LWT - Food Science and Technology 97 (2018) 648–655

Contents lists available at ScienceDirect

LWT - Food Science and Technology

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

Inoculation strategies to improve persistence and implantation of commercial *S. cerevisiae* strains in red wines produced with prefermentative cold soak

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ARTICLE INFO

Keywords:
Persistence
Cold soak
Yeast strain
Saccharomyces cerevisiae
Red wine

ABSTRACT

Prefermentative cold soak is a winemaking technique aimed at enhancing aroma and colour extraction in red wines. This study aimed at evaluating implantation and persistence rates of commercial active dry yeast strains (ADY) in wines produced with cold soak using two different inoculation strategies. Cold soak was conducted at 4 ± 1 °C, 8 ± 1 °C and 12 ± 1 °C for 7 days. Two yeast strains (Lalvin ICV D254 and Lalvin Rhône 2056) were inoculated before and after cold soak. Implantation of Lalvin ICV D254 at the end of cold soak was higher when conducted at 8 °C and 4 °C, whereas it was undetectable (< 5%) at 12 °C. Lalvin Rhône 2056 showed implantation percentages at the end of cold soak ranging from 40% to 100%, with higher percentages in cold soak conducted at 12 °C. Moderate persistence of ADY at the end of alcoholic fermentation was observed in all treatment inoculated before cold soak. In the treatments inoculated after cold soak, the persistence of Lalvin ICV D254 ranged from 12 to 38% whereas Lalvin Rhône 2056 was less than 5% at the end of alcoholic fermentation. Overall, persistence of both strains improved in inoculation before cold soak relative to inoculation after cold soak.

1. Introduction

Wine alcoholic fermentation was traditionally carried out by native microorganisms naturally present in the grape juice. However, the use of commercial *S. cerevisiae* strains to induce a controlled alcoholic fermentation is nowadays a common winemaking practice, which is currently used to improve fermentation reproducibility as well as the uniformity and quality of the final wine (Fernández-Espinar, López, Ramón, Bartra, & Querol, 2001).

Selected yeast strains are marketed as active dry yeasts (ADY) and nowadays there is a wide offer according to the varietal, wine style or production goal sought. Although it is commonly assumed that the dominance and persistence of the inoculated yeast is maintained throughout the fermentation process with a predictable influence on the final product, several authors have indicated that competition and dominance of commercial *S. cerevisiae* over indigenous yeasts may not be as consistent as previously thought (Maro, Ercolini, & Coppola, 2007; Santamaria, Garijo, Lopez, Tenorio, & Gutierrez, 2005). Lange et al. (2014), defined inoculum implantation as the percentage of relative abundance of the inoculum 24 h after inoculation, and inoculum persistence as the percentage of relative abundance at the end of alcoholic fermentation. Delteil (2001) defined as effective persistence when more than 80% of the isolates show the same molecular pattern as the inoculated yeast strain. Conversely, an unsuccessful persistence occurs when the implantation rate is less than 50%. Indeed, relatively low implantation and persistence rates of the inoculum (< 60%) have been previously reported (Clavijo, Calderon, & Paneque, 2011; Medina et al., 2013). Because it is not common practice to monitor implantation rates of the inoculated yeasts during alcoholic fermentation, it is unknown how often a relatively low implantation/persistence of the

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https://doi.org/10.1016/j.lwt.2018.07.063
Received 6 March 2018; Received in revised form 13 July 2018; Accepted 30 July 2018
Available online 30 July 2018
0023-6438/

inoculum occurs (Barrajón, Arevalo-Villena, Rodriguez-Aragon, & Briones, 2009; Delteil, 1992).

Implantation and persistence of ADY depends on different factors such as yeast rate addition, rehydration protocol, and inoculation temperature (Ferranini, Bocca, & Cavazza, 2007; Soubeyrand, Julien, & Sablayrolles, 2006). Moreover, both the qualitative and quantitative composition of native yeast population in the fresh grape must prior to inoculation may be the key factor to determine the successful implantation. The yeast population typically found in the grape must mainly consist on indigenous yeasts present on the grapes when harvested or introduced through the winery and cellar equipment during destemming and crushing operations (Andorra, Landi, Mas, Guillamón, & Esteve-Zarzoso, 2008; Mercado, Dalcero, Masuelli, & Combina, 2007). Moreover, some pre-fermentative winemaking practices, including thermomaceration, flash pasteurization and cold soak, could influence the native yeast composition of the must prior to the onset of alcoholic fermentation (Albertin et al., 2014; Hierro, Gonzalez, Mas, & Guillamón, 2006). Cold soak is a widely used winemaking practice aimed at increasing aroma and colour extraction in red wines (Casassa & Harbertson, 2014). Briefly, prefermentative cold soak consist on allowing the contact of skins, seeds and must prior to the onset of alcoholic fermentation, in a nonalcoholic or low-alcohol environment. The fermentative activity is reduced by chilling the must and solids to a temperature that would prevent the development of *S. cerevisiae* (Casassa & Sari, 2015). In a previous study from our group, we showed that the temperature at which prefermentative cold soak is conducted affects the natural composition of native yeast species, thereby changing the number and proportion of *Saccharomyces* and non-*Saccharomyces* populations as a function of the cold soak temperature used (Maturano et al., 2015).

In order to prevent a possible deviation during alcoholic fermentation of red wines subjected to pre-fermentative cold soak, winemakers add ADY either prior to the onset of this process or, most commonly, just after it, at or before the onset of alcoholic fermentation. However, as the percentage of implantation and persistence of inoculated yeasts is rarely assessed in a winery setting, it is unknown which of these strategies is better. Therefore, the aim of the present study was to evaluate the effectiveness of two inoculation strategies, namely inoculation prior to cold soak and inoculation at the end of pre-fermentative cold soak and prior to the onset of alcoholic fermentation. To that end, implantation and persistence rates of ADY inoculated in red wines produced with cold soak conducted at three different temperatures were determined.

2. Materials and methods

2.1. Commercial yeast strains and rehydration procedures

Two commercial active dry *Saccharomyces cerevisiae* yeasts, Lalvin ICV D254 and Lalvin Rhône 2056 (Lallemand Inc., Montreal Canada), were assayed. Both strains were selected considering specific oenological traits, including their tolerance to low temperatures and their ability to dominate and compete in fermenting musts. The two strains have the following oenological traits: fermentation temperature range (Lalvin ICV D254: 12-28 °C and Lalvin Rhône 2056: 15-28 °C) and *Killer* character (Lalvin ICV D254: *killer* neutral and Lalvin Rhône 2056: *Killer* active). Both strains have a moderate fermentative vigour and medium nitrogen demand (Lallemand Company, 2017).

Rehydration was carried out according to the manufacturer's recommendation (Lallemand Company, 2014). Briefly, an amount of water (at 37 °C) 10 times the weight of the yeast was added to the dry yeast; the mixture was stirred gently, and then left for 20 min to rehydrate. The yeast starter was acclimatised by progressively adding must to avoid a temperature difference higher of 10 °C between that of the yeast starter and the must to be inoculated.

2.2. Experimental design

Cabernet Sauvignon grapes were harvested from a vineyard located in El Pedernal (32°05′ South, 68°86' West), San Juan province, Argentina. At the moment of harvest grape clusters were fully ripen and showed no signs of insect damage or fungal infections. Grape clusters were manually harvested, immediately transported to the laboratory and then crushed and destemmed, followed by the addition of 50 mg/L SO_2. The fresh grape must composition at the moment of crushing was as follows: 24° Brix (237.8 g/L reducing sugars); titratable acidity 5.5 g/L as tartaric acid; and pH 3.5. Fermentations were carried out in 5 L plastic fermentors with each containing 3 kg of fresh grape must. Treatments were carried out in triplicate (n = 3). Pre-fermentative cold soak was carried out for 7 days at three different temperatures: 4 ± 1 °C, 8 ± 1 °C, and 12 ± 1 °C. Temperatures were maintained by placing the fermentors in a refrigerated chamber. At the end of cold soak musts were warmed up to room temperature to induce the start alcoholic fermentation.

Both strains of ADY (Lalvin ICV D254 and Lalvin Rhône 2056) were inoculated using two different strategies: inoculation was performed either *before* the onset of pre-fermentative cold soak (*i.e.* before cold soak, BCS) or at the end of pre-fermentative cold soak once the must was warmed up and *prior* to the onset of alcoholic fermentation (*i.e.* after cold soak, ACS). The inoculation rate addition was set at 25 g of ADY/100 L for both yeast strains (as manufacturer recommendation) to obtain a final concentration of about 2×10^6 cells/mL in the must. Alcoholic fermentation was carried out at controlled temperature (24 ± 2 °C). After alcoholic fermentation, wines were racked, free SO_2 was adjusted to 35 mg/L to ensure 0.8 mg/L of molecular SO_2 in order to avoid malolactic fermentation and stored at 4 °C for 30 days to allow tartaric stabilisation, bottled without filtration and horizontally stored at 18 °C.

2.3. Sampling and yeast molecular identification

The implantation and persistence of the inoculated ADY was verified as follows. One hundred mL samples were taken at different times throughout the winemaking process: one day after inoculation (D1), at the end of the cold soak (D7) and at the end of alcoholic fermentation (D17). Decimal dilutions (0.1 mL) were plated onto Wallerstein Laboratory (WL) nutrient agar medium (Oxoid, Hampshire, UK), supplemented with 0.2 g/L of dichloran (Fluka A.G., St. Gallen, Switzerland) and 0.5 g/L of chloramphenicol (Sigma Aldrich, Saint Louis, MO, USA) to inhibit the growth of moulds and bacteria, respectively. Petri dishes were incubated at 28 °C for 48–72 h. Plates were assessed for total viable yeast counts and were examined daily until the colonies were large enough to allow visual discrimination between the different types of colonies as reported by Pallmann et al. (2001). A total of 10–20 colonies compatible with *Saccharomyces* colony type were recovered. Isolates were purified by streak plating, sub-cultured on Malt Extract Agar and incubated at 28 °C for 48–72 h as previously described (Maturano et al., 2015).

Discrimination between *Saccharomyces* and non-*Saccharomyces* yeasts was established by the ability of each isolate to grow on L-lysine medium (Oxoid, Basingstoke, UK). All isolates unable to grow with L-lysine as sole nitrogen source were categorized as being *Saccharomyces* (Fleet, 1993). *Saccharomyces* isolates were subsequently differentiated at strain level by interdelta PCR analysis and compared with molecular patterns obtained from commercial strains as previously described (Legras & Karst, 2003). Total DNA extraction was performed according to Hoffman and Winston (1987). Oligonucleotide primers, delta 12 (5′-TCAACAATGGAATCCCAAC-3′) and delta 21 (5′-CATCTTAACACCG TATATGA-3′), were used to amplify total genomic DNA between the repeated interspersed delta sequences. Thermocycler parameters used were as follows: 95 °C for 4 min, followed by 35 cycles at 95 °C for 30 s, 46 °C for 30 s, 72 °C for 45 s and 72 °C for 10 min. PCR products were

separated on 1.2% agarose gels in 1X TBE buffer and stained with 5 μg/mL ethidium bromide. Gel was visualised by UV transillumination using the Gel Doc 1000 Video Gel Documentation System (Bio-Rad, Richmond, USA).

2.4. Basic chemical analysis

Initial and final chemical analysis of musts and wines, including titratable acidity, pH, reducing sugars, Brix, volatile acidity, glycerol and ethanol were determined by FOSS WineScan FT 120 MIR spectrophotometer (FOSS, Hillerod, Denmark).

2.5. Data analysis

Analysis of variance (One-Way ANOVA) of the chemical wine parameters was performed independently for each strain treatment using a professional version of InfoStat software (FCA-UNC, Córdoba, Argentina). Fisher-LSD Test ($p < 0.05$) was used to compare each chemical parameter in wines inoculated before and after cold soak and temperature into the same yeast strain treatments.

3. Results

At the end of alcoholic fermentation, basic chemical analyses were carried out in the wines, with results reported in Table 1. Only few differences were found in these parameters when the same ADY strain was analysed for the treatments inoculated before and after cold soak. Volatile acidity was higher in wines inoculated with Lalvin ICV D254 after cold soak than before cold soak conducted at 12 °C during. Titratable acidity was also higher in wines inoculated with Lalvin ICV D254 after cold soak than before cold soak conducted at 8 °C (Table 1). A higher ethanol concentration was recorded in wines inoculated with Lalvin Rhône 2056 before than after cold soak conducted at 8 °C (Table 1). Moreover, glycerol concentration was generally higher in wines where the two lower temperatures of cold soak were applied, namely 4 °C and 8 °C, and this was independent of the inoculation strategy and commercial yeast strain used (Table 1).

A total of 1080 *Saccharomyces* colonies were isolated, purified and molecularly typed by interdelta PCR. Fig. 1 shows the total yeast population counts during cold soak and alcoholic fermentation in treatments carried out following the two inoculation strategies. Both before and after cold soak, the inoculated treatments showed an increase in their respective populations during cold soak carried out at the two higher cold soak temperatures (8 and 12 °C) (Fig. 1). No increase or a small decline in total yeast counts were recorded at the end of cold soak conducted at 4 °C (Fig. 1). For the treatments inoculated after cold soak, it was possible to assess the distribution of *Saccharomyces* and non-*Saccharomyces* populations within the total yeast population (Fig. 1B). Expectedly, the predominance of non-*Saccharomyces* populations in grape must was observed. However, at the end of the cold soak treatments an increase of *Saccharomyces* relative to that of non-*Saccharomyces* was recorded in all temperatures studied (Fig. 1B–D7).

All treatments inoculated with ADY before cold soak demonstrated a moderate to high implantation rates, ranging from 44 to 100% 24 h after inoculation (Table 2; BCS -D1). Strain Lalvin ICV D254 showed a moderate implantation in cold soak conducted at 12 °C, whereas implantation rate was 100% at the two lower cold soak temperatures (4 and 8 °C) at day 1. The implantation percentage of Lalvin ICV D254 was higher in the lower cold soak temperatures at the end of the cold soak as well (Table 2). At the end of cold soak conducted at 12 °C the strain Lalvin ICV D254 was below the limit of detection, and a high diversity of native *S. cerevisiae* was recorded instead, showing seven different molecular patterns (Fig. 2A). The same seven native strains were detected at the end of alcoholic fermentation in co-existence with the ADY strain, which represented 22% of the final yeast population (Fig. 2A). Moreover, Lalvin ICV D254 inoculated before cold soak conducted at 8 °C allowed an implantation rate of 40% at the end of the cold soak. At this temperature, three other native strains isolated at percentages ranging from 10 to 30% were found (Fig. 2B). Persistence of this commercial strain was 38% whereas the other four different native yeast patterns grouped together represented 62% of the total yeast population at the end of alcoholic fermentation (Fig. 2B). The highest implantation percentage at the end of cold soak was observed in cold soak conducted at 4 °C (D7) for Lalvin ICV D254 inoculated before cold soak, but at the end of alcoholic fermentation a persistence rate of only 44% of the commercial strain was observed (Fig. 2C).

The ADY strain Lalvin Rhône 2056 inoculated before cold soak showed satisfactory implantation rates (88–90%) on day 1 and its implantation at the end of cold soak ranged from 40% to 100%, being higher in the treatments in which cold soak was conducted at higher temperatures (Table 2). However, implantation percentages for this strain at the end of alcoholic fermentation were only moderate

Table 1
Chemical analysis of Cabernet Sauvignon wines fermented with *S. cerevisiae* strains inoculated before (BCS) and after (ACS) cold soak conducted at three different temperatures. Average values followed by the standard deviation (n = 3).

Inoculation time	Parameters	Lalvin ICV D254			Lalvin Rhône 2056		
		CS4	CS8	CS12	CS4	CS8	CS12
BCS	Ethanol (% v/v)	14.10 ± 0.23ab	13.31 ± 0.04a	13.83 ± 0.65ab	14.40 ± 0.11ab	15.02 ± 0.03c	14.73 ± 0.33bc
	Titratable acidity (g/L tartaric acid)	4.52 ± 0.01a	4.54 ± 0.04a	4.66 ± 0.04a	4.78 ± 0.32a	4.66 ± 0.01a	4.68 ± 0.11a
	Volatile acidity (g/L acetic acid)	0.78 ± 0.01bc	0.64 ± 0.06a	0.72 ± 0.02ab	0.73 ± 0.06a	0.70 ± 0.13a	0.77 ± 0.06a
	pH	3.63 ± 0.01a	3.65 ± 0.03a	3.68 ± 0.01a	3.60 ± 0.01ab	3.61 ± 0.01ab	3.65 ± 0.06b
	Glycerol (g/L)	9.86 ± 0.07b	10.00 ± 0.27bc	8.91 ± 0.06a	10.28 ± 0.06bc	10.38 ± 0.04bc	9.03 ± 0.01a
ACS	Ethanol (% v/v)	13.93 ± 0.59ab	13.94 ± 0.11ab	14.31 ± 0.11b	14.11 ± 0.20a	14.09 ± 0.04a	14.68 ± 0.36bc
	Titratable acidity (g/L tartaric acid)	4.62 ± 0.01a	4.80 ± 0.13b	4.57 ± 0.03a	4.98 ± 0.03a	4.73 ± 0.13a	4.72 ± 0.04a
	Volatile acidity (g/L acetic acid)	0.83 ± 0.04c	0.67 ± 0.05a	0.81 ± 0.01c	0.74 ± 0.05a	0.80 ± 0.03a	0.79 ± 0.01a
	pH	3.62 ± 0.01a	3.60 ± 0.09a	3.62 ± 0.01a	3.59 ± 0.01ab	3.54 ± 0.01a	3.61 ± 0.03ab
	Glycerol (g/L)	9.76 ± 0.25b	10.46 ± 0.27c	9.05 ± 0.18a	10.21 ± 0.13b	10.60 ± 0.14c	9.01 ± 0.10a

Values within each yeast strain and parameter followed by different letters are significantly different according to Fisher-LSD Test ($p < 0.05$).

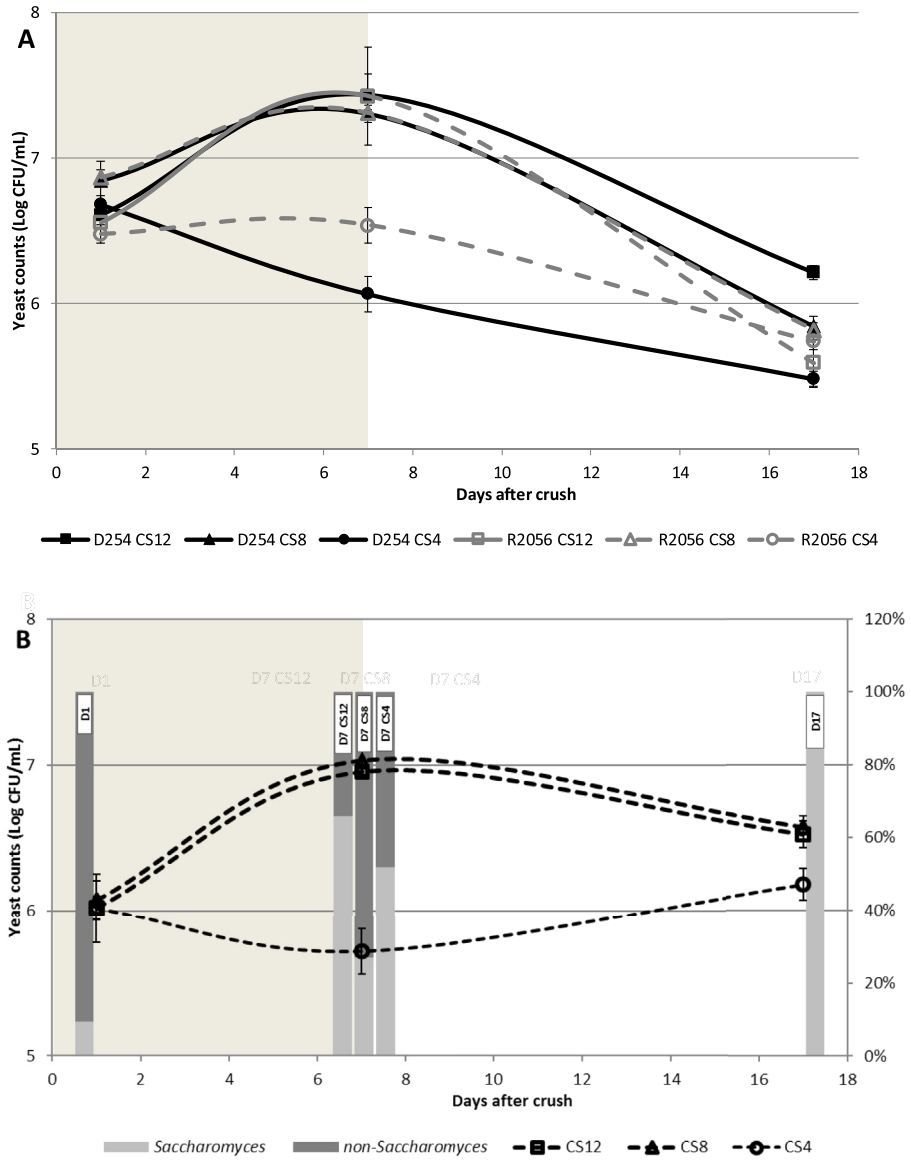

Fig. 1. Total yeast population and *Saccharomyces*/non-*Saccharomyces* percentages during cold soak and alcoholic fermentation in red wine inoculated with commercial strains (Lalvin ICV D254 and Lalvin Rhône 2056) before (A) and after (B) cold soak conducted at three different temperatures (CS12, CS8 and CS4) for 7 days. The cold soak period is shown in grey colour.

Table 2
Percentages of implantation and persistence of commercial *S. cerevisiae* strains inoculated before (BCS) and after (ACS) cold soak conducted at three different temperatures in Cabernet Sauvignon must and wines. CS (cold soak), AF (alcoholic fermentation) Average values followed by the standard deviation (n = 3).

Treatment	Process stage	Sampling time	Lalvin ICV D254			Lalvin Rhône 2056		
			CS4	CS8	CS12	CS4	CS8	CS12
BCS	CS	Day 1	100 ± 0	100 ± 0	50 ± 2	88 ± 2	90 ± 0	80 ± 4
		Day 7	100 ± 0	40 ± 4	< 5	67 ± 4	40 ± 2	100 ± 0
	AF	Day 17	44 ± 2	38 ± 2	22 ± 4	40 ± 4	20 ± 2	25 ± 4
ACS	AF	Day 17	12 ± 6	38 ± 4	30 ± 2	< 5	< 5	< 5

(Table 2). This suggests that higher implantation percentages at the end of cold soak may not necessarily result in concomitantly higher implantation percentages of the same strain at the end of alcoholic fermentation. Again, a fairly large diversity of native strains was found to co-exist with the inoculated Lalvin Rhône 2056 strain at the end of fermentation, where five to seven different molecular patterns were simultaneously isolated at percentages ranging from 10 to 25%, without any clear dominance of any of them (Fig. 2).

Yeast survival at the end of cold soak was generally different for the two commercial strains according to the three cold soak temperatures studied. Cold soak at 4 °C favoured the survival of Lalvin ICV D254, whereas cold soak conducted at 12 °C favoured that of Lalvin Rhône 2056 (Fig. 2). However, the persistence at the end of alcoholic fermentation was similar for the two strains regardless the temperature of

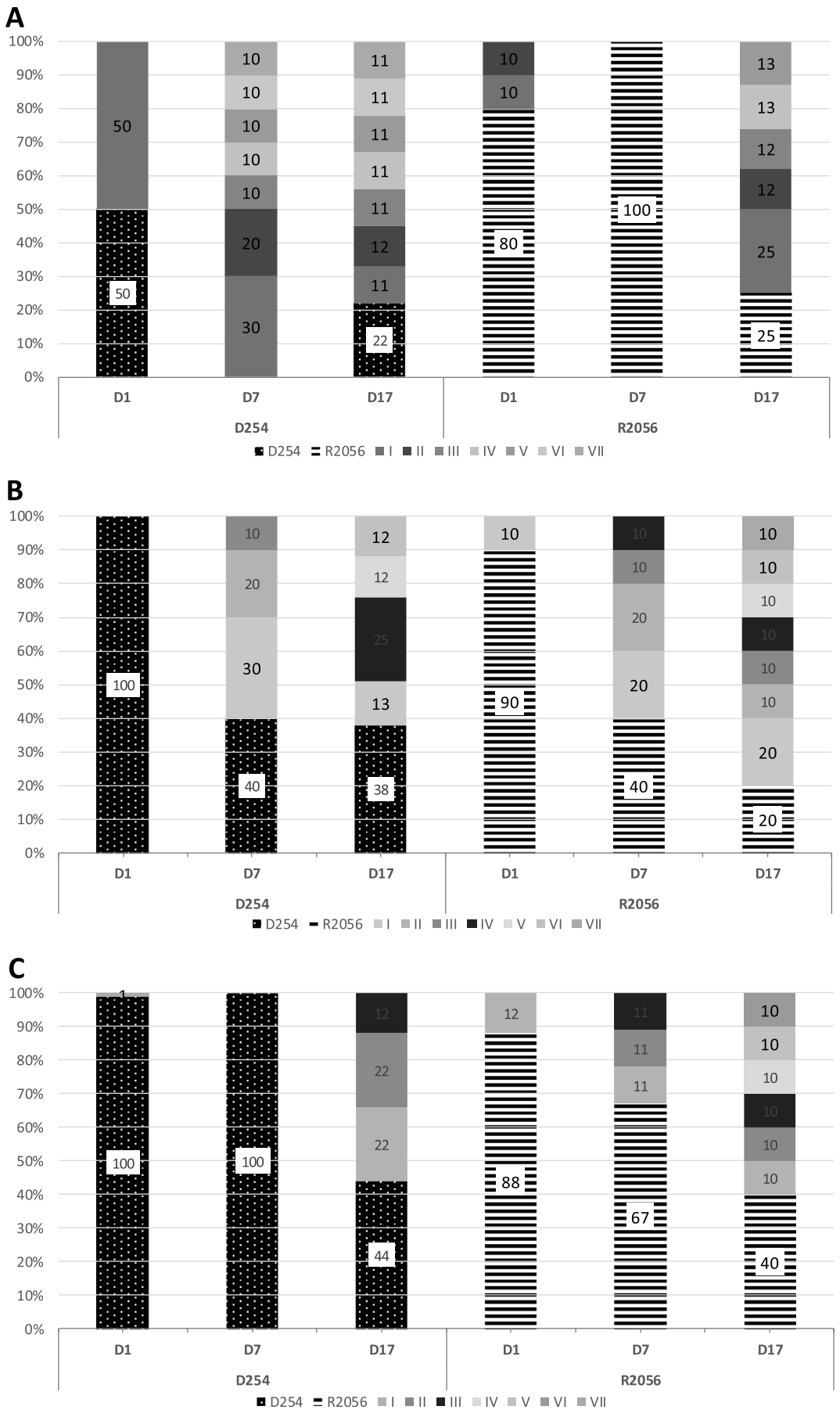

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Fig. 2. Diversity of *Saccharomyces* strains (in percentage) in red wine inoculated with commercial strains Lalvin ICV D254 and Lalvin Rhône 2056 before cold soak (BCS) conducted at three different temperatures A) 12 ± 1 °C; B) 8 ± 1 °C; C) 4 ± 1 °C including the start of cold soak (D1), the end of the cold soak (D7) and the end of the alcoholic fermentation (D17). Different interdelta PCR molecular patterns are indicated with Roman numerals.

the cold soak (Table 2 and Fig. 2).

Regarding the treatments inoculated after cold soak, the persistence at the end of alcoholic fermentation was different for each strain (Table 2). For example, when Lalvin ICV D254 was inoculated after the cold soak, persistence ranged from 12 to 38% at the end of alcoholic fermentation. Persistence percentages at 4 °C, 8 °C and 12 °C were slightly higher, similar and smaller, respectively, than treatments inoculated before cold soak (Table 2). In contrast, the persistence of the Lalvin Rhône 2056 strain at the end of alcoholic fermentation was less than 5% when inoculated after cold soak, independently of the cold soak temperature assayed (Table 2). In general, a higher diversity of native *S. cerevisiae* strains was detected in non-inoculated grape must at the beginning and the end of the cold soak and also at the end of alcoholic fermentation regardless of the temperature of cold soak. Five to eight different native strains were detected, but no clear dominance of any of them was observed by the end of alcoholic fermentation (Fig. 3).

Overall, the persistence of both commercial strains studied here, namely Lalvin ICV D254 and Lalvin Rhône 2056, was better when these were inoculated before the onset of cold soak (Table 2). However, persistence of the two commercial strains was typically below 44%, regardless of the inoculation strategy used.

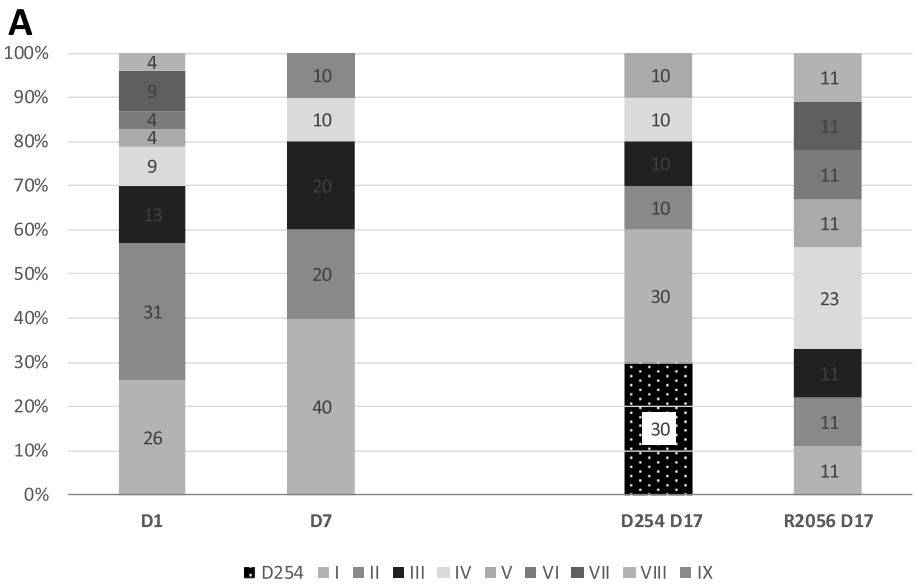
4. Discussion

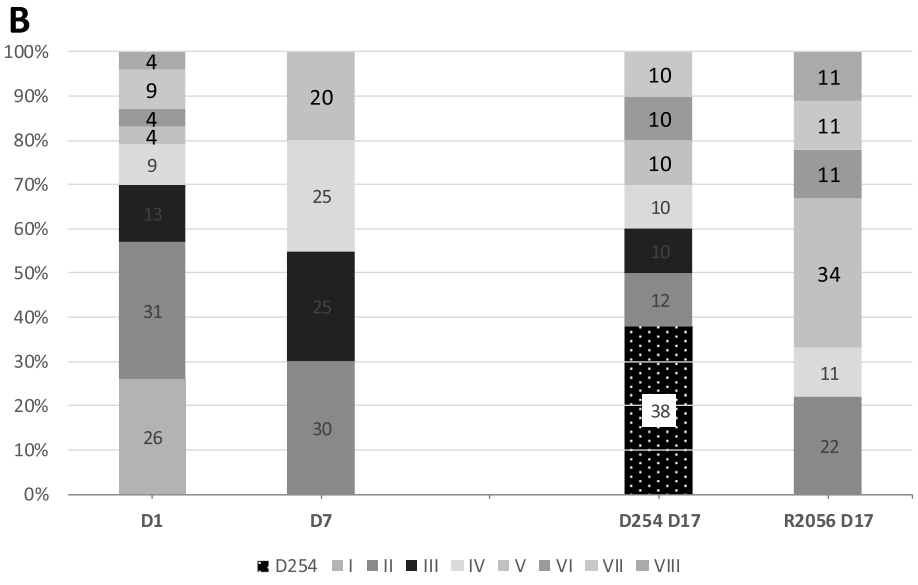
The two commercial yeasts assayed in our study demonstrated a different behaviour during cold soak and alcoholic fermentation. Strain Lalvin Rhône 2056 appeared as more tolerant and competitive than strain Lalvin ICV D254 at temperatures used during cold soak (12 °C, 8 °C and 4 °C). For example, at the end of cold soak conducted at 12 °C, Lalvin Rhône 2056 showed a higher implantation percentage (100%) whereas Lalvin ICV D254 was undetectable at the end of this stage. This situation was overturned when must was warmed up to induce alcoholic fermentation and inoculation occurred after cold soak, whereby Lalvin ICV D254 showed similar persistence percentages than Lalvin Rhône 2056 (22% and 24%, respectively). The difference in competitiveness of the two commercial strains at different temperatures was more evident when they were inoculated at the end of cold soak. Indeed, in the treatments inoculated after cold soak (ACS), Lalvin Rhône 2056 was unable to compete with the native yeast populations during alcoholic fermentation and, as a result, it was undetectable at the end of the fermentation period.

As previously discussed by Lange et al. (2014), a low inoculum implantation/persistence in wine fermentations can be attributed to factors affecting the competitive interaction between the inoculum and other yeasts. These factors include yeast strain (sensitivity to ethanol, presence/absence of the *killer* factor), temperature, nutrition, and must acidity (Barrajón et al., 2009; Blanco, Miras-Avalos, & Orriols, 2012; Fleet, 2008; Lopes, Rodríguez, Sangorrín, Querol, & Caballero, 2007). Also, yeast strains are known to perform differently at different temperatures (Medina et al., 2013). The two commercial strains selected in this study are described by the manufacturer as tolerant to low temperatures, with the minimum fermentation temperature recommended for Lalvin ICV D254 being 12 °C and for Lalvin Rhône 2056 being 15 °C (Lallemand Company, 2017). Counter to expectations, Lalvin Rhône 2056 inoculated before cold soak at 12 °C seemed to be more competitive than Lalvin ICV D254 during cold soak. As previously mentioned, Lalvin Rhône 2056 demonstrated 100% dominance at the end of cold soak, whereas Lalvin ICV D254 was below the detection limit at the end of cold soak conducted at 12 °C. Moreover, both commercial strains seemed to successfully tolerate and survive the lowest cold soak temperature selected for our study (4 °C). This finding suggests that early inoculation before cold soak could be a promising strategy to improve persistence of the selected strain at the end of alcoholic fermentation in wines submitted to very low temperatures during cold soak. As a diverse population of microbes may occur during the pre-fermentative phase of red wines (Hall, Zhou, Qian, & Osborne, 2017), it is also plausible that the early inoculation with a commercial inoculum resulted in wines with less volatile acidity compared with wines inoculated after cold soak, as it was observed in early inoculation with Lalvin ICV D254 before cold soak conducted at 8 °C.

We also observed an increase in the total yeast population counts during cold soak conducted at 8 °C and 12 °C, together with a change in the dynamics of *Saccharomcyes* and non-*Saccharomyces* percentages. Moreover, the composition of native *Saccharomyces* strains in musts inoculated after cold soak was diverse, as we recorded eight different molecular patterns, as well as a succession of different *Saccharomyces* strains from day 1 to day 7 during cold soak. Four and five different subpopulations of native *Saccharomyces* strains were observed, which depended on the temperature of the cold soak. Both the increase in the total population counts during cold soak and the high diversity of the native *Saccharomyces* population observed in the present study may have hindered the persistence of the commercial yeast inoculated, and this effect was more evident in the treatments inoculated after cold soak. In line with this, a previous study from our group demonstrated low persistence of a commercial ADY Lalvin ICV D254 inoculated at the end of cold soak conducted at 14 ± 1 °C (Maturano et al., 2015). Nevertheless, other studies in which alcoholic fermentation was conducted with ADY inoculation have demonstrated that inoculated commercial yeasts were responsible for the fermentation even though they did not significantly suppress the development of native populations during the first stages (Querol, Barrio, Huerta, & Ramón, 1992; Schütz & Gafner, 1993). In the present study, the strategy based on inoculation before cold soak improved the persistence of commercial strains at the end of alcoholic fermentation. Nevertheless, even if persistence of the commercial strains was improved, the diversity of the native *Saccharomyces* strains was still maintained.

The overall low persistence rates of the commercial strains herein studied was unexpected. As previously demonstrated, implantation of commercial starters is not always successful (Clavijo et al., 2011; Medina et al., 2013). Lange et al. (2014) compared implantation/persistence of different yeast inoculants among three Canadian wineries in British Columbia including 2 and 4-years comparisons. These authors found clear year-to-year variation in the inoculum implantation/persistence but they did not identify any obvious fermentation factors that would point to a causative reason for the relatively low inoculum implantation/persistence. Therefore, the overall low implantation rates found in our study may be tied to non-fermentation factors, including (but not limited to) the basic chemical composition of the must, the native yeast microflora of the clusters at harvest or the increased of native yeast population recorded during the cold soak conducted at the higher temperatures. In a related work, viable populations of non-*Saccharomyces* yeast were observed during two cold soak treatments (traditional cold soak at 8 °C during 7 days and cold soak with dry ice at 5 ± °C during 7 days). The authors observed that inoculation with a commercial *Saccharomyces cerevisiae* strain previous to cold soak (Lalvin EC-1118) appeared as effective in maintaining viable *Saccharomyces* populations during cold soak, which in combination with CO_2, avoided off-odours in the resulting wines. In line with our current findings, the results of this work suggest it is advisable to early inoculate with a commercial culture of *Saccharomyces cerevisiae* to control unwanted non-*Saccharomyces* populations (Casassa & Sari, 2015).





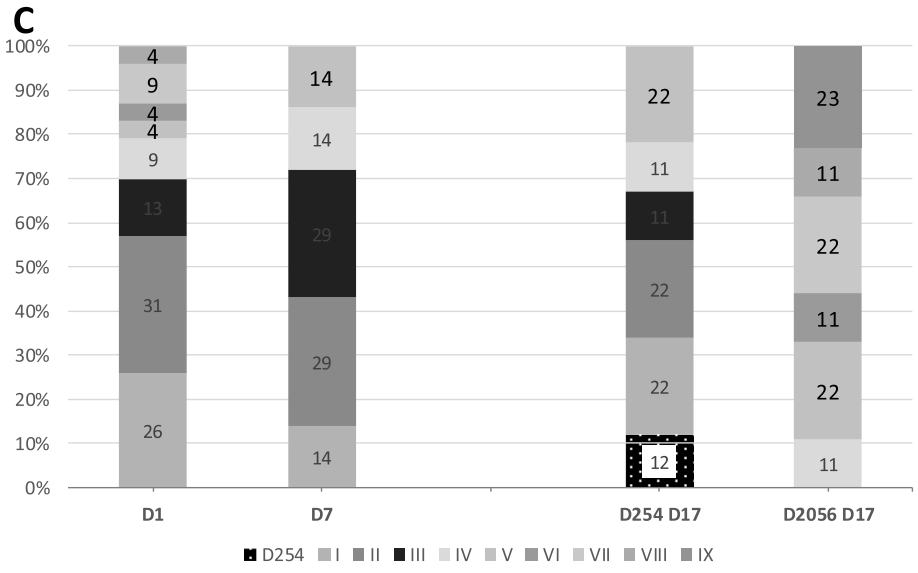


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Fig. 3. Diversity of *Saccharomyces* strains (in percentage) in red wine inoculated with commercial strains Lalvin ICV D254 and Lalvin Rhône 2056 after cold soak (ACS) conducted at three different temperatures A) 12 ± 1 °C; B) 8 ± 1 °C; C) 4 ± 1 °C including the start of cold soak (D1), the end of the cold soak (D7) and the end of the alcoholic fermentation (D17). Different interdelta PCR molecular patterns are indicated with Roman numerals.

5. Conclusions

Higher persistence of both commercial strains was observed when inoculated before cold soak, whereas low or no persistence was observed when inoculation of the strains was performed after cold soak. Although the results of the present study suggest that none of the inoculation strategies used could ensure an implantation/persistence completely successful, inoculation before the onset of cold soak may still be recommended as a sound practice if the use of cold soak, regardless of the temperature regime, is going to be part of the winemaking protocol. The two commercial strains used in this study (Lalvin ICV D254 and Lalvin Rhône 2056) showed different implantation/persistence according to the cold soak temperatures assessed, highlighting the importance of the choice of an appropriate yeast strain when cold soak is going to be part of the winemaking protocol.

Acknowledgements

This work was supported by INTA Project PNAIyAV 1130032. M.V. Mestre is a postgraduate fellowship from the National Scientific and Technical Research Council (CONICET-Argentina). The authors declare no conflict of interest.

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