreal, Canada). Alcoholic fermentation was performed at controlled temperature (24 ± 2 °C). A control treatment was included for each grape cultivar and consisted of inoculation of fresh musts with *S. cerevisiae* Lalvin D254, with simultaneous maceration and alcoholic fermentation. Temperature was monitored during prefermentative and fermentative phases by *iButton*® temperature data logger (Maxim Integrated, San Jose CA, United States) placed inside each tank.

Must and wine samples were taken from each treatment at different stages: fresh must upon crushing, after 2, 5 and 7 days during prefermentative cold soak, and at the beginning, in the middle (density 1050–1040 g/L) and at the end of the alcoholic fermentation (density 995–990 g/L). All samples were immediately submitted to microbial and chemical analysis. After alcoholic fermentations had finished, the wines were settled and racked. The wines were physically and chemically stabilised, bottled without filtration and stored at 18 °C.

2.2. 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 moulds 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). A proportional and representative number of each colony type was recovered. An average of 20 colonies were isolated from each sample, representing about 150 yeast isolates collected during each cold soak treatment. In total, 612 and 598 colonies were collected from Cabernet Sauvignon and Malbec assays, respectively. 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. Molecular yeast identification

2.3.1. DNA extraction

Yeasts were grown in 10 mL YEPD medium at 25 °C during 24–48 h. Yeast DNA was extracted according to Hoffman and Winston (1987). DNA concentration and quality were determined by electrophoresis on a 0.7% agarose gel (Invitrogen by Life Technology, Carlsbad CA, United States).

2.3.2. Sequencing of the 26S D1/D2 rDNA domain

Partial 26S-rRNA gene sequences (D1/D2 domains) were amplified using NL-1 (5′-GCATATCAATAAGCGGAGGAAAAG-3′) and NL-4 (5′-GGTCCGTGTTTCAAGACGG-3′) primers according to Kurtzman and Robnett (2003). Briefly, amplification was performed using an Eppendorf Mastercycler Gradient (Eppendorf, Hamburg, Germany) with an initial denaturation at 95 °C for 5 min, followed by 40 PCR cycles with denaturation at 94 °C for 1 min, annealing at 55.5 °C for 2 min and extension at 72 °C for 2 min. An additional extension at 72 °C for 10 min was carried out at the end of the 40 cycles. The amplified fragments were purified with a Pure Link PCR purification kit (Invitrogen by Life Technology, Carlsbad CA, United States) according to the manufacturer's instructions. Both strands of the rDNA region were sequenced with the Sanger capillary sequencing method, using a Premix BigDye Terminator v 3.1 Cycle Sequencing kit (Applied Biosystems, Warrington, UK). 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 identities of 99% or higher.

2.3.3. Implantation of commercial Saccharomyces cerevisiae yeast strains

S. cerevisiae Lalvin D254 strain implantation was analysed 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 intraspecific differentiation by interdelta PCR (Legras and Karst, 2003).

2.4. Monitoring and chemical analysis of alcoholic fermentation

The performance of the alcoholic fermentation was monitored by daily measurements of density and temperature. Sugar consumption and volatile acidity were recorded at the microbiological sampling times according to Nazrala et al. (2009). Fermentations were considered completed once reducing sugars were below 1.8 g/L. Ethanol, residual reducing sugars, titratable acidity, volatile acidity and pH of the wines were analysed according to the Official Methods established by the International Organisation of Vine and Wine (O.I.V., Organisation Internationale de la Vigne et du Vin, 2005).

2.5. Sensory analysis

Sensory analysis was carried out about 4 months after bottling by 13 trained panellists from the Sensorial Analysis Group of the Wine Research Centre at INTA. Wines were equilibrated at room temperature (22 °C) before pouring 50-mL samples into ISO 3591 International Standards Organization (I.S.O., International Standards Organization 3591, 1977) wine glasses. All wines were tasted blindly and the sample order was randomly assigned. During the first session, nine sensory properties were retained by consensus among panellists: colour intensity, violet hue, fruity, spicy, vegetal character, bitterness, acidity, astringency and concentration. During the second session, the intensity of each descriptor was measured using a non-structured scale (Reynolds et al., 2001).This procedure was followed for evaluation of both Cabernet Sauvignon and Malbec wines during separate sessions.

2.6. Data analysis

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 analysed by repeated measures analysis of variance (ANOVA), using IBM SPSS software (version 19.0, Chicago, United States). ANOVA was also performed for each of the sensory properties of the experiments. Principal components analysis (PCA) was used to simplify interpretation of the sensorial data and is presented in biplot graphs. This analysis was performed using a professional version of InfoStat software (FCA-UNC, Córdoba, Argentina).

3. Results

3.1. Total yeast counts during prefermentative cold soak and alcoholic fermentation

Total yeast counts, temperature profiles, sugar consumption and volatile acidity during prefermentative cold soak and alcoholic fermentation of Cabernet Sauvignon and Malbec wines are shown in Figs. 1 and 2, respectively. Prefermentative cold soak was carried out at three different temperatures during 7 days (168 h) with Cabernet Sauvignon and Malbec grape cultivars. Average assay temperatures during prefermentative cold soak treatments were 14 ± 1 °C, 8 ± 1 °C and 2.5 ± 1 °C. In control wines (without prefermentative cold soak) grape juices were directly inoculated with active dry yeast at an initial concentration of 2×10^6 cfu/mL observing an early start of alcoholic fermentation (Figs. 1a and 2a). The initial native yeast populations in fresh musts of Cabernet Sauvignon and Malbec were $6.61 \pm 0.33 \times 10^2$ and $2 \pm 0.25 \times 10^3$ cfu/mL, respectively. The effect of the temperature used for prefermentative cold soak on the development of total yeast populations is shown in Figs. 1 and 2. Total yeast counts increased progressively during the prefermentative stage in musts kept at 14 ± 1 °C, and the final population at the end of the cold soak was higher than 10^6 cfu/mL. The treatments demonstrated significant sugar consumption during prefermentative cold soak, which correlated well with the

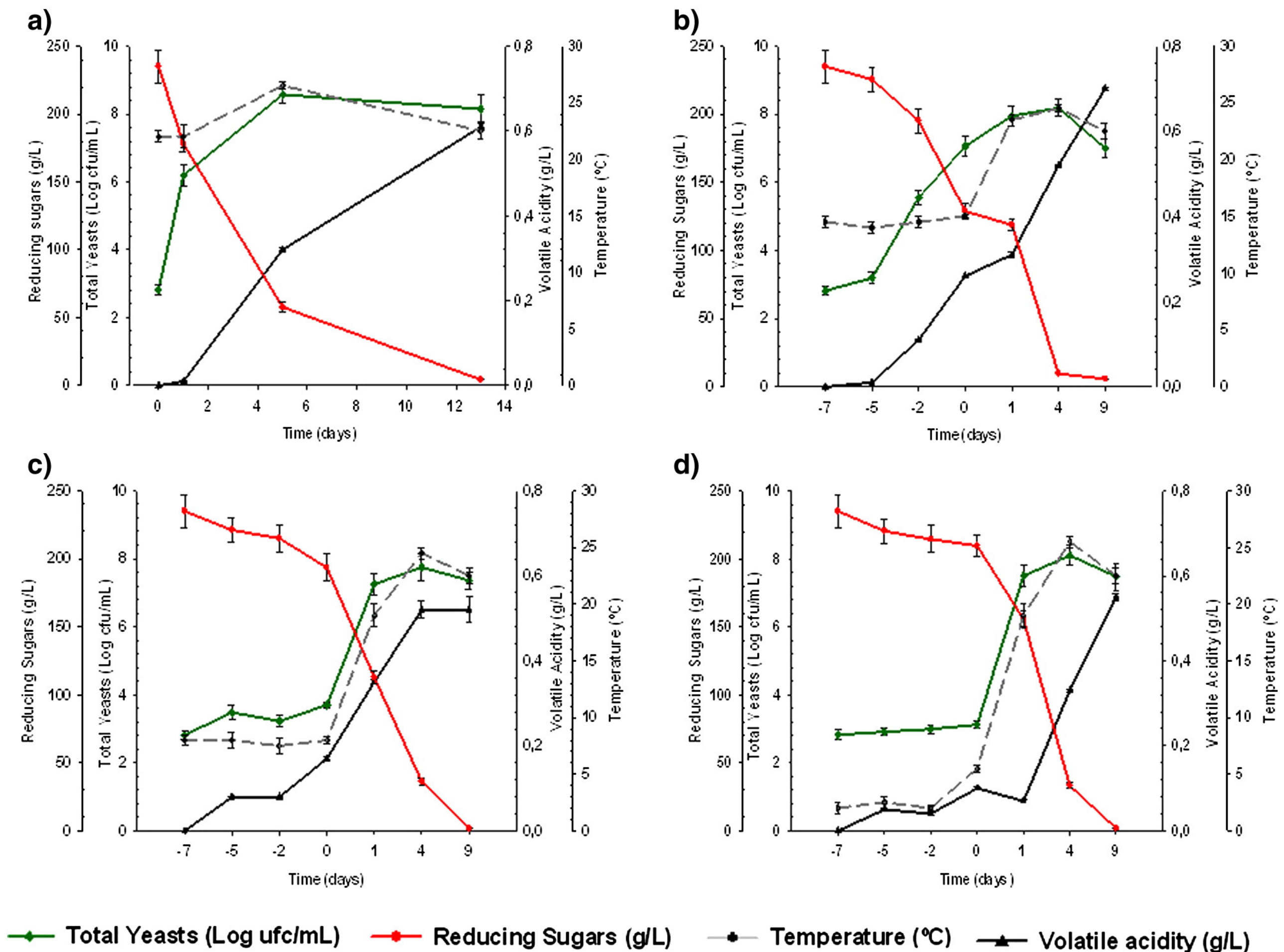

Fig. 1. Evolution of the total yeast population (cfu/mL), reducing sugar (g/L), volatile acidity (g/L) and temperature (°C) during prefermentative cold soak and alcoholic fermentation in Cabernet Sauvignon: a) Control without prefermentative cold soak; b) 14 ± 1 °C; c) 8 ± 1 °C, d) 2.5 ± 1 °C. Inoculation with active dry yeast was done day 1.

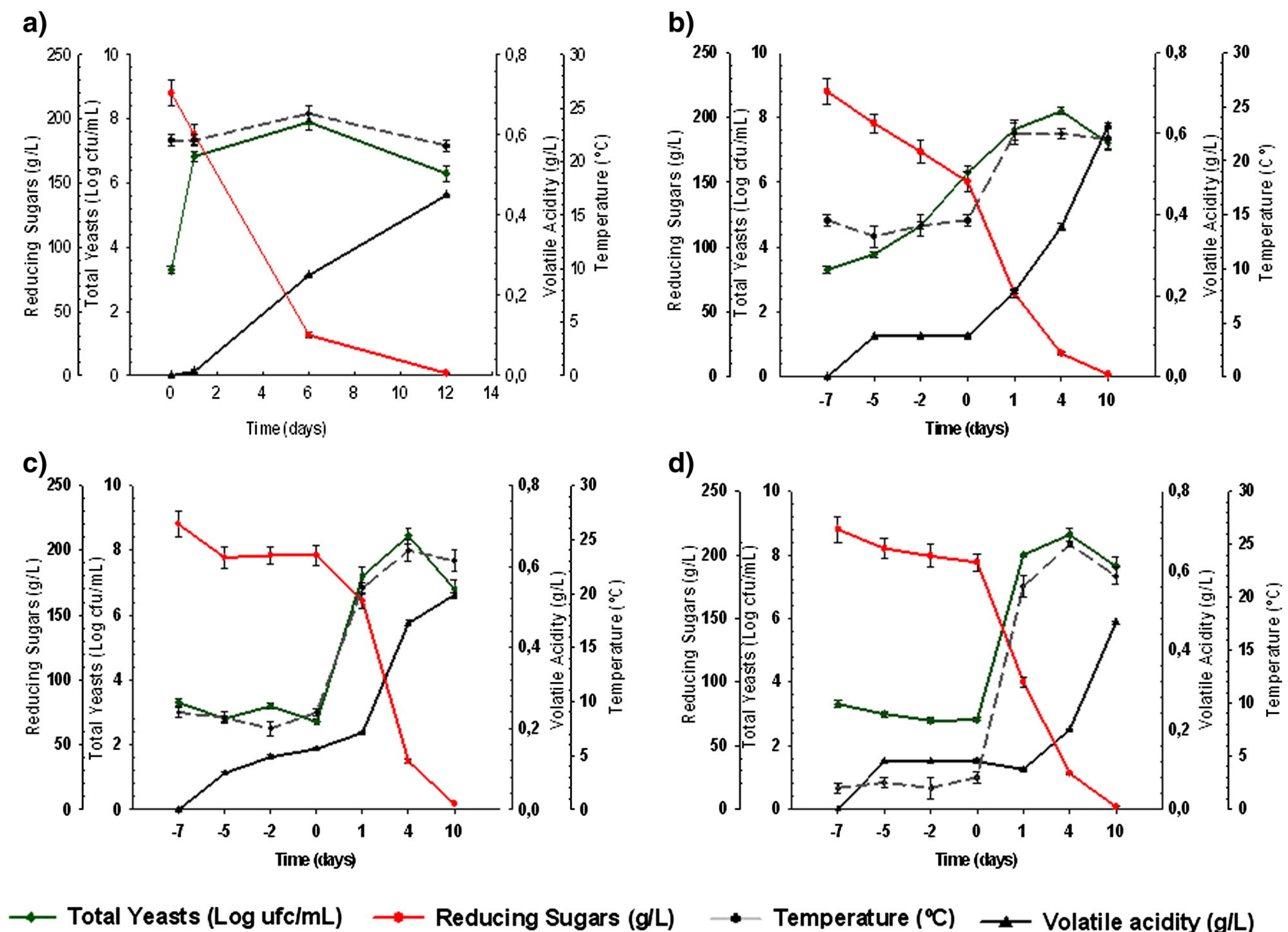

Fig. 2. Evolution of the total yeast population (cfu/mL), reducing sugar (g/L), volatile acidity (g/L) and temperature (°C) during prefermentative cold soak and alcoholic fermentation in Malbec: (a) Control without prefermentative cold soak; (b) 14 ± 1 °C; (c) 8 ± 1 °C; (d) 2.5 ± 1 °C. Inoculation with active dry yeast was done day 1.

increase in total yeast populations. Consumption of reducing sugars during the prefermentative cold soak conducted at 14 ± 1 °C in Cabernet Sauvignon was 107 ± 5.35 g/L and in Malbec 69.67 ± 3.89 g/L (Figs. 1b and 2b). Cold soak treatments carried out at 8 ± 1 °C showed moderate fluctuations in yeast counts during the prefermentative stage, with an overall increase in the total yeast count of 14.55% for Cabernet Sauvignon and 7.20% for Malbec at the end of the cold soak (Figs. 1c and 2c). As expected, no increase in yeast population was recorded at the end of the prefermentative cold soak in either must maintained at 2.5 ± 1 °C (Figs. 1d and 2d). Sugar consumption at the end of the prefermentative cold soak at 8 ± 1 °C and 2.5 ± 1 °C treatments varied between 10.80 and 17.68 g/L (Figs. 1 cd and 2 cd). This observation suggests that active metabolism could be present in some yeast species despite temperatures as low as 2.5 ± 1 °C. The same metabolism could also be evidenced with the production of acetic acid during the prefermentative cold soak (Figs. 1 cd and 2 cd). Acetic acid production during the prefermentative stages increased at higher cold soak temperatures (Figs. 1 bcd and 2 bcd).

Inoculation with commercial yeast was carried out to favour the start of alcoholic fermentation after prefermentative treatments had finished. Total yeast counts rapidly increased, exceeding 1×10^7 cfu/mL 24 h after inoculation (Figs. 1b–d and 2b–d). Alcoholic fermentations finished in 10 days in all musts with prefermentative cold soak (residual sugars < 2 g/L), whereas control treatments needed 13 days to reach the same level of residual sugars (Figs. 1a and 2a).

3.2. Diversity of yeast species during prefermentative cold soak and alcoholic fermentation

Yeast species diversity and development were monitored during prefermentative cold soak and alcoholic fermentation. A total of 12 species belonging to 9 genera were identified in Cabernet Sauvignon, and a total of 10 yeast species belonging to 5 genera were identified in Malbec (Figs. 3 and 4). As expected, an elevated percentage and a high diversity of non-*Saccharomyces* yeast species was found in fresh grape juice during the early stages of prefermentative cold soak. The predominant non-*Saccharomyces* species in fresh Cabernet Sauvignon must were *Hanseniospora uvarum* (15.15%), followed by *Candida zemplinina* (12.35%), *Cryptococcus albidus* (12%) and *Pichia manshurica* (12%), whereas *Pichia occidentalis* (4.55%) and *Metschnikowia pulcherrima* (1.55%) were found at a minor percentage (Fig. 3). The main non-*Saccharomyces* species isolated from fresh Malbec must were *H. uvarum* (21.4%), *C. zemplinina* (12.65%), *P. occidentalis* (10.25%) and *P. kluyveri* (10%), followed by *H. vinae* (9%) and *M. pulcherrima* (3.8%) (Fig. 4). Unexpectedly, more than 30% of the total yeasts in both grape cultivars isolated from grape juice belonged to *S. cerevisiae* species (Figs. 3 and 4).

The relative proportions of the yeast species changed noticeably during prefermentative cold soak. Highest yeast species diversity was observed during prefermentative cold soak conducted at 14 ± 1 °C (Figs. 3a and 4a). At this temperature, *H. uvarum* significantly increased its population during the prefermentative stage, reaching 70–90% of

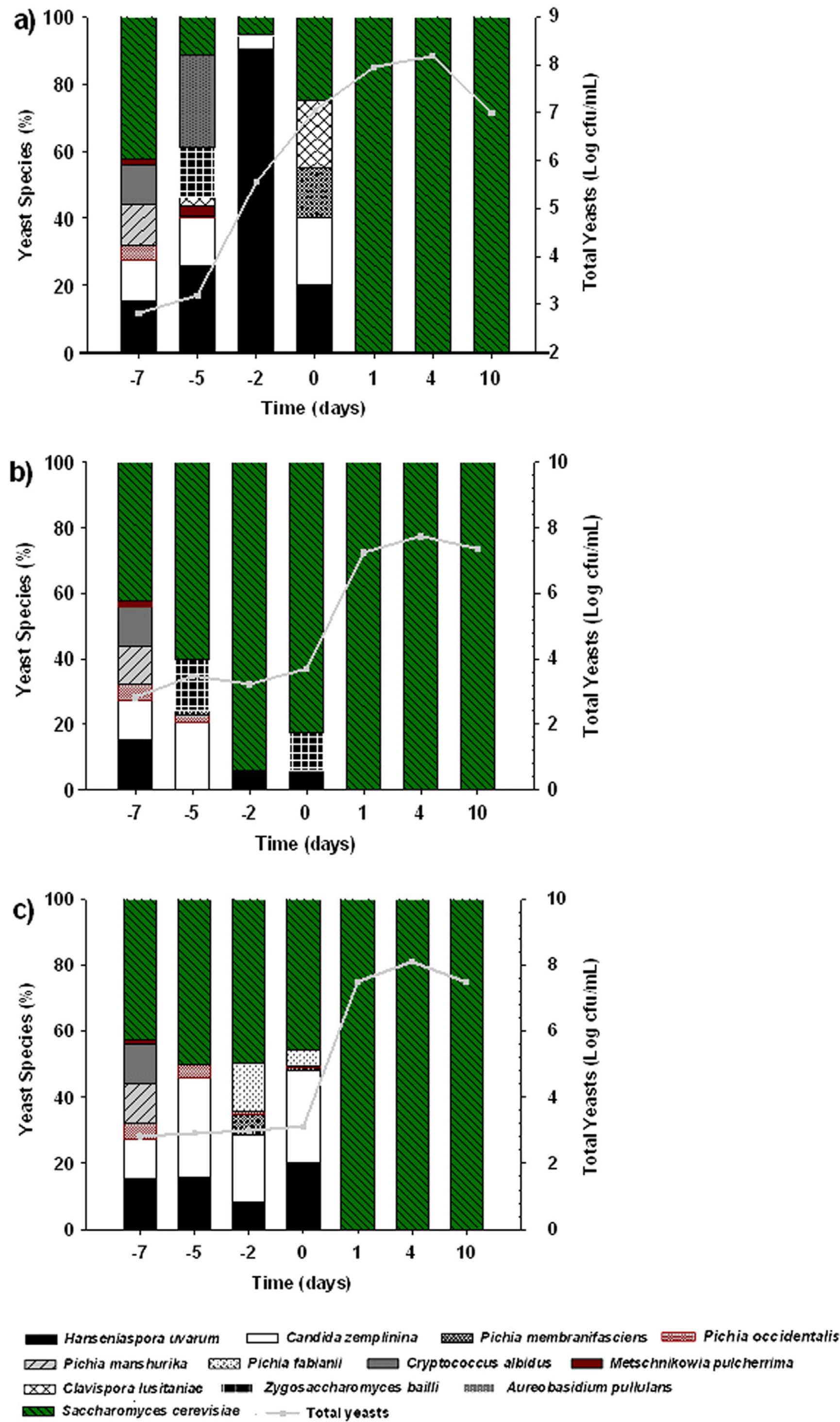

Fig. 3. Dynamics of yeast species during prefermentative cold soak (CS) and alcoholic fermentation in Cabernet Sauvignon: (a) 14 ± 1 °C; (b) 8 ± 1 °C; (c) 2.5 ± 1 °C. Inoculation with active dry yeast was done day 1.

the total yeast population on the fifth day of cold soak. In contrast, *C. zemplinina* and *P. occidentalis* populations maintained stable during this period (Figs. 3a and 4a). Grape must maintained at 14 ± 1 °C during the prefermentative cold soak showed a particular yeast diversity that was not observed at the other cold soak temperatures. *Aurobasidium pullulans* and *Clavispora lusitaniae* were only isolated from prefermentative Cabernet Sauvignon samples, while *Candida californica* and *Metschnikowia fruticola* were isolated from Malbec samples. Prefermentative cold soak conducted at 14 ± 1 °C allowed a quantitative and qualitative modification in the yeast populations, and consequently, at the beginning of the alcoholic fermentation yeast populations had changed completely.

S. cerevisiae was the predominant species recorded in samples during prefermentative cold soak maintained at 8 ± 1 °C, comprising 72–82% of the total yeast population at the end of the cold soak period. *Zygosaccharomyces bailii, H. uvarum, C. zemplinina* and *P. occidentalis* were also isolated, but at lower percentages (Figs. 3b and 4b).

The predominant non-*Saccharomyces* species isolated from must samples during the prefermentative cold soak performed at 2.5 ± 1 °C were *C. zemplinina, P. occidentalis* and *H. uvarum* (Figs. 3c and 4c).

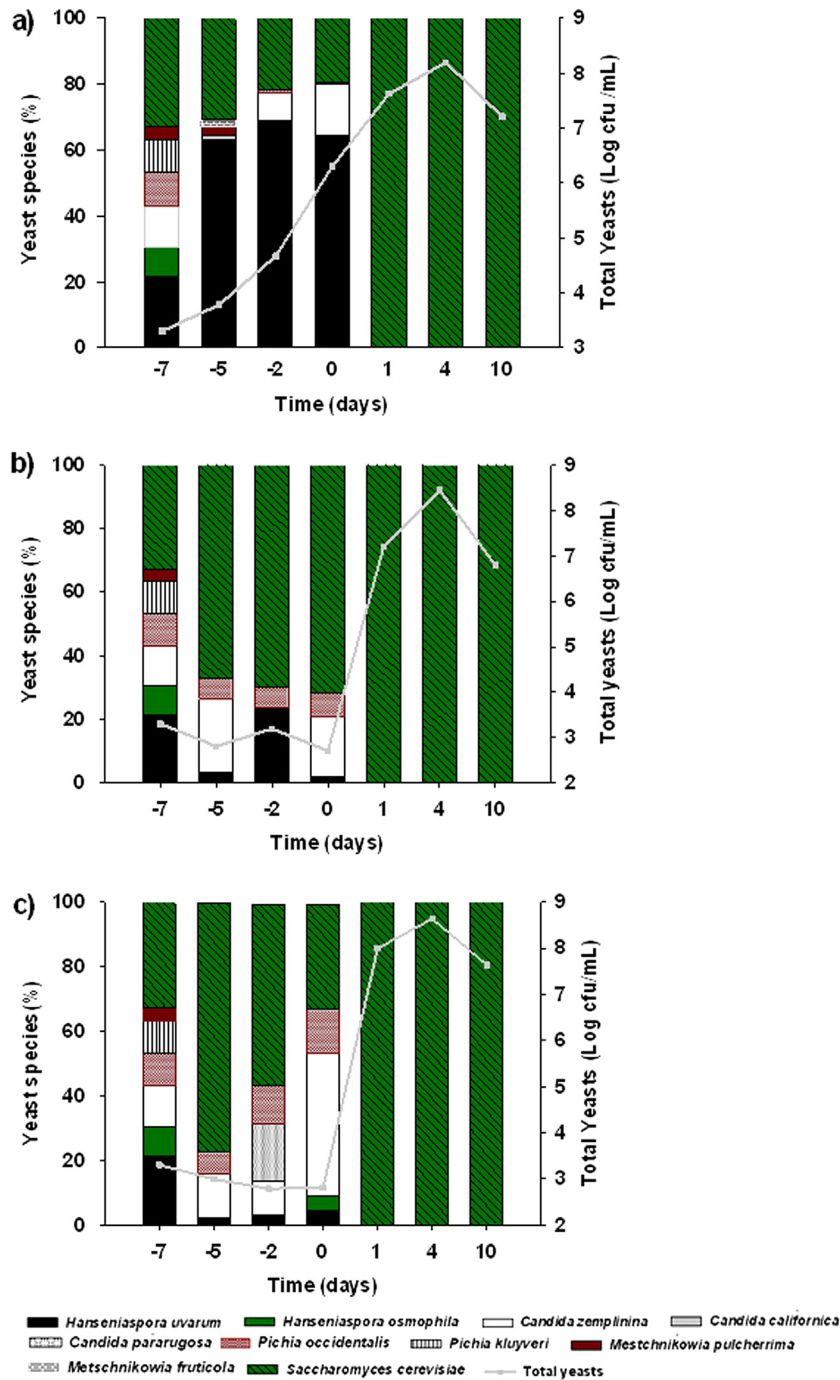

Fig. 4. Dynamics of yeast species during CS and alcoholic fermentation in Malbec: (a) 14 ± 1 °C; (b) 8 ± 1 °C; (c) 2.5 ± 1 °C. Inoculation with active dry yeast was done day 1.

Neither the yeast population size nor the species diversity was significantly modified during the prefermentative cold soak at this temperature.

At the end of the prefermentative cold soak, grape musts were inoculated with a commercial *S. cerevisiae* starter resulting in complete dominance of this species in all the samples taken after inoculation (Figs. 3 and 4). However, due to the high proportion of native *S. cerevisiae* recorded at the end of cold soak treatments carried out at higher temperatures, intraspecific analysis of *S. cerevisiae* was carried out in order to assess implantation of the commercial strain. Molecular data showed that only 32–56% of the *S. cerevisiae* yeasts isolated at the end of the alcoholic fermentation after prefermentative cold soak at 14 ± 1 °C coincided with the commercial yeast molecular pattern. In this treatment, only one pattern different to that of D254 was identified as dominant competing with the commercial strain at the end of the fermentation in both musts (Malbec and Cabernet Sauvignon). The fact of finding the same prevailing pattern in both fermentations could suggest a cellar origin for this strain. A higher percentage of implantation of the commercial strain (75–95%) was found at the end of alcoholic fermentation in musts previously cold soaked at 8 ± 1 °C, together with five different molecular patterns in minor proportion. Complete dominance of the commercial yeast strain was recorded at the end of the alcoholic fermentation in must with prefermentative cold soak performed at 2.5 ± 1 °C (data not shown).

Table 1
Chemical analysis of wines obtained after prefermentative cold soak performed at different temperatures.

Grape cultivar	Cabernet Sauvignon				Malbec			
Parameter (units)	Control	14 ± 1 °C	8 ± 1 °C	2.5 ± 1 °C	Control	14 ± 1 °C	8 ± 1 °C	2.5 ± 1 °C
Residual sugars (g/L)	<1.8	<1.8	<1.8	<1.8	<1.8	<1.8	<1.8	<1.8
Alcohol grade (% v/v)	14.1 ± 0.1	14.3 ± 0.1	14.0 ± 0.1	14.0 ± 0.1	14.8 ± 0.1	14.7 ± 0.1	14.5 ± 0.1	14.8 ± 0.1
Titratable acidity (g/L tart. ac.)	4.95 ± 0.06	5.55 ± 0.02*	4.65 ± 0.04	4.5 ± 0.06	5.25 ± 0.08	6.08 ± 0.06*	5.33 ± 0.03	5.48 ± 0.04
Volatile acidity (g/L acetic ac.)	0.61 ± 0.05	0.79 ± 0.06*	0.52 ± 0.04	0.55 ± 0.01	0.45 ± 0.04	0.62 ± 0.07*	0.53 ± 0.06	0.47 ± 0.02
pH	4.1 ± 0.1	4.1 ± 0.1	4.1 ± 0.1	4.1 ± 0.1	3.9 ± 0.1	3.8 ± 0.1	3.9 ± 0.1	3.8 ± 0.1

* Statistical difference between prefermentative temperatures within the same chemical parameter and grape cultivar according to Tukey test ($p < 0.05$). Control means without prefermentative cold soak.

3.3. Chemical and sensory analysis of wines

Table 1 shows chemical parameters of the wines produced after prefermentative cold soak treatments. Titratable and volatile acidity were significantly higher in wines with prefermentative cold soak conducted at 14 ± 1 °C than at the lower temperatures. Other chemical wine parameters assayed, such as ethanol, residual reducing sugars and pH, were not statistically different between treatments within the same grape cultivar (Table 1).

Sensory analysis was performed 4 months after the wines were bottled. The main goal of this analysis was to assess the impact of the changes in yeast population dynamics developed during prefermentative cold soak and of the potential production of off-flavours and aromas, on the sensory properties of the wines. Off-flavours were not detected during sensory analysis in any of the wines analysed. Cabernet Sauvignon wines without prefermentative cold soak showed statistically significant differences with wines submitted to prefermentative cold soak for six of the nine different descriptors evaluated (colour intensity, violet hue, fruity, vegetal character, concentration and acidity) (data not shown). PCA revealed that the first two principal axes, PC1 and PC2, accounted for 96% of the variation in Cabernet Sauvignon sensorial values, with 73% and 23%, respectively (Fig. 5). PC1 shows a clear separation between wines with and without prefermentative cold soak independently of the temperature used in the prefermentative stage. Differentiation between prefermentative treatments can be explained by PC2 (bitterness and spicy). Cabernet Sauvignon wines with lower CS temperatures (8 ± 1 °C and 2.5 ± 1 °C) appear to be defined by vegetal character, colour intensity, violet hue, concentration and astringency, whereas wines with prefermentative cold soak at higher temperature (14 ± 1 °C) are more associated with acidity (Fig. 5). Similarly, Malbec wines showed significant differences for five of the nine descriptors assayed (colour intensity, fruity, spicy, vegetal character and astringency) (data not shown). A two-dimensional plot of Malbec wines after PCA explained 88% of the variation: PC1: 60% and PC2: 28%. In this case, the variables explained by PC1 were responsible for the main differences between cold soak and control treatments whereas PC2 explained the difference between prefermentative treatments (vegetal character, bitterness and fruity) (Fig. 6).

4. Discussion

Total yeast counts and yeast species present in grape juices shortly after crushing were in line with previous reports on healthy grapes (Combina et al., 2005a; Fleet and Heard, 1993; Ribéreau-Gayon et al., 2006). It is well known that the presence of *S. cerevisiae* on grapes is rare and only occurs at very low numbers in freshly crushed musts (Combina et al., 2005a; Mortimer and Polsinelli, 1999; Pretorius, 2000). The present study recorded an unexpectedly high percentage of *Saccharomyces* (more than 30%) in fresh grape musts. In agreement with Andorrà et al. (2008) and Mercado et al. (2007), *Saccharomyces*

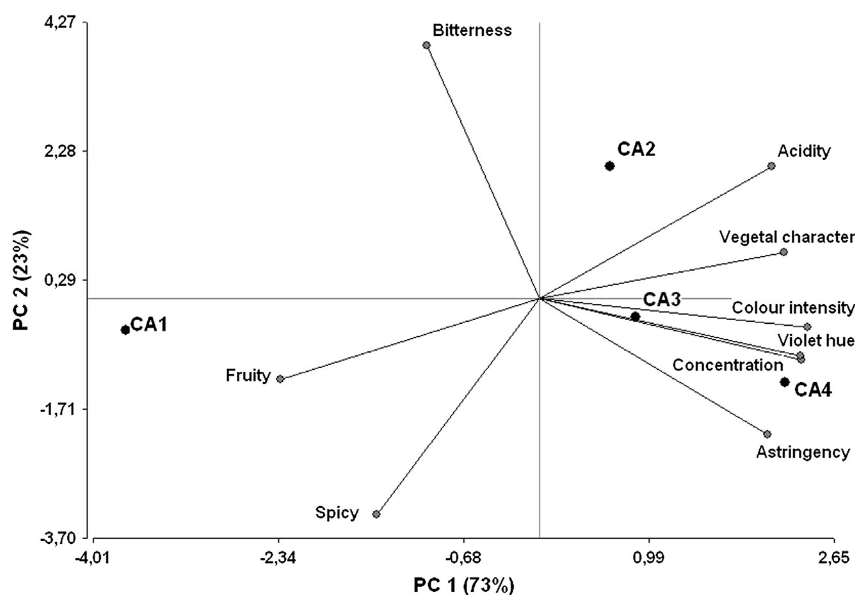

Fig. 5. Biplot of the two principal components for sensory analysis of Cabernet Sauvignon (CA) wines obtained with different treatments. CA1: control; CA2: cold soak at 14 ± 1 °C; CA3: cold soak at 8 ± 1 °C, CA4: cold soak at 2.5 ± 1 °C.

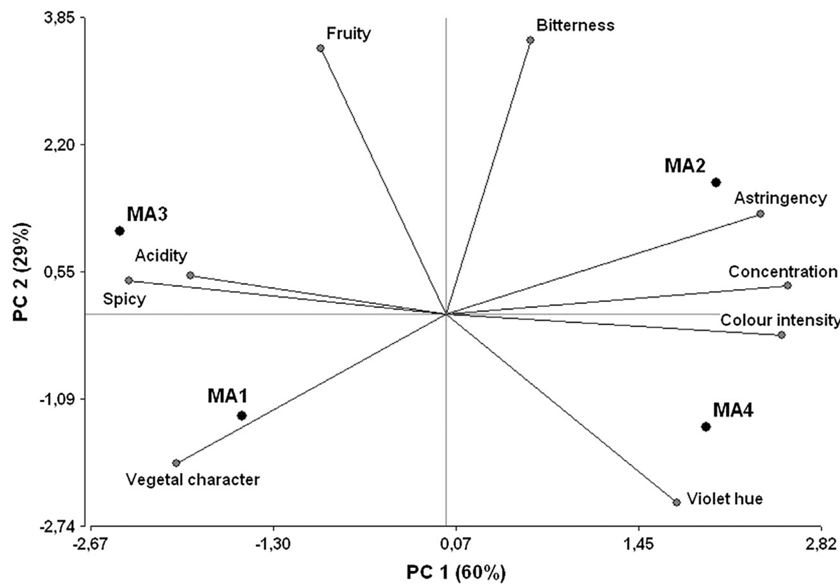

Fig. 6. Biplot of the two principal components for sensory analysis of Malbec (MA) wines obtained with different treatments. MA1: control; MA2: cold soak at 14 ± 1 °C; MA3: cold soak at 8 ± 1 °C; MA4: cold soak at 2.5 ± 1 °C.

could have been introduced through contact of the must with the winery equipment or other winery surfaces. Under the experimental conditions of the present study, i.e., an experimental winery that had previously processes grapes and fermented wines inoculated with commercial yeast starters, the higher *Saccharomyces* proportion could be partially explained. Moreover, the low population of total yeast present in fresh grape must could have contributed to make these cellar originated *Saccharomyces* even more evident in the final yeast percentages.

Prefermentative cold soak presented both qualitative and quantitative changes in the yeast populations as a function of the different temperatures assayed. The influence of wild yeasts on the organoleptic quality of wines is generally related to their presence and persistence during vinification (Comitini et al., 2011). Prefermentative cold soak carried out at 14 ± 1 °C showed a significant increase in total yeast counts, and accordingly, this was reflected in the sugar consumption patterns (Figs. 1b and 2b). At this working temperature, the growth of several yeast species (mostly *H. uvarum*) and the concomitant sugar consumption was evidenced. Therefore, the use of the term "prefermentative" for the 14 ± 1 °C treatment appears as unwarranted in the context of the present study. Nevertheless, we have decided to keep the term because of its widespread use in commercial wineries. In commercial settings, the term prefermentative is generally used in spite of applying similar working temperatures as the ones reported for the 14 ± 1 °C treatment, because this temperature range is operatively attained in commercial fermenters. Our results are consistent with other studies that have reported the effect of this temperature on the total wine yeasts during the prefermentative stage (Albertin et al., 2014; Hierro et al., 2006; Zott et al., 2008). It has been shown that growth and survival of certain non-*Saccharomyces* species in fermentations carried out at temperatures below 20 °C improved (Andorrá et al., 2010; Heard and Fleet, 1988; Mendoza et al., 2009). Temperatures of 14 ± 1 °C applied during prefermentative cold soak facilitated selective growth of *Hanseniaspora* species, mainly *H. uvarum*, and to a lesser extent *Candida* species, especially *C. zemplinina.* Several other authors have reported development of these yeast species at low temperatures. Mendoza et al. (2009) found that in mixed cultures of *Saccharomyces cerevisae* and *Kloeckera apiculata*, the highest *K. apiculata* biomass was obtained at 15 °C, whereas maximum *S. cerevisiae* biomass was observed at 25 °C. Heard and Fleet (1988) observed that growth and survival of *K. apiculata* was better in fermentations carried out below 20 °C and the strain was dominant in fermentations at 10 °C. In agreement with these observations, others studies applying different prefermentative low temperatures (10 to 15 °C) concluded that cold soak favoured the development of *H. uvarum* irrespective of the temperatures assayed (Albertin et al., 2014; Cuénat et al., 1996). It should be mentioned, however, that there is considerable controversy concerning the effect of growth of apiculate yeasts on the organoleptic quality of wines (Ciani et al., 2006; Maturano et al., 2012; Moreira et al., 2005). Most likely, contributions or detriments to wine quality associated with *H. uvarum* mainly depend on the size of the population. The high proportion of *Hanseniaspora* observed during prefermentative cold soak in the current study can be related to increased acidity and bitterness of the wines.

The second non-*Saccharomyces* species that increased in number during prefermentative cold soak was *C. zemplinina*, a species originally described in botrytised grapes (Sipiczki, 2003). It is reported to be osmotolerant and fructophylic, and it generally produces low amounts of acetic acid, together with significant quantities of glycerol (Rantsiou et al., 2012). In the present work, this species was favoured by lower temperatures. This observation is consistent with Zott et al. (2008), who reported dominance of *C. zemplinina* during cold maceration at 4 °C and 10 °C in red must. Other studies have described that *C. zemplinina* produced high concentrations of pyruvic acid and increasing levels of vitisin A in wines fermented with sequential and pure cultures of *C. zemplinina*/*S. cerevisiae* was recorded (Mangani et al., 2011). In the present study, high proportions of *C. zemplinina* and *S. cerevisiae* populations present during prefermentative maceration could enhance colour parameters (intensity and violet hue) as evidenced in Cabernet Sauvignon and Malbec grapes macerated at the lowest temperature.

Five *Pichia* species were also present during prefermentative cold soak. Some *Pichia* species are considered undesirable because volatile phenols and biofilm production (Barata et al., 2008; Saez et al., 2011). However, the importance of certain *Pichia* species has attracted the researcher's interest because they can potentially contribute to positive aromas and mouthfeel attributes in wines (Bourdichon et al., 2012). Diverse non-*Saccharomyces* genera, such as *Candida, Hanseniaspora* and *Pichia,* produce higher levels of total polysaccharides in fermenting media than *S. cerevisiae* (Domizio et al., 2011, 2014). In accordance with

this fact, Cabernet Sauvignon wines performed with prefermentative cold soak at the highest temperature demonstrated a negative relationship between the non-*Saccharomyces* population size and astringency.

In the present study, all treatments showed an increasing proportion of the *S. cerevisiae* population during the final stage of prefermentative cold soak, but the predominance of this species was more noticeable in must macerated at 8 ± 1 °C. This later would suggest that some cryotolerant *S. cerevisiae* strains could be present in the must. There is a wide diversity within *S. cerevisiae* species and cryotolerant *S. cerevisiae* strains are well known in brewing and oenology. Also, mechanisms involved in adaptation to low temperatures have been demonstrated (Giudici et al., 1998; Massera et al., 2011; Salvado et al., 2012).

The high proportion of native *S. cerevisiae* in musts after cold soak treatment affected the dominance of the commercial starter added. Consequently, alcoholic fermentation of wines previously cold soaked at 14 ± 1 °C is mainly carried out by a native *S. cerevisiae* strain. This is another factor that can affect the sensory perception of wines.

One of the most important findings in the present study is the evidence that the temperature used to conduct prefermentative cold soak really affects the natural proportion of native yeast species. In general, the two grape juices assayed displayed similar yeast population dynamics during the prefermentative stage, and they were related to the temperature. Our study also evidences the complexity of factors and interactions that contribute to develop colour and aroma attributes of wines. Temperature regulation is obviously an important tool in wine making as it shifts the population from *Saccharomyces* to non-*Saccharomyces* yeasts.

Conflict of interest

No conflict of interest declared

Acknowledgements

This work was supported by the Regional Viticulture Project MZASJ 1252101 from the National Institute of Agricultural Technology (INTA). Dr.Y.P. Maturano is a postdoctoral fellow of the National Council of Sciences and Technical Research (CONICET-Argentina). We are grateful to Dr. Luis F. Casassa for his scientific contribution to improve the manuscript.

References

Albertin, W., Miot-Sertier, C., Bely, M., Marullo, P., Coulon, J., Moine, V., Colonna-Ceccaldi, B., Masneuf-Pomarede, I., 2014. Oenological prefermentation practices strongly impact yeast population dynamics and alcoholic fermentation kinetics in Chardonnay grape must. Int. J. Food Microbiol. 178, 87–97.

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.

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

Barata, A., González, S., Malfeito-Ferreira, M., Querol, A., Loureiro, V., 2008. Sour rot-damaged grapes are sources of wine spoilage yeasts. FEMS Yeast Res. 8, 1008–1017.

Barata, A., Malfeito-Ferreira, M., Loureiro, V., 2012. The microbial ecology of wine grape berries—review. Int. J. Food Microbiol. 153, 243–259.

Bourdichon, F., Casaregola, S., Farrokh, C., Frisvad, J.C., Gerds, M.L., Hammesf, W., Harnett, J., Huys, G., Laulund, S., Ouwehand, S., Powell, I.B., Prajapati, J.B., Seto, Y., Ter Schure, E., Van Boven, A., Vankerckhoven, V., Zgoda, A., Tuijtelaars, S., Bech, Hansen E., 2012. Food fermentations: microorganisms with technological beneficial use. Int. J. Food Microbiol. 154, 87–97.

Casassa, L.F., Harbertson, J., 2014. Extraction, evolution, and sensory impact of phenolic compounds during red wine maceration. Annu. Rev. Food Sci. Technol. 5 (1), 83–109.

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.

Ciani, M., Beco, L., Comitini, F., 2006. Fermentation behaviour and metabolic interactions of multistarter wine yeast fermentations. Int. J. Food Microbiol. 108 (2), 239–245.

Combina, M., Mercado, L., Borgo, P., Elia, A., Jofre, V., Ganga, A., Martinez, C., 2005a. Yeast associated to Malbec grapes berries from Mendoza, Argentina. J. Appl. Microbiol. 98, 1055–1061.

Comitini, F., Gobbi, M., Domizio, P., Romani, C., Lencioni, L., Mannazzu, I., Ciani, M., 2011. Selected non-*Saccharomyces* wine yeasts in controlled multistarter fermentations with *Saccharomyces cerevisiae*. Food Microbiol. 28 (5), 873–882.

Cuénat, P., Lorenzini, F., Breguy, R., Zufferey, E., 1996. La maceration préfermentaire a froid du Pinot noir. Aspects technologiques et microbiologiques. Rev. suisse vitic. Arboric. Hortic. 4, 259–265.

Domizio, P., Romani, C., Lencioni, L., Comitini, F., Gobbi, M., Mannazzu, I., Ciani, M., 2011. Outlining a future for non-*Saccharomyces* yeasts: selection of putative spoilage wine strains to be used in association with *Saccharomyces* cerevisiae for grape juice fermentation. Int. J. Food Microbiol. 147 (3), 170–180.

Domizio, P., Liu, Y., Bisson, L.F., Barile, D., 2014. Use of non-*Saccharomyces* wine yeasts as novel sources of mannoproteins in wine. Food Microbiol. 43, 5–15.

Fleet, G.H., 2003. Yeast interaction and wine flavour. Int. J. Food Microbiol. 86, 11–22.

Fleet, G.H., Heard, G.M., 1993. Yeasts-growth during fermentation. In: Fleet, G.H. (Ed.), Wine Microbiology and Biotechnology. Harwood Academic Publishers, Chur, pp. 27–54.

Fugelsang, K.C., Edwards, C.G., 2007. Wine Microbiology - Practical Applications and Procedures. Springer-Verlag, Heidelberg, Germany.

Giudici, P., Caggia, C., Pulvirenti, A., Rainieri, S., 1998. Karyotyping of *Saccharomyces* strains with different temperature profiles. J. Appl. Microbiol. 84, 811–819.

Granchi, L., Ganucci, D., Messini, A., Vincenzini, M., 2002. Oenological properties of *Hanseniaspora osmophila* and *Kloeckera corticis* from wines produced by spontaneous fermentations of normal and dried grapes. FEMS Yeast Res. 2, 403–407.

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

Hoffman, C.S., Winston, F., 1987. A ten-minute DNA preparation from yeast efficiently release autonomous plasmids for transformation of *E. coli*. Gene 57, 267–272.

I.S.O. - International Standards Organization 3591, 1977. Sensory analysis. Apparatus. Wine-tasting glass. ISO Editons, Switzerland.

Kurtzman, C.P., Robnett, C.J., 2003. Phylogenetic relationships among yeasts of the *Saccharomyces* complex determined from multigene sequence analyses. FEMS Yeast Res. 3 (4), 417–432.

Legras, J.L., Karst, F., 2003. Optimization of interdelta analysis for *Saccharomyces cerevisiae* strain characterization. FEMS Microbiol. Lett. 221, 249–255.

Mangani, S., Buscioni, G., Collina, L., Bocci, E., Vincenzini, M., 2011. Effects of microbial populations on anthocyanin profile of Sangiovese wines produced in Tuscany, Italy. Am. J. Enol. Vitic. 62, 487–494.

Marais, J., 2003. Effect of different wine-making techniques on the composition and quality of Pinotage wine—I: low-temperature skin contact prior to fermentation. S. Afr. J. Enol. Vitic. 24, 70–75.

Massera, A., Assof, M., Sturm, M.E., Sari, S., Jofré, V., Cordero-Otero, R., Combina, M., 2011. Selection of indigenous *Saccharomyces cerevisiae* strains to ferment red musts at low temperature. Ann. Microbiol. 62, 367–380.

Maturano, Y.P., Rodríguez Assaf, L.A., Toro, M.E., Nally, M.C., Vallejo, M., Castellanos de Figueroa, L.I., Vazquez, F., 2012. Multi-enzyme production by pure and mixed cultures of *Saccharomyces* and non-*Saccharomyces* yeasts during wine fermentation. Int. J. Food Microbiol. 155 (1), 43–50.

Mendoza, L.M., Manca de Nadra, M.C., Farías, M.E., 2007. Kinetics and metabolic behaviour of a composite culture of *Kloeckera apiculata* and *Saccharomyces cerevisiae* wine related strains. Biotechnol. Lett. 29, 1057–1063.

Mendoza, L.M., Manca de Nadra, M.C., Bru, E., Farías, M.E., 2009. Influence of wine-related physicochemical factors on the growth and metabolism of non-*Saccharomyces* and *Saccharomyces* yeasts in mixed culture. J. Ind. Microbiol. Biotechnol. 36, 229–237.

Mercado, L., Dalcero, A.M., 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, 403–412.

Moreira, N., Mendes, F., Hogg, T., Vasconcelos, I., 2005. Alcohols, esters and heavy sulphur compounds production by pure and mixed cultures of apiculate wine yeasts. Int. J. Food Microbiol. 103 (3), 285–294.

Mortimer, R.K., 2000. Evolution and variation of the yeast *Saccharomyces* genome. J. Genome Res. 10, 403–409.

Mortimer, R.K., Polsinelli, M., 1999. On the origins of wine yeast. Res. Microbiol. 150, 199–204.

Nazrala, J., Paladino, S., Vila, H., Lucero, C., 2009. Manual de técnicas analíticas para mostos y vinos. Centro Regional Mendoza-San Juan- INTA. Ediciones INTA, Mendoza, Argentina, pp. 1–61.

O.I.V. - Organisation Internationale de la Vigne et du Vin, 2005. Recueil des méthodes internationales d'analyse des vins et des moûts. OIV Editions, Paris, France.

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

Parish, M.E., Carroll, D.E., 1985. Indigenous yeasts associated with muscadine (*Vitis rotundifolia*) grapes and musts. Am. J. Enol. Vitic. 36 (2), 165–169.

Pretorius, I.S., 2000. Tailoring wine yeast for the new millennium: novel approaches to the ancient art of winemaking. Yeast 16, 675–729.

Rantsiou, K., Dolci, P., Giacosa, S., Torchio, F., Tofalo, R., Torriani, S., Cocolin, L., 2012. *Candida zemplinina* can reduce acetic acid produced by *Saccharomyces cerevisiae* in sweet wine fermentations. Appl. Environ. Microbiol. 78 (6), 1987–1994.

Reynolds, A., Cliff, M., Girard, B., Kopp, T.G., 2001. Influence of fermentation temperature on composition and sensory properties of Semillon and Shiraz wines. Am. J. Enol. Vitic. 52, 235–240.

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, 2nd Ed. John Wiley and Soons, Chichester.

Sacchi, K., Bisson, L., Adams, D., 2005. A review of the effect of winemaking techniques on phenolic extraction in red wines. Am. J. Enol. Vitic. 56, 197–206.

Saez, J.S., Lopes, C.A., Kirs, V.E., Sangorrín, M., 2011. Production of volatile phenols by *Pichia manshurica* and *Pichia membranifaciens* isolated from spoiled wines and cellar environment in Patagonia. Food Microbiol. 28, 503–509.

Salvado, Z., Chiva, R., Rozès, N., Cordero-Otero, R., Guillamón, J.M., 2012. Functional analysis to identify genes in wine yeast adaptation to low-temperature fermentation. J. Appl. Microbiol. 113, 76–88.

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

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