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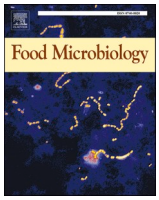

Influence of different stress factors during the elaboration of grape must's *pied de cuve* on the dynamics of yeast populations during alcoholic fermentation

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ABSTRACT

The *pied de cuve* (PdC) technique involves using a portion of grape must to undergo spontaneous fermentation, which is then used to inoculate a larger volume of must. This allows for promoting autochthonous yeasts present in the must, which can respect the typicality of the resulting wine. However, the real impact of this practice on the yeast population has not been properly evaluated. In this study, we examined the effects of sulphur dioxide (SO_2), temperature, ethanol supplementation, and time on the dynamics and selection of yeasts during spontaneous fermentation to be used as PdC. The experimentation was conducted in a synthetic medium and sterile must using a multi-species yeast consortium and in un-inoculated natural grape must. *Saccharomyces cerevisiae* dominated both the PdC and fermentations inoculated with commercial wine yeast, displaying similar population growth regardless of the tested conditions. However, using 40 mg/L of SO_2 and 1% (v/v) ethanol during spontaneous fermentation of Muscat of Alexandria must allowed the non-*Saccharomyces* to be dominant during the first stages, regardless of the temperature tested. These findings suggest that it is possible to apply the studied parameters to modulate the yeast population during spontaneous fermentation while confirming the effectiveness of the PdC methodology in controlling alcoholic fermentation.

1. Introduction

Alcoholic fermentation is characterized by a succession of microorganisms (mostly yeasts) that follow a typical pattern determined by the changes in the medium (Fleet, 2003). The grape must is a very limiting medium where many microorganisms are not able to proliferate, due mostly to high osmolarity, low pH, and a certain imbalance between sugars and nitrogen sources (Varela et al., 2004). However, some adapted microorganisms can survive and start a hurdle race only a few can finish, being recognized *Saccharomyces cerevisiae* (Sce) as the main one (Bauer and Pretorius, 2000). The first aspect of this race is that most of the yeast starts actively fermenting the must under an excess of sugar (even in the presence of oxygen due to the Crabtree effect), which in turn produces a further nutrient decrease and increases the unbalance between carbon and nitrogen sources. Presumably, the driving force behind this behaviour is most likely competition with other microorganisms to consume faster and convert sugar into biomass, in semi-anaerobic niches, and a wide temperature range (Goddard, 2008; Hagman and Piškur, 2015). In addition, the main product of alcoholic fermentation is ethanol to which many yeast are sensitive and consequently disappear as soon as its concentration increases. Both ecological interactions and physicochemical parameters are the main factors in determining the prevalence during fermentation and the contribution of each species to the final wine (Bagheri et al., 2020).

The control of this microbiological succession during alcoholic fermentation has been the objective of the winemakers since the beginning of modern winemaking. Several tools have been applied to modify or control the process highlighting the use of sulphur dioxide (SO_2) and the management of different temperatures during fermentation. SO_2 has been used since the Roman times and its different properties make it almost indispensable, mainly because of its antioxidasic, antioxidant and antiseptic activities (Boulton et al., 1999). Many microorganisms are susceptible to the toxicity of SO_2 and its use has been extended on time until our days (Fleet, 2003). Temperature is another of

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the tools that has the winemaker to regulate the dynamics of microorganisms and it has been proposed as one of the mechanisms of Sce to overrun their competitors during alcoholic fermentation (Albergaria and Arneborg, 2016; Salvadó et al., 2011). Goddard (2008) demonstrated that Sce can modify the environment to provide a favorable niche, for example, by increasing the temperature during fermentation. At temperatures above 25 °C, easily reachable during alcoholic fermentation, Sce has a clear advantage whereas, at lower temperatures, other non-Sce yeast might proliferate as well. Nowadays, winemakers tend to lower the fermentation temperature as low as 10–16 °C to preserve aroma compounds in wine or to favour the growth of non-*Saccharomyces* (non-Sce) yeast that might provide complexity to the final product, especially for white wines (Beltran et al., 2008; Edwards and Aplin, 2022). The inherent risk of favouring less fermentative yeasts is nutrient depletion before alcoholic fermentation is finished (Gobert et al., 2017; Roca-Mesa et al., 2020).

Finally, the microbial succession can be also controlled by the main achievement in general of the fermentation industry: the use of starter cultures. Different starter cultures have been used, but the main advancement was the development of the Dry Wine Yeast (DWY), which provided a very cellar-friendly application (Fleet, 2008; Mas et al., 2016; Suárez-Lepe and Morata, 2012). Currently, a reasonable but limited number of strains are available as commercial DWY. Nevertheless, the use of these DWY has produced concern due to uniformity in the sensorial profile of wines by the inoculation of one single strain (Belda et al., 2017). Additionally, the use of indigenous yeast can improve the microbial biodiversity in the selected region and contribute the *low input* winemaking strategies with differentiated flavours and aromas. This fact is important in a highly competitive market for the winery industry aiming at wine appellation and production of unique wines linked to the *terroir* concept in the context of organic and biodynamic wine production (Comitini et al., 2017). *Terroir* concept refers to the regional or even local characteristics of climate, soil, grape variety, and even the *microbial terroir*, which could be specific to a given plot (Bokulich et al., 2012; Bozoudi and Tsaltas, 2016; Mas et al., 2020).

Alternatively, this winemaker's tendency to get away from the use of DWY has led to the recovery of the traditional technique known as *pied de cuve* (PdC) as a method to both incorporate regional traits and some kind of microbiological control of the fermentation (Mas and Portillo, 2022). PdC is a French term that refers to a method of indirect inoculation through an inoculum made from must that is already fermenting (Clavijo et al., 2011; Li et al., 2012; Ubeda Iranzo et al., 2000). The fermenting must can be obtained either by DWY inoculation on a small volume of must or from vats fermenting spontaneously using grape berries that, usually, are harvested earlier. Most of the studies have been conducted in sparkling wines using the addition of nitrogen sources, different commercial DWY strains and grape varieties, or ethanol addition (Benucci et al., 2016; Martí-Raga et al., 2015; Moschetti et al., 2016). However, there are very few studies that analyse the best conditions of the PdC to provide an appropriate yeast population to be used as inoculum, and most of them just used one modality of PdC or evaluated the effect of one parameter over the kinetic and population of the fermentation (Abdo et al., 2020; Börlin et al., 2020; Morgan et al., 2019; Moschetti et al., 2016; Li et al., 2012).

The present work aims to analyse the effect of different tools on the hands of the winemakers to determine the appropriate conditions to provide a PdC with the optimal population of yeast to be inoculated into the grape must. According to our design, the optimal yeast population of PdC should harbor a high number of cells, allow the influence of autochthonous yeast diversity at the beginning of the fermentation and, finally, the selection of vigorous or moderate fermentative species to ensure the correct ending of the alcoholic fermentation. With this aim, fermentations have been carried out using the PdC technique involving the use of synthetic and reconstituted concentrated must inoculated with a defined yeast consortium under different stressing conditions of SO_2, temperature, and the presence of ethanol. After that, we validated the results through the combination of some of these parameters in uninoculated PdC of Muscat of Alexandria must.

2. Material and methods

2.1. Laboratory experimentation

Laboratory fermentations were prepared either in the synthetic must medium (SM; Riou et al., 1997) or reconstituted concentrated must (RCM). The SM had 300 mg/L of yeast-assimilable nitrogen (YAN), and 200 g/L of total sugar (100 g/L of glucose and 100 g/L fructose) and its pH was adjusted to 3.3. The RCM was purchased sterile at The Syrup Factory (Reus, Spain) and once diluted at 1:4 with sterile water, it had 152 mg/L of YAN, 107 g/L of glucose, and 123 g/L of fructose.

The laboratory fermentations involved three steps (Fig. 1. A): i) preparation of a yeast consortium, inoculated at a concentration of 2×10^6 cells/mL into the SM or RCM to initiate an inoculated preliminary fermentation or *pied de cuve* (PdC); ii) conducting the PdC under each stress conditions or a combination of them iii) inoculation of 2% (v/v) of the PdC into a new RCM must to perform the alcoholic fermentation (AF).

We think that the SM allows for better monitoring of the fermentation without the interference of the matrix. However, RCM was chosen for the analysis of the combination of the selected parameters and the alcoholic fermentation because it represents a matrix more similar to the natural must than the SM.

2.1.1. Yeast consortium used for laboratory fermentations

Laboratory fermentations were inoculated with a yeast consortium consisting of a representation of the most abundant yeast species at Catalonia fresh must and beginning of alcoholic fermentation. These yeasts and their relative proportion in fresh must were determined based on previous studies that used molecular biology and next-generation sequencing techniques (Abdo et al., 2020; Lleixà et al., 2018; Portillo and Mas, 2016; Wang et al., 2015b). The strain *Saccharomyces cerevisiae* CECT 13132 and six non-Sce yeast: *Hanseniaspora uvarum* CECT 10389*; Starmerella bacillaris* CECT 11109*;* the saprophytic yeast-like *Aureobasidium pullulans* CECT 2660;*Lachancea thermotolerans* Lt2 and *Torulaspora delbrueckii* Td5 (Agrovin S.A, Ciudad Real, Spain); *Metschnikowia pulcherrima* CECT 13131 were selected to create a consortium representative of the most abundant yeasts. Some of these strains were previously isolated from the Priorat region (Catalonia) with a Qualified Denomination of Origin (DOQ) (Padilla et al., 2016) and deposited in the Spanish Type Culture Collection (CECT).

Yeasts were characterized morphologically with Wallerstein Laboratory Nutrient Agar (WLN) medium (Difco Laboratories, Detroit, MI, USA). This medium allows for the differentiation of these species based on their morphology and their identification was confirmed by ITS-PCR (Esteve-Zarzoso et al., 1999) during the setting up of this study. For each essay, a single colony of each yeast was grown overnight in a YPD liquid medium (1% yeast extract, 2% peptone, 2% dextrose, all from Biogenetics, Milan, Italy) at 28 °C. After 24 h, the cultures were refreshed in YPD liquid and then, were co-inoculated for each of the designed essays with a total population of 2×10^6 cells/mL. The composition of the consortium used for laboratory inoculated fermentations was as follows: 50% *H. uvarum*, 20% *S. bacillaris*, 15% *A. pullulans*, 4% *L. thermotolerans*, 5% *M. pulcherrima*, 4% *T. delbrueckii*, 2% *S. cerevisiae*. However, *A. pullulans* disappeared during the first hour of fermentation, so it was removed from the mixture. Consequently, the proportions of the other species were adjusted accordingly: 50% *H. uvarum*, 30% *S. bacillaris*, 5% *L. thermotolerans*, 5% *M.pulcherrima*, 8% *T. delbrueckii*, and 2% *S. cerevisiae.*

2.1.2. Pied de cuve preparation

The different PdCs using stress conditions individually or combined were carried out in triplicate in 500 mL glass bottles, each filled with

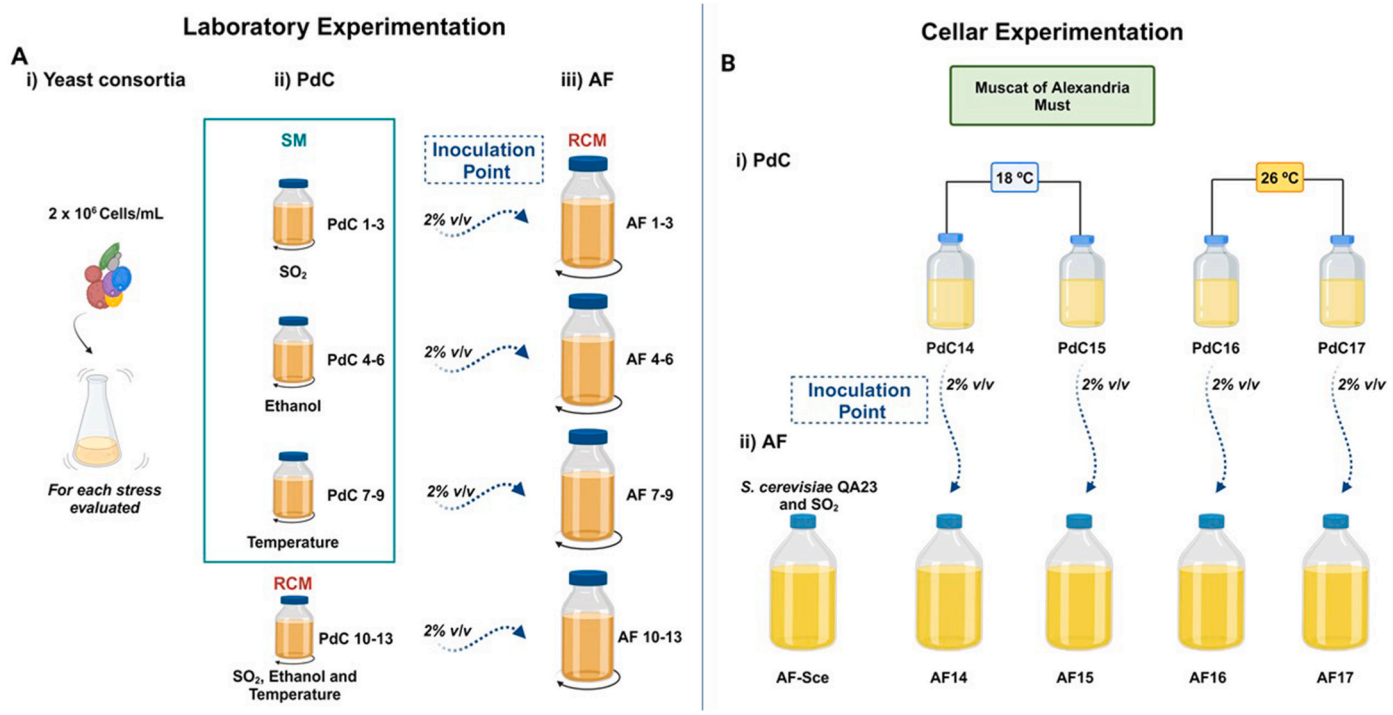

Fig. 1. Diagram illustrating the process followed to prepare the different *pied de cuve* (PdC) and alcoholic fermentation (AF) at laboratory experimentation (A) and cellar experimentation (B). Every condition was tested in triplicated. Figure created with BioRender.com.

400 mL of SM or RCM and agitated at 120 rpm.

2.1.2.1. Evaluation of single parameters. We evaluated the effect of different parameters individually on the performance of PdC using SM inoculated with the yeast consortium described in the previous subsection (Fig. 1. A).

We selected the optimal parameters in the PdC to obtain a good fermentation performance (high yeast cell number allowing the presence of non-Sce and good rates of sugar consumption in time). For each parameter, three different PdCs were prepared and fermented at 22 °C. These parameters included SO_2 concentrations of 0, 40, and 80 mg/L (PdC1, PdC2, PdC3, respectively) added as sodium metabisulphite. The selected SO_2 concentration was tested at different ethanol levels of 0, 0.5, and 1% (v/v) resulting in PdC4, PdC5, and PdC6, respectively. The selected SO_2 and ethanol concentrations were tested at temperatures of 16, 22, and 28 °C corresponding to PdC7, PdC8, and PdC9, respectively.

2.1.2.2. Evaluation of the combination of parameters. After identifying the optimal parameters in the PdC using SM to obtain a good fermentation performance, we evaluated the combination of those parameters in RCM to perform different PdCs (Fig. 1. A). These combinations included 4 different conditions: without and with the addition of 40 mg/L of SO_2 and 1% (v/v) of ethanol at 18 °C (PdC10 and PdC11, respectively), and at 26 °C (PdC12 and PdC13, respectively). Those temperatures were the fermentation temperatures at the cellar for white and red grape varieties, respectively.

2.1.3. Laboratory alcoholic fermentations

The time of the inoculation of PdC into RCM to perform the main AF was evaluated for 48 and 72 h at 22 °C in the presence or absence of 40 mg/L of SO_2 and 1% (v/v) ethanol. The best time for the inoculation of the PdC was considered when a high cell density and a drop in the must density indicated a clear start for the fermentation. We selected these factors as the criteria for the inoculation of PdC in subsequent experiments. Additionally, we consider that the selected time of inoculation allowed the imposition of Sce at the end of the AF.

After the selection of the best time for PdC inoculation into RCM to perform the AF, PdCs1-13 prepared under each condition (with individual or combined parameters using SM or RCM, respectively) were inoculated at 2% (v/v) into new RCM to perform the main alcoholic fermentation (AF1-13). All the AFs were carried out under standardized conditions of 22 °C and 120 rpm (Fig. 1. A). An additional AF was inoculated with 2×10^6 cells/mL of the commercial DWY.

S. cerevisiae strain QA23 (Lallemand Inc., Montreal, Canada) was used as the control for each tested condition (AF-Sce).

2.2. Cellar experimentation

The cellar experimentation was performed at the experimental cellar of the University Rovira I Virgili using Muscat of Alexandria must harvested from the 2022 vintage. The natural must had 199.8 g/L total reducing sugars, pH 3.47, and YAN 102.5 mg/L. During grape pressing, 40 mg/L of SO_2 and 20 mg/L of pectolytic enzymes (Lallzyme, Lallemand Inc., Montreal, Canada) were added. The must was then allowed to settle down for at least 24 h at 8 °C. After 24 h of must settling, the total and free SO_2 levels were less than 5 mg/L.

The cellar experimentation included two steps (Fig. 1. B): i) fresh natural must was subjected to spontaneous fermentation in triplicate (PdC) under 4 specific conditions. ii) 2% (v/v) of each of the 4 PdCs were inoculated into new fresh natural must to perform the main AF, also in triplicate.

2.2.1. PdC preparation using natural must

The combined parameters selected to perform the PdC in RCM at laboratory experimentation were also tested in un-inoculated fresh natural must to prepare 4 different PdCs (Fig. 1. B). For that, 700 mL of fresh must was subjected to spontaneous fermentation in triplicate under 4 different conditions: without and with the addition of 40 mg/L of SO_2 and 1% (v/v) of ethanol at 18 °C (PdC14 and PdC15, respectively), and 26 °C (PdC16 and PdC17, respectively). As mentioned previously, these temperatures were the usual for white and red vinifications, respectively at the cellar. The 1% (v/v) of ethanol

supplementation in the cellar experimentation was done by adding the corresponding volume of wine containing 10% (v/v) of ethanol.

2.2.2. Alcoholic fermentations using natural must

2% (v/v) of the combined triplicates of each of the 4 different PdC (PdC14-17) was inoculated to 3 L of fresh natural must contained in 5 L bottles to perform the AFs (AF14-17) of freshly pressed grape must prepared as described in the previous subsection. The time for the inoculation of the PdC was done based on the results of the previous evaluation (subsection 2.1.2.2). The four different AFs were carried out in triplicate at 18 °C (Fig. 1. B).

Additionally, a fresh batch of clarified must supplemented with 40 mg/L of SO_2 was inoculated with 2×10^6 cells/mL of the commercial DWY *S. cerevisiae* strain QA23 (AF-Sce). The AF-Sce was also performed in triplicate and incubated at 18 °C to serve as a control of the AF performed with 2% (v/v) of the 4 different PdCs.

2.3. Fermentation monitoring

2.3.1. Fermentation kinetics and chemical analysis

Fermentations were monitored daily by measuring must density (electronic densitometer, Densito 30PX Portable Density Meter; Mettler Toledo, Barcelona, Spain) and considered to be finished when must density was lower than 1000 g/L and residual sugars were enzymatically determined to be less than 2 g/L.

Musts were chemically analysed before AF. Total sugars and YAN were quantified using the Y15 Bioanalyzer with the corresponding enzymatic kits (BioSystems S.A, Barcelona, Spain).

2.3.2. Yeast population dynamics

The yeast population of AF was sampled at four different stages, based on the density of the must: the beginning (BF; 1100-1080 g/L), middle (MF; 1050-1040 g/L), and final fermentation stage (FF; <1000 g/L). In the case of PdC, the time when the 2% (v/v) was inoculated into new must (IP) was included for sampling. Nevertheless, both PdCs and AFs were monitored till the end of their fermentation.

Various methods were used to track the population depending on the experimentation. In the case of laboratory experimentation, we used microscopy counting using a Neubauer chamber and plating of 100 μL of each triplicate of the samples serially diluted in three different solid culture media. The total viable yeast population was counted using a YPD solid medium (17 g/L agar). The WLN medium was used to count and morphologically differentiate the known species used for laboratory experimentation. To slow down the growth of Sce and to make it easier to monitor the non-Sce species, the lysine-agar medium (LYS, Oxoid Ltd, Basingstoke, UK) was used (De Angelo and Siebert, 1987). All plates were incubated at 28 °C, and the colony counting (CFU/mL) in WLN medium was done after 4 days to visualize differences between species' morphologies. The YPD and LYS media were incubated for 3 and 5 days, respectively, before colony counting (CFU/mL). Additionally, we performed a test of qPCR analysis with some PdCs prepared using SM inoculated with the yeast consortia. The objective of this test was to compare the results obtained by CFU counting in WLN medium with the qPCR analysis of the same samples. Based on the results of this test, qPCR was discarded for laboratory experimentation because it did not detect of yeast decreasing at the final stage of the fermentations as compared with CFU counting.

In the case of cellar experimentation, we used total cell count by microscopy, viable yeast on YPD and LYS medium (CFU/mL), and the quantitative PCR (qPCR) to quantify and follow the general trend of the most abundant yeast genera detected previously at Catalonian cellars by next-generation sequencing and molecular techniques. In the case of AFs performed to evaluate the best time for PdC inoculation, three different PdCs were prepared: without any additions, with 40 mg/L of SO_2 and 1% (v/v) ethanol, and with 1% (v/v) ethanol. For these PdCs, just CFU/mL were determined in WLN medium for each of the inoculated strains at the end of the fermentation.

2.3.3. Quantitative PCR analysis

Those samples selected for qPCR analysis were used for DNA extraction and qPCR using primers that targeted the genera of the main yeast described previously in Catalonian must fermentation (Abdo et al., 2020; Lleixà et al., 2018; Portillo and Mas, 2016; Wang et al., 2015b).

Cell pellets from 1 mL sample were washed twice with sterile distilled water before DNA extraction using the DNeasy Plant Mini Kit following the manufacturer's protocol (Qiagen, Hilden, Germany). qPCR was performed in a QuantStudioTM 5 real-time PCR instrument (Applied Biosystems by Thermo Fisher Scientific, Waltham, MA, USA). TB Green TM Premix Ex TaqTM II (Takara Bio Inc., Kusatsu, Japan) was used following the manufacturer's instructions (Applied Biosystems, Foster City, CA, USA). The primers used for the quantification of each species were those described previously for Sce at the species level and *Hanseniaspora* at the genus level (Hierro et al., 2007); for *S. bacillaris* (Andorrà et al., 2010a), for *T. delbrueckii* (Zott et al., 2010), for *M. pulcherrima* and *L. thermotolerans* (García et al., 2017). The cell concentrations of each DNA sample were determined in triplicate by automatically calculating the cycle threshold (CT). To create standard curves for each species, a tenfold serial dilution of DNA 10^7 to 10^2 cells/mL was used, and each dilution was assayed in triplicate. The average of the CT measurements was used to quantify the cell concentrations. The program developed by Thermo Fisher Scientific was used to visualize the results in Biosystems, and parameter values for the calibration curves for each species can be found in Supplementary Table S1.

2.4. Molecular typing and diversity of S. cerevisiae isolates from natural must

The biological replicates of the different PdCs and the corresponding AF, were sampled and inoculated on solid YPD medium plates. Ten colonies were randomly picked from all the biological replicates from the PdC at the selected time for PdC inoculation into new must (IP). In the case of AF, the sampling points were BF, MF, and FF as described at the population dynamics section. Cell lysis of the isolates was performed as previously described by García et al. (2017). Briefly, the lysis consisted of a couple of washes of pellets from colonies (cultured by 48 h) with sterile water, and then samples were disrupted by 3 cycles of 1 min at a Mini-bead-beater-16 (Biospec Products, Inc., Bartlesville, OK, USA). After 2 min of centrifugation at 10.000 rpm, just 400 μL of supernatant was recovered and conserved at −20 °C until molecular identification.

2.4.1. Differentiation of Sce and non-Sce isolates

In the first place, the isolates were considered to be different depending on their morphology in WLN medium. After that, isolates were identified as Sce or non-Sce yeast based on the amplicon size of the ITS-5.8S rDNA region (Esteve-Zarzoso et al., 1999). For those isolates with different morphology but the same amplicon size of the ITS-5.8S rDNA region, additional DNA digestions using HinfI, HaeIII, and CfoI enzymes were performed, as described in the referenced article. The PCR products were separated on a 1.5% agarose gel stained with 0.04 μL/mL of GreenSafe Premium (Nzytech, Lisboa, Portugal) in 1X Tris-borate-EDTA buffer. The DNA ladder marker used was 100-bp (Thermo Fischer Scientific Inc., Madrid, Spain) and the electrophoresis was conducted at 100 V for 1 h and the gels were visualized under UV light.

2.4.2. Diversity analysis of Sce isolates

Those colonies identified as Sce by their amplicon size were genetically differentiated by an Interdelta-PCR analysis. The Interdelta-PCR analysis for*S. cerevisiae* strains typification was performed using the primers delta 12 and 21 and the PCR program previously described (Legras and Karst, 2003). All the PCR reactions were performed in a

thermocycler 2720 Thermal Cycler (Applied Biosystems, Thermo Fischer Scientific Inc., Madrid, Spain). The PCR products were visualized as explained in the previous subsection and the images of the Interdelta profiles were saved for their analysis. After gels were imaged-scanned, the clustering of profiles was done using the GelJ v 2.0 program (Department of Mathematics and Computer Science at the University of La Rioja, Logroño, Spain). Dark images of poor-quality agarose gels were discarded after the first draft of the dendrogram comparing the different Interdelta profiles. The final dendrogram was constructed with the unweighted pair group method with arithmetic mean (UPGMA) (Guzzon et al., 2018). The matrix of similarity was constructed on DICE coefficients known as the similarity coefficient explained by Nei and Li (1979). A band was deemed to be present within a population if it had been successfully amplified in at least one individual from that population. The criterion chosen to determine the cut-off level for grouping the Sce isolates into the same strains was a 90% or higher similarity in their Interdelta-PCR profiles. The reproducibility of the Interdelta polymorphism was checked by including duplicates of 11 profiles selected from different agarose gels.

To assess the diversity of Sce strains during PdC fermentations in natural must, the total number of Interdelta-PCR patterns obtained from the aforementioned sampled points was used to calculate the Shannon index (H'), evenness (J) and Simpson index using the formulas described in Börlin et al. (2016).

2.5. Statistical analysis and area under the curve

All the fermentations were performed in triplicate to improve the consistency of the results. Statistical analysis of qPCR data and area under the curve (AUC) was performed using ANOVA and the Tukey test with XLSTAT version 2022.5.1 software (Addinsoft, Paris, France). A *p*-value of less than 0.05 was considered statistically significant. The area under the curve (AUC) was calculated to assess significant differences in the fermentation performance. This was achieved by analysing the decrease in density during fermentation and then integrating the values between two consecutive time points (Ruiz de Villa et al., 2023).

3. Results

3.1. Selection of parameters from laboratory experimentation in SM

The evaluation of the effect of different parameters on PdC performance was carried out in SM inoculated with a consortium of 6 non-Sce and one Sce strains (Fig. 1. A). These parameters were SO_2, ethanol supplementation, and temperature and their impact on the dynamics of the inoculated yeasts and the fermentation kinetics were evaluated during PdCs fermentations (PdC1-9), and the corresponding AFs (AF1-9) inoculated with the 2% (v/v) of those PdCs (Fig. 2). The objective was to select those parameters that allowed the presence of non-Sce at the beginning of the AF while achieving the highest possible yeast population. Additionally, we aimed for the completion of fermentation within a reasonable timeframe thanks to the selection of moderate and vigorous fermentative yeasts.

3.1.1. Impact of sulphur dioxide supplementation on PdC

The PdC1-3 evaluated the effect of different concentrations of SO_2 over the dynamic of the inoculated yeast consortium and the fermentation kinetics. Only the highest concentration (80 mg/L SO_2) affected

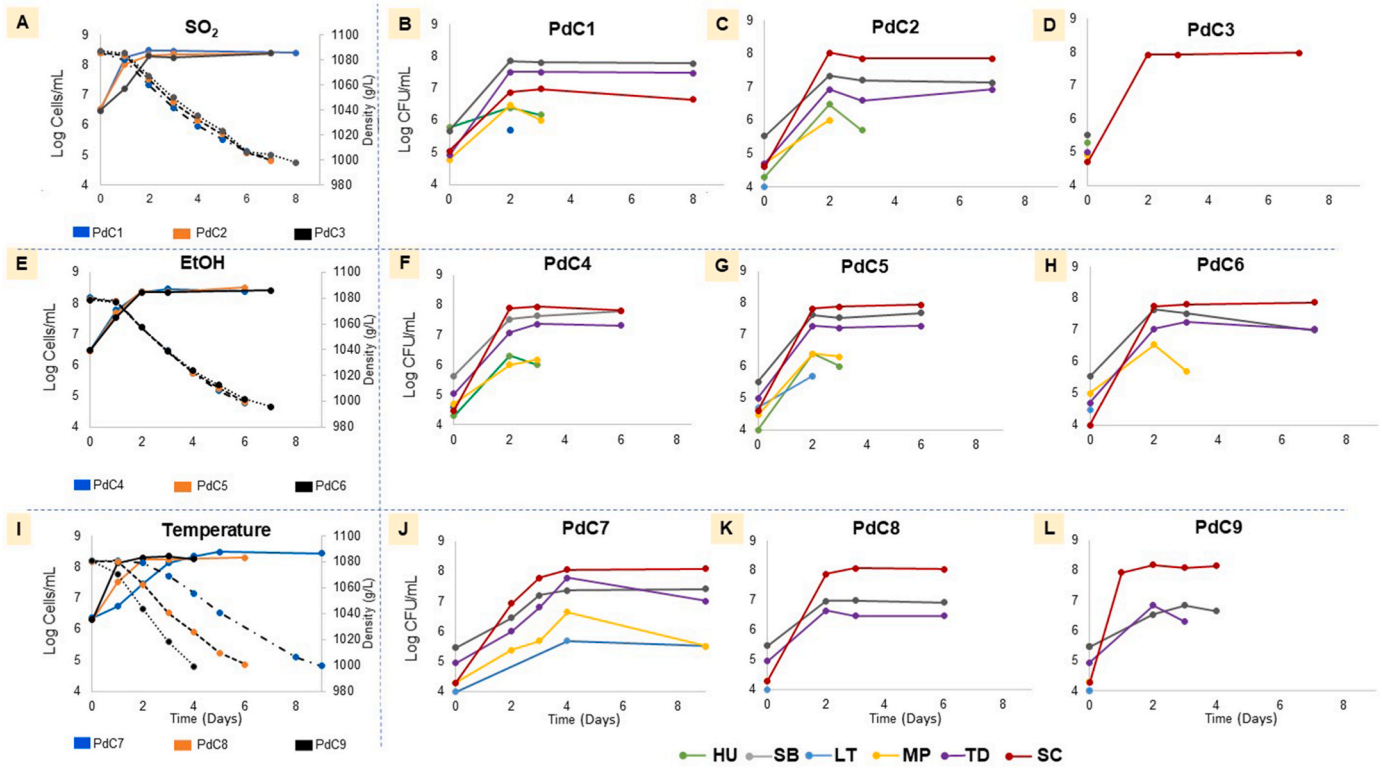

Fig. 2. Impact of sulphur dioxide (SO_2, A), ethanol (EtOH, E) and temperature (I) on fermentation kinetics and total yeast population during preparation of PdCs using synthetic must (SM). Solid lines stand for the number of cells quantified at the microscope while dashed lines stand for the density (g/L). PdC1, PdC2, PdC3 were fermented at 22 °C with SO_2 concentrations of 0, 40, and 80 mg/L, respectively, added as sodium metabisulphite. PdC4, PdC5, and PdC6 were fermented at 22 °C with 40 mg/L SO_2 h and 0, 0.5, and 1% (v/v) of ethanol. PdC7, PdC8, and PdC9 had 40 mg/L SO_2 and 1% (v/v) of ethanol and were fermented at 16, 22, and 28 °C, respectively. CFU/mL in WLN medium for the non-*Saccharomyces* (non-Sce) and *Saccharomyces* (Sce) strains in each prepared PdC evaluating the effect of SO_2 (B–D), ethanol (F–H) and temperature (J–L). Every value in the graphs corresponds to the mean of biological triplicates. HU, *Hanseniaspora uvarum; SB, Starmerella bacillaris;* LT, *Lachancea thermotolerans;* MP, *Metschnikowia pulcherrima;* TD, *Torulaspora delbrueckii*; SC, *Saccharomyces cerevisiae*.

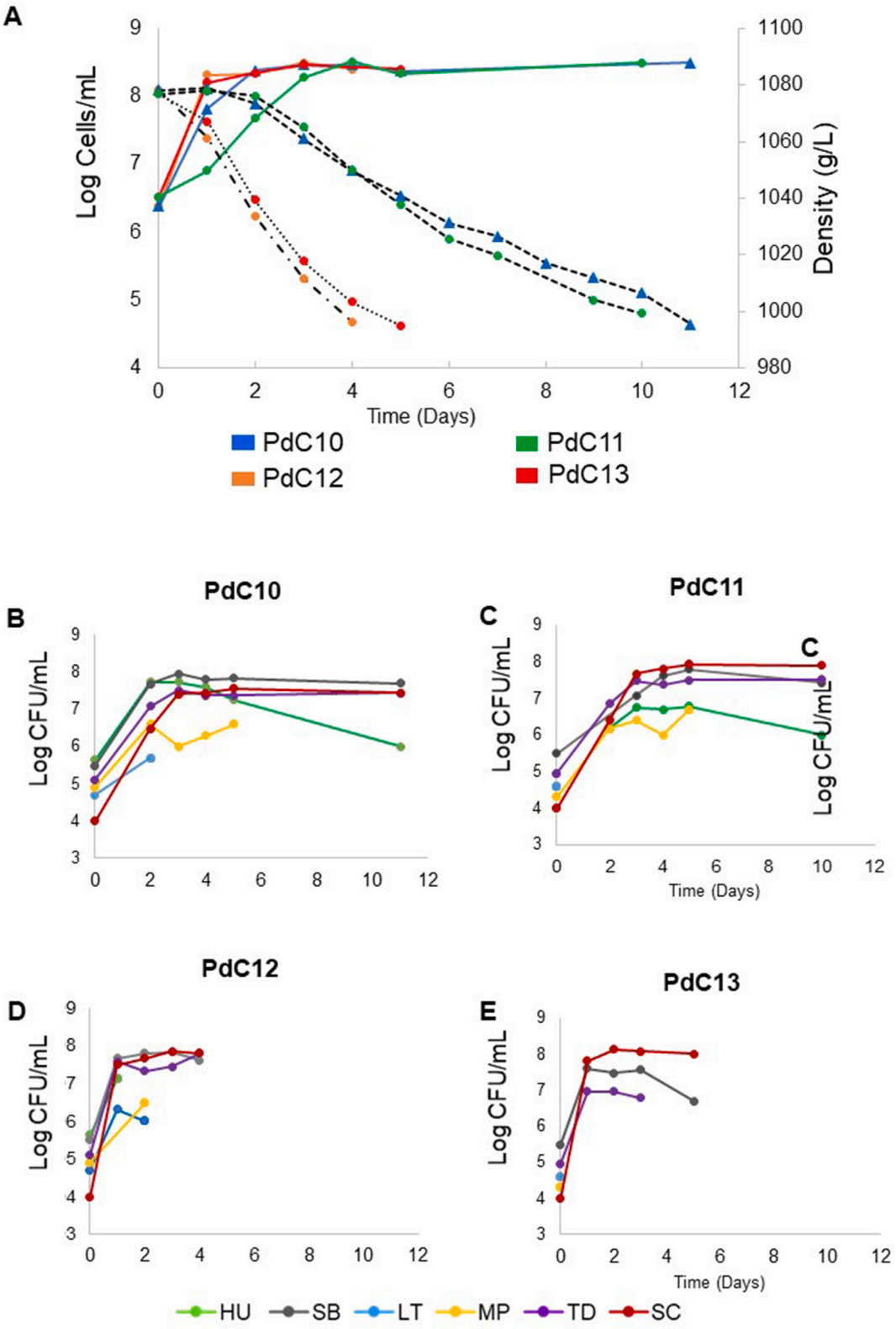

Fig. 3. (A) Combined effect of sulphur dioxide (SO_2), ethanol and temperature on PdC10-13 using RCM medium. The PdC10 and PdC12 were carried out spontaneously without SO_2 or ethanol at 18 and 26 °C, respectively. PdC11 and PdC13 were supplemented with 40 mg/L of SO_2 and 1% (v/v) ethanol. The yeast population is represented by the number of cells quantified under the microscope (solid lines) and fermentation kinetic is represented by the density (g/L) of the fermenting must (dashed lines) during the PdCs. (B, C, D and E) CFU/mL count in WLN medium of the non-Sce and Sce strains inoculated in PdC using RCM. Every value in the graphs corresponds to the mean of biological triplicates. HU, *Hanseniaspora uvarum*; SB, *Starmerella bacillaris*; LT, *Lachancea thermotolerans*; MP, *Metschnikowia pulcherrima*; TD, *Torulaspora delbrueckii*; SC, *Saccharomyces cerevisiae*.

the overall dynamics of the yeast population and resulted in a notable delay of the yeast growth for the first 48 h of the PdC3 (Fig. 2. A). However, the maximum population for PdC1-3 was attained on the second day and did not show significant differences. The presence of SO_2 also affected fermentation kinetics, causing a one-day delay in completing PdC3 (Fig. 2. A). When examining the impact of SO_2 on each yeast species inoculated in the PdCs (measured as CFU/mL recovered on WLN solid medium) the inoculated Sce strain dominated at both PdC2 and PdC3 (Fig. 2. B and Fig. 2. C). Nevertheless, 40 mg/L of SO_2 allowed some of the non-Sce strains to be present until the end of the PdC2 (*S. bacillaris* and *T. delbrueckii*), while culturable *M. pulcherrima* and *H. uvarum* were detected up to 48 h of PdC2. On the other hand, PdC3 selected the inoculated Sce strain exclusively (Fig. 2. D).

Based on SO_2 supplementation results, the rest of the parameters were tested using 40 mg/L of SO_2 to have an advantageous selection of Sce fermentative yeasts while keeping most non-Sce strains that could maintain the microbial diversity of the PdC.

3.1.2. Impact of ethanol supplementation on PdC

After analysing the SO_2 results, the next step was to evaluate the effect of adding ethanol to PdCs. Concentrations of 0%, 0.5%, or 1% (v/v) of ethanol were used in the PdC4, PdC5 and PdC6, respectively, using 40 mg/L SO_2 and 22 °C. The effect of ethanol was negligible in both the growth rate and the maximum total yeast population as determined by microscopy counting of PdCs samples (Fig. 2. E). Furthermore, similar fermentation kinetics were observed for PdC4 and PdC5. However, PdC6 increased the time to complete the fermentation process by one day, indicating a significant difference ($p < 0.05$) (Fig. 2. E).

As observed by CFU counts on WLN medium of PdC5 and PdC6 (Fig. 2. G and Fig. 2. H) the Sce strain was not affected by the ethanol concentration at all but, within the non-Sce strains, some resulted being more sensitive than others (Fig. 2. H). *H. uvarum, S. bacillaris*, *M. pulcherrima* and *T. delbrueckii* were not affected in PdC5. However, culturable cells of *H. uvarum* and *L. thermotolerans* were not detected in PdC6 (Fig. 2. H).

Based on these results, it seems that 1% (v/v) ethanol allowed the presence of some of the inoculated non-Sce till the end while selecting a high number of fermentative yeasts. Thus, 1% (v/v) of ethanol was selected to supplement the PdC from now on.

Additionally, we performed a test with qPCR analysis to monitor the inoculated yeast in some of the PdCs (PdC1-6) using SM (Fig. S1) and compared the results to the Neubauer and CFU counting. Although the total yeast number assessed by qPCR (Fig. S1) was similar to Neubauer counting (Fig. 2 A and B), the qPCR results did not detect the decrease in the number of culturable non-Sce reported by the CFU counts at the final stage of the fermentation. Therefore, for laboratory inoculated fermentations, we chose to primarily rely on the Neubauer and CFU/mL count methods to determine the total number and dynamics of yeast during fermentation, respectively.

3.1.3. Impact of temperature on PdC

The effect of the temperature on PdCs using SM was also evaluated resulting in significantly different ($p < 0.05$) fermentation kinetics as shown in Fig. 2. I. Thus, the PdC7, PdC8 and PdC9 finished on the 4th, 6th' and 9th days, respectively (Fig. 2. C). The lower temperature also delayed the growth rate of the total yeast population. However, all three PdCs reached a similar maximum number of yeasts ($2.2–3.1 \times 10^8$ cells/mL) and PdC8 and PdC9 achieved that maximum during the second day (Fig. 2 H and I). The abundance of non-Sce organisms was found to be impaired at higher temperatures, as shown in Fig. 2. J. In PdC7, viable, *M. pulcherrima*, *S. bacillaris*, and *T. delbrueckii* were detected till the end of the fermentation. However, in PdC8 and PdC9, only the latter two non-Sce species were detected, and their numbers decreased at higher temperatures (Fig. 2. K and Fig. 2. L). The Sce strain number, on the other hand, remained unaffected by any of the tested temperatures (Fig. 2. J-L).

Lower temperature favoured the non-Sce permanence at the PdC and higher temperature shortened the fermentation time and the lag phase of *S. cerevisiae* growth. Therefore, we decided to test the selected SO_2 and ethanol concentrations using RCM at both temperatures but adjusting them to the temperatures used at the cellar for white and red vinifications (18 °C and 26 °C, respectively).

3.2. Combination of selected parameters using RCM

After identifying the parameters that favour the presence of non-Sce yeasts and good fermentation performance in SM, those parameters were applied using RCM (Fig. 1. A).

Fig. 3 shows the PdCs using RCM without SO_2 or ethanol supplementation and fermented either at 18 °C (PdC10) or 26 °C (PdC12). The results of these fermentations were compared to RCM supplemented with 40 mg/L of SO_2 and 1% (v/v) ethanol incubated at either 18 or 26 °C (PdC11 and PdC13, respectively). The yeast population reached its maximum during the first day in PdC12 and PdC13, regardless of the addition of SO_2 and ethanol. On the other hand, PdC10 reached its maximum of cells on the second day while PdC11 reached it during the fourth day (Fig. 3. A). The stronger effect on the fermentation kinetics was also exerted by the temperature leading to the end of the fermentation on the 4th and 5th days for PdC12 and PdC13, respectively. This order was inverted at 18 °C, and the supplemented PdC (PdC11) finished the 10th, while PdC10 finished one day later. The CFU/mL counts for each strain on WLN medium were consistent with the individual parameters evaluated (Fig. 3. B-E). In PdC10, the non-Sce strains were favoured over the Sce strain (Fig. 3. B). When SO_2 and ethanol were added in PdC11, it resulted in a moderate inhibition of the growth of most of the non-Sce but less inhibition than at 26 °C. The Sce strain maximum population was favoured by supplementation with SO_2 and ethanol at 18 °C (Fig. 3. C), but its growth rate was slower than at 26 °C (Fig. 3. E). At the highest temperature (PdC12 and PdC13), the Sce population remained unaffected by the addition of SO_2 and ethanol to the RCM. In PdC12, *T. delbrueckii* and *S. bacillaris* were present till the end of fermentation in a high proportion, while the other non-Sce strains decreased on the first or second day (Fig. 3. D-E). However, when the RCM was supplemented at the highest temperature (PdC13), the populations of *T. delbrueckii* and *S. bacillaris* stopped growing on the first day, and *T. delbrueckii* disappeared by the 3rd or 4th day of fermentation. Furthermore, the rest of the non-Sce strains were not detected during fermentation in PdC13 (Fig. 3. E).

3.3. Time effect of PdC inoculation

The effect of the time selected for the addition of PdC to fresh must at 2% (v/v) to perform the AF was tested either for 48 or 72 h using RCM. The PdCs were carried out at 22 °C and subjected or not to the influence of the SO_2/ethanol concentrations selected in the previous experiments. The results indicated that there were no significant differences in fermentation kinetics between AFs inoculated with the PdCs using combined parameters (40 mg/L SO_2 and 1% (v/v) ethanol) at 48 or 72 (Supplementary Fig. S2). The proportion of the recovered strains on WLN medium at the end of the AFs showed slight differences due to the inoculation time of PdC (Fig. 4). Notably, when PdC was supplemented with SO_2 and ethanol, 95–100% of the CFU/mL were Sce at the end of the AF. Based on these findings, the 48-h time point appeared to be a good time to incorporate spontaneous fermentation to the fresh must at 2% (v/v) because a higher proportion of Sce was recovered at the end of the AF. Additionally, during the monitoring of the PdCs performed at 22 °C (PdC1-6 and PdC8) that is also the temperature used for the evaluation of inoculation time, we realized that at 48 h all the PdCs had reached over 10^8 cells/mL and experienced a decrease in must density of, at least, 15 g/L, regardless of the treatment (Fig. 3. A). As a result, we used these parameters as the main criteria to inoculate the PdC into the fresh must at cellar experimentation because spontaneous PdCs usually

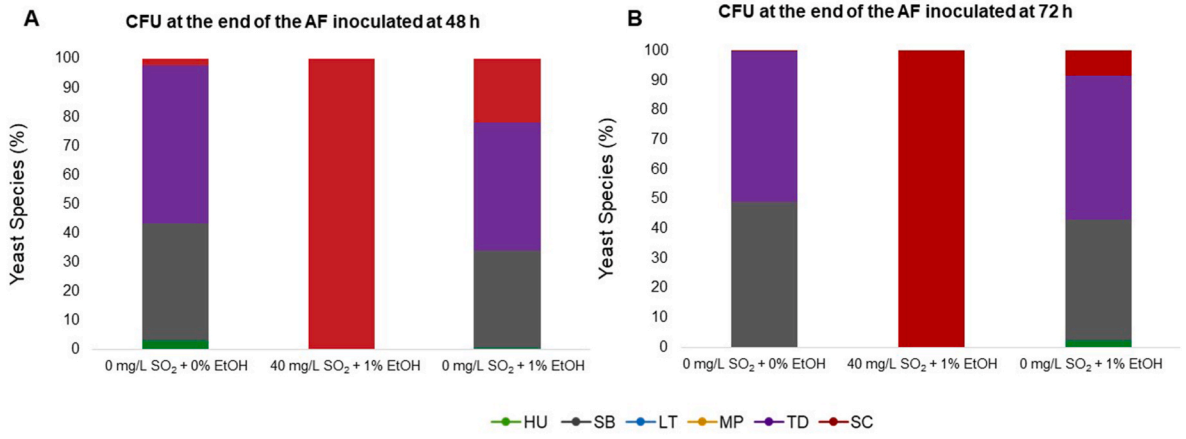

Fig. 4. Impact of inoculation time of PdC on the yeast population measured as CFU/mL on WLN medium at the end of alcoholic fermentation (AF) using RCM. The AFs were inoculated at 48 h (A) or 72 h (B) with PdCs prepared with or without the influence of sulphur dioxide (SO_2) and ethanol (EtOH). Every value in the graphs corresponds to the mean of biological triplicates. HU, *Hanseniaspora uvarum*; SB, *Starmerella bacillaris*; LT, *Lachancea thermotolerans*; MP, *Metschnikowia pulcherrima*; TD, *Torulaspora delbrueckii*; SC, *Saccharomyces cerevisiae*.

experience a delay in the starting of AF.

3.4. Impact of different PdCs on the performance of AF during laboratory experimentation

The PdC1-13 prepared as described in subsection 2.1.3 were inoculated at 48 h into new RCM to perform the corresponding AFs (AF1-13).

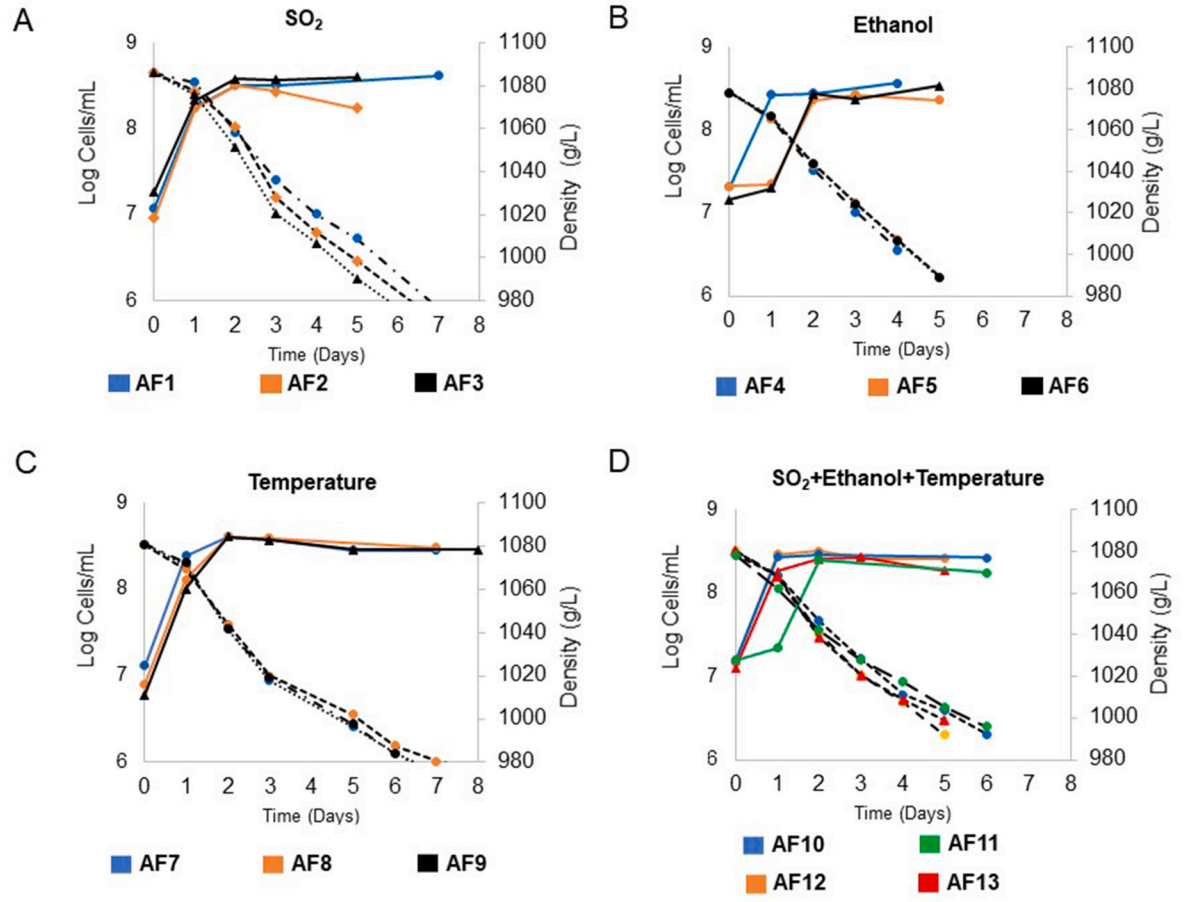

Fig. 5. Alcoholic fermentations (AFs) inoculated with different PdCs using reconstituted concentrated must (RCM). Yeast population dynamics as total cells/mL by microscopy count (solid lines) and fermentation kinetics as the density of sugar in g/L (dashed lines). AF1-3 were inoculated with PdC1-3 evaluating the effect of SO_2 (A), AF4-6 were inoculated with PdC4-6 evaluating the effect of ethanol (B), AF7-9 were inoculated with PdC4-5 evaluating the effect temperature (C), and AF10-13 were inoculated with PdC10-13 evaluating the combined effect parameters (D). Every value in the graphs corresponds to the mean of the values from biological triplicates.

These AFs were carried out without additional treatment at 22 °C. For each PdC's treatment, a DWY-inoculated fermentation was included as a control (AF-Sce).

The fermentation kinetics and growth rate of the yeast population in the AFs were found to be quite similar under each of the conditions tested. The AF finished within 5–8 days (Fig. 5). These findings suggest that the PdC methodology is effective in controlling the start of the fermentation. AF1 showed a delay of one day in the fermentation respect

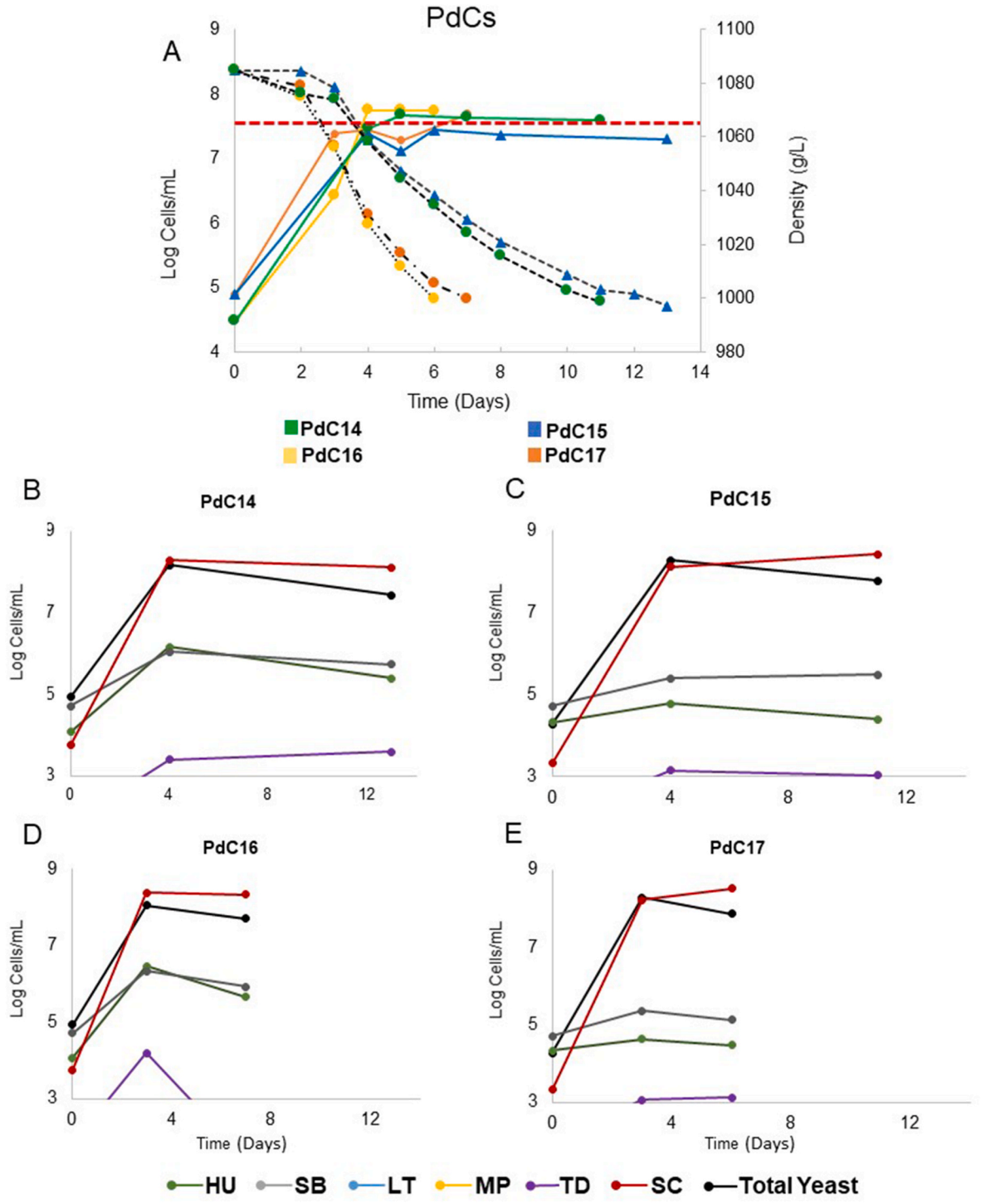

Fig. 6. (A) Sulphur dioxide (SO_2), temperature, and ethanol effect on total yeast population and fermentation kinetic during PdC14-17 using natural must of Muscat of Alexandria during cellar experimentation. Solid lines stand for the number of yeasts quantified under the microscope while dashed lines stand for the density (g/L) of the fermenting must. The red dashed line indicates the time point selected to inoculate these PdCs on the fresh must. The PdC14 and PdC16 were carried out spontaneously without SO_2 or ethanol at 18 and 26 °C, respectively. PdC15 and PdC17 were supplemented with 40 mg/L of SO_2 and 1% (v/v) ethanol. (B–E) most relevant yeast genera (Sce and non-Sce) quantified by qPCR in PdC14-17 using natural must. Every value in the graphs correspond to the mean of the values from biological triplicates and, additionally, the qPCR analysis resulted in technical triplicates. HU, *Hanseniaspora*; SB, *Starmerella bacillaris*; LT, *Lachancea thermotolerans*; MP, *Metschnikowia pulcherrima*; TD, *Torulaspora delbrueckii*; SC, *Saccharomyces cerevisiae*. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

AF3 (Fig. 5. A). However, there were no significant differences observed in the kinetics of AF5 and AF6, where SO_2 was combined with ethanol in PdC5 and PdC6 (as shown in Fig. 5. B). Nevertheless, AF5 and AF6 reached the maximum yeast population with a delay of 24 h with respect AF4 (as shown in Fig. 5. B). No significant differences were observed either in the kinetics or total yeast population when evaluating the effect of inoculation of PdCs prepared under different temperatures over AF7, AF8, and AF9 (Fig. 5. C). In the case of the combination of the selected parameters to perform the PdCs, the supplementation of ethanol and SO_2 did not affect significantly the kinetics of AF10, AF11, AF12 and AF13 (Fig. 5. D). However, the lower temperature of the PdCs induced AF10 and AF11 to finish the 6th day instead of the 5th day observed for AF12 and AF13. Besides, AF10 and AF12 achieved the maximum yeast population on the second day instead of the first day (Fig. 5. D).

3.5. Natural must validation at cellar experimentation

3.5.1. Monitoring of cellar PdCs

The PdCs were carried out spontaneously at 18 and 26 °C (PdC14 and PdC16, respectively) and compared to PdCs of also non-inoculated must supplemented with 40 mg/L of SO_2 and 1% (v/v) ethanol (PdC15 and PdC17). The density and total yeast population of four spontaneous PdCs (PdC14-17) were monitored daily till the end of their fermentation (Fig. 6. A). These PdCs were inoculated at 2% (v/v) into clarified Muscat of Alexandria must to perform the AF14-17 following the criteria of the best time point for inoculation (IP). This time point was reached during the 3rd day at 26 °C and the 4th day at 18 °C (Fig. 6. A).

The PdC fermentation kinetics were fastest at the highest temperature (PdC16 and PdC17) finishing the fermentation on the 6th day or a day later if supplemented with SO_2 and ethanol. The PdCs at 18 °C finished either the 10th (PdC14) or the 13th day (PdC15), also depending on supplementation. This trend was similar to the same combination of parameters in PdC using RCM (see Fig. 3. A).

Yeast population dynamics of PdCs were monitored by qPCR (Fig. 6 B-E). targeting the most abundant genera described in previous studies (Lleixà et al., 2018; Portillo and Mas, 2016; Wang et al., 2015b).

As mentioned earlier, we had chosen CFU counting to monitor the yeast population in laboratory experiments because we inoculated known yeast species, allowing us to easily track their morphology in the WLN medium.

In the case of AFs performed with PdCs using un-inoculated natural must, we used qPCR instead to asses yeast dynamics because other yeast species might be involved. In this case, the morphological identification of the colonies in WLN was not reliable. During the beginning of the PdCs, *H. uvarum* and *S. bacillaris* were identified as the predominant yeasts (Fig. 6. B-E). However, they were eventually overtaken by Sce at the IP (3rd day for PdC16 and PdC17, 4th day for PdC14 and PdC15). Nevertheless, fermentations without SO_2 and ethanol (Fig. 6. B and D) had a higher number of non-Sce cells than the ones supplemented with SO_2 and ethanol at the IP (Fig. 6. C and E).

3.5.2. Impact of PdC inoculation on AFs at the cellar

The fermentations inoculated with the different PdC (AF14-17) were conducted in natural must without SO_2 correction at 18 °C (Fig. 7). Additionally, a separated batch of must was inoculated with a commercial DWY Sce strain (AF-Sce) to serve as control. The results showed that the fermentation kinetics and rate of the AFs were similar with all of them finishing within 10–14 days. The AF-Sce took longer to consume the sugar during the initial days of fermentation but still finished in the first place (Fig. 7). The AF-Sce fermentation process was completed on the 10th day. The AF17 exhibited just one-day delay compared to AF-Sce and showed faster kinetics than the remaining AFs by either 2 or 3 days (Fig. 7).

The inoculation with the PdC in the fresh must resulted in the establishment of robust microbial populations, which served as a strong starter for initiating fermentation.

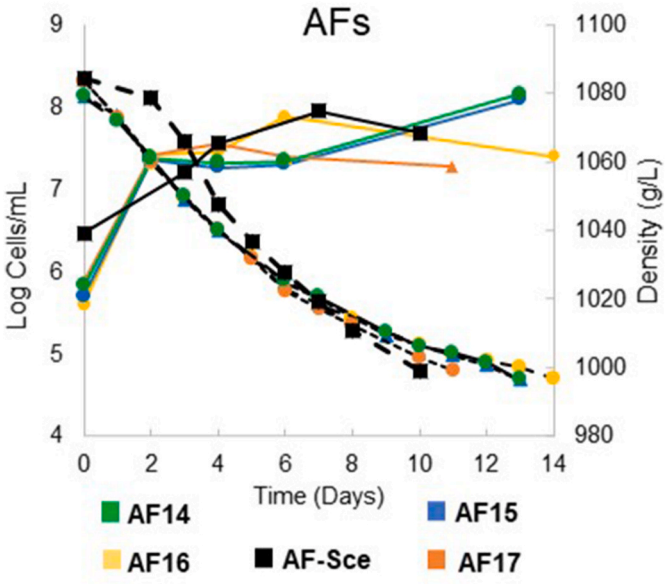

Fig. 7. Effect of PdC14-17 inoculation on total yeast population and fermentation kinetic during the corresponding alcoholic fermentation (AF14-17) using natural must Muscat of Alexandria during cellar experimentation. The inoculated control with Sce QA23 (AF-Sce) is also represented. Solid lines stand for the number of yeasts quantified under the microscope while dashed lines stand for the density (g/L) of the fermenting must. Every value in the graphs corresponds to the mean of the values from biological triplicates.

Significant differences were appreciated in the total yeast population among the treatments analysed by qPCR (Table 1). AF14 and AF15, revealed a higher yeast population and *T. delbrueckii* at the beginning of the fermentation and a significantly higher number of *T. delbrueckii* at the end. Nevertheless, yeast population values were similar at the end of all the AFs. Notably, the inoculation of PdCs made that the beginning of AF14, AF15, AF16 and AF17 presented a significantly higher yeast population than the AF-Sce. The main differences accounted for the quantification of *S. bacillaris* and *T. delbrueckii* (Table 1). In the case of AF-Sce, the relative proportion of non-Sce was lower than at AF14-17 due to the inoculation of the commercial Sce.

3.6. Diversity of S. cerevisiae strains on natural must

Ten colonies were randomly picked from YPD plates inoculated with all the triplicate samples of the fermentation stages indicated previously: IP and BF, MF and FF of the AF. This selection resulted in 40 colonies for each of the 4 treatments (with and without supplementation of 40 mg/L SO_2 and 1% (v/v) ethanol at 18 °C and 26 °C) plus 30 colonies for the AF-Sce, which lacks of PdC. An Interdelta-PCR analysis was conducted which grouped the 190 colonies into 70 different profiles of Sce. We analysed the diversity and relative abundance of these profiles in the PdCs and AFs (Fig. 8. A). Diverse Interdelta profiles were observed in PdCs, with only one common profile (profile 4) at the inoculation point (IP), even when the same natural must have been used. Also, the Sce composition in the PdC seems to have no influence on the profile composition observed in the AF inoculated with them. These results could indicate either that the Sce community is rapidly changing in time or that the isolates representation was insufficient to capture the Sce diversity. The specific Interdelta profiles selected at each fermentation were dependent on the selective pressure applied. For instance, profile 3 was only observed in PdCs at 26 °C (PdC16 and PdC17), profile 7 at 18 °C (PdC14 and PdC15), and profiles 8 and 11 when SO_2 and ethanol were added (PdC15 and PdC17). In all four AFs, there was no clear dominance of any specific profile at the FF, showing high intraspecific diversity till the end of the fermentation. The commercial strain QA23

Table 1
Total yeast population and main yeast genera quantified by qPCR during AF in natural musts inoculated with different PdCs (AF14-17) or with a commercial strain (AF-Sce). Different subindices letters indicate significantly different values ($p < 0.05$).

Time	tARGET	AF14	AF15	AF16	AF17	AF-SCE
BF	Total Yeast	3.42E+06^{b}	6.11E+06^{c}	1,28E+06^{a}	1.41E+06^{a}	1.09E+06^{a}
	Hanseniaspora	1.25E+05^{a}	2.26E+05^{c}	7.59E+04^{a}	2.01E+05^{bc}	5.35E+04^{a}
	S. bacilliaris	3.50E+05^{a}	6.87E+05^{c}	2.37E+05^{ab}	4.46E+05^{a}	7.77E+04^{b}
	T. delbrueckii	5.65E+03^{b}	8.45E+03^{c}	1.50E+03^{a}	1.80E+03^{a}	1.54E+02^{d}
	S. cerevisiae	1.39E+07^{ab}	2.33E+07^{b}	6.06E+06^{a}	6.92E+06^{a}	2.06E+07^{b}
MF	Total *Yeast*	5.54E+07^{a}	1.01E+08^{ac}	5.59E+07^{a}	1.46E+08^{bc}	1.84E+08^{b}
	Hanseniaspora	4.69E+04^{a}	8.19E+04^{bc}	4.26E+04^{a}	6.68E+04^{ab}	1.06E+05^{c}
	S. bacilliaris	5.73E+05^{a}	7.28E+05^{a}	5.34E+05^{a}	8.00E+05^{a}	8.73E+05^{a}
	T. delbrueckii	1.42E+04^{c}	2.43E+04^{d}	5.69E+03^{ab}	1.03E+04^{bc}	2.24E+03^{a}
	S. cerevisiae	1.67E+08^{a}	3.60E+08^{b}	1.75E+08^{a}	3.62E+08^{b}	1.45E+08^{a}
FF	Total Yeast	5.85E+07^{ab}	9.77E+07^{b}	6.02E+07^{ab}	8.89E+07^{b}	4.15E+07^{a}
	Hanseniaspora	4.77E+04^{ab}	4.67E+04^{ab}	5.65E+04^{a}	2.80E+04^{b}	2.65E+04^{b}
	S. bacilliaris	5.42E+05^{a}	6.29 + 05^{a}	5.10E+05^{a}	7.12E+05^{a}	4.59E+05^{a}
	T. delbrueckii	6.45E+03^{b}	8.62E+03^{b}	2.29E+03^{a}	3.28E+03^{a}	5.47E+02^{a}
	S. cerevisiae	1.50E+08^{a}	2.42E+08^{a}	2.05E+08^{a}	2.03E+08^{a}	2.48E+08^{a}

BF beginning fermentation, MF Middle fermentation, and FF final fermentation. The results of the post-hoc test are noted with the letters (a, b, c, d) that indicate a significant difference in population yeast within the treatments at different fermentation stages, deviation standard error of the mean of triplicates is also indicated.

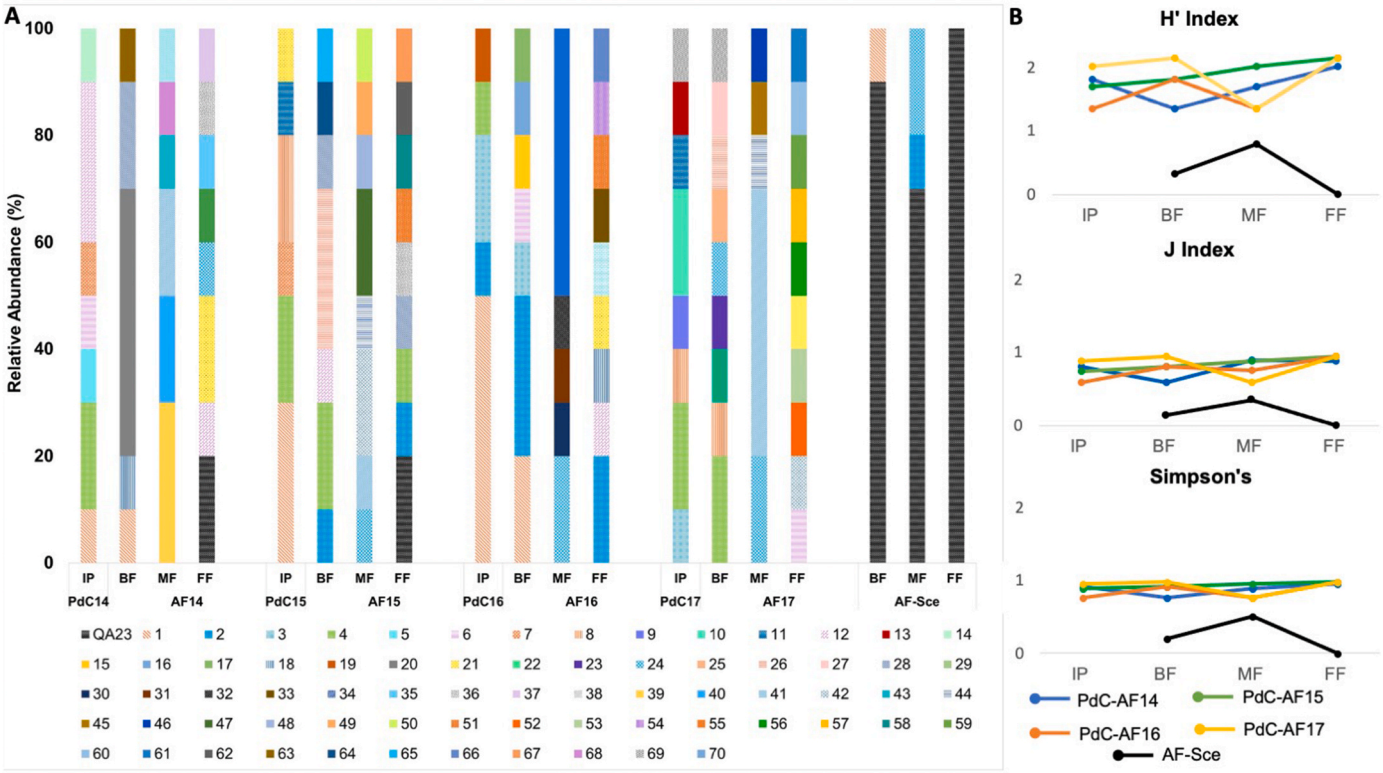

Fig. 8. (A) Relative abundance of Interdelta profiles of *Saccharomyces* colonies isolated from YPD plates medium. IP stands for the PdCs sampled at the inoculation point whereas BF, MF, and FF correspond to the beginning, middle, and final fermentation stages of the AFs inoculated with different PdCs. PdC14 and PdC 15 correspond to the PdCs prepared without or with the addition of SO_2 and ethanol at 18 °C, respectively; PdC16 and PdC17 correspond to PdCs prepared without or with the addition of SO_2 and ethanol at 26 °C, respectively. (B) Diversity indexes at each fermentation stage.

inoculated in the AF-Sce was the most abundant detected during all stages of this fermentation (Fig. 8. A). Noticeably, the DWY QA23 only represented up to 20% of the Sce strains at the end of the AF14 and AF15 and did not overtake any of the AFs. In any case, there was a greater diversity of Sce in the PdC-inoculated fermentations than in the AF.

The total number of isolates and the abundance of the different Interdelta profiles at each sampling point were used to calculate the diversity indexes Shannon (H'), Simpson's, and evenness (J). Results show that Sce diversity was similar at the IP for the four PdCs, with the highest value observed in PdC17 (Fig. 8. B). The diversity dropped at the beginning of AF16 and AF17, increased towards the middle, and decreased again at the end of the fermentation. AF14 and AF15 showed an increase in diversity at the beginning till the end of the AF. Nevertheless, all the PdCs showed lower diversity at the IP than at the end of the corresponding AFs, which were similar between them. The diversity of the AF-Sce was much lower from the beginning than the PdCs and other AFs, it increased towards the middle and dropped to 0 at the end of the fermentation as the result of the takeover by the strain QA23 (Fig. 8. B).

Finally, different and some identical Interdelta profiles of Sce strains were grouped at 90% similarity by a dendrogram into 26 clusters (Supplementary Fig. 3). The clustering did not follow either the different

treatments of the PdC or the stage of the fermentations. However, the clustering was reliable because 11 identical profiles from different images were able to group at 100% similarity and confirmed the selection of the 70 different profiles used to calculate the relative abundance of Sce strains and their diversity at each fermentation stage.

4. Discussion

Wineries are increasingly interested in exploring strategies that involve minimal intervention during fermentations, aiming to promote native microbiota from the environment to add value to their wine product. Pursuing microbiological complexity in wines through spontaneous fermentations has yielded positive outcomes in terms of fostering diversity of species and strains associated with the *terroir* (Börlin et al., 2020; Mas and Portillo, 2022). This microbial diversity may result in added complexity to the wine's sensorial perception (Alexandre 2020; Esteve-Zarzoso et al., 1998; Gamero et al., 2016; Romano et al., 2003). However, the effectiveness of this fermentation method has been undermined by issues of contamination and fermentation interruption, unpredictable results, or formation of biogenic amine during fermentation (Capece et al., 2012; Tristezza et al., 2013; Vázquez et al., 2023).

PdC, an antique winemaking technique, has recently gained attention as a fascinating area of exploration the utilization of native microbiota *fingerprint* to be inherited and transmitted into the freshly inoculated must. This is achieved by inoculating a high number of actively fermenting yeasts, which also provides certain microbial control (Börlin et al., 2020; Mas and Portillo, 2022). Recently, the effect of the PdC method was proposed as a viable alternative for controlling spontaneous fermentation has sparked interest in exploring its impact on the fermentative process and the diversity of Sce strains. Börlin et al. (2020) found that PdC fermentations were conducted more efficiently than spontaneous fermentations and similarly to the inoculated with DWY. However, they did not find any significant sensory differences and only one modality of PdC was applied (Börlin et al., 2020).

Currently, there is a lack of scientific basis and information on how to prepare PdC to obtain optimal results and how the different parameters during preliminary fermentation select the microbial population of the natural musts. To address this gap, we conducted a pioneering study using various must matrices to assess the effect of several factors on the yeast population through selective pressure during both the PdC and the subsequent AF. Moreover, these factors evaluated can be conveniently incorporated into everyday cellar routines.

Our laboratory experimentation used a yeast consortium that was prepared based on the yeasts that were previously detected in Catalonian musts (Lleixà et al., 2018; Padilla et al., 2016; Portillo and Mas, 2016; Wang et al., 2015a, 2015b) and it was inoculated in SM. Increasing SO_2 concentrations resulted in the domination of Sce without affecting the speed of the fermentation. However, not adding SO_2 led to a lower Sce population while using 40 mg/L instead of 80 mg/L of SO_2 allowed some non-Sce yeasts to be detected further during the PdC. Similar results have been reported by other studies that analysed the effect of SO_2 on yeast population dynamics during AF (Cocolin and Mills, 2003; Pateraki et al., 2014). Non-Sce yeasts are more sensitive to SO_2 compared with Sce yeasts (Albertin et al., 2014; Constanti et al., 1998; Divol et al., 2012; Henick-Kling et al., 1998). Additionally, it has been described that Sce can release metabolites that can modify the environment and give them a competitive edge over the non-Sce yeasts (Wang et al., 2016). It should be emphasized that the use of this optimized PdC leads to a significant decrease in the concentration of SO_2 compared to the commonly used levels of 60–80 mg/L SO_2 in the cellar routine during the winemaking process. This reduction aligns with the current European political trend of cutting down the consumption or exposure of allergenic substances in the food industry, like SO_2. This is because it has been linked to a variety of negative health effects (Vally and Misso, 2012). It is important to note that SO_2 plays a significant role in exerting control over microbial populations while simultaneously acting as an antioxidant agent, which is supported by other researchers (Esparza et al., 2020; Nikolantonaki et al., 2014). However, viable alternatives to SO_2 have been recently explored in the form of microbiological, chemical, and physical approaches (Mas et al., 2020).

Limited research has been conducted on the impact of temperature in un-inoculated must (Andorrà et al., 2010b; Hierro et al., 2007). Most of the studies have examined the behaviour of a consortium of non-Sce and Sce yeast aiming to simulate spontaneous fermentation under lab conditions. These studies have consistently demonstrated that Sce performs better at higher temperatures compared to non-Sce yeasts (Alonso-del-Real et al., 2017; Hierro et al., 2007). However, in the absence of inoculation or when mixed populations are inoculated, *S. bacilliaris* exhibits favorable performance throughout the fermentation process (Andorrà et al., 2010b; Ciani and Comitini, 2006; Sharf and Margalith, 1983). Our experiments confirm that PdCs at higher temperatures favoured Sce over the non-Sce (except *S. bacilliaris* and *T. delbrueckii*) and the fermentation kinetics are slower at lower temperatures. Sce's maximum number is not affected at the tested temperatures, but the non-Sce are impaired at 22–28 °C. It is known that Sce can grow at a wide range of temperatures, even higher than 32 °C. This is an essential trait that explains its dominance during non-refrigerated wine fermentation (Salvadó et al., 2011). Thus, the use of lower temperatures may be a strategy to allow the non-Sce to contribute to wine complexity, although nutrient competition should be considered to avoid sluggish fermentations (Gobert et al., 2017; Roca-Mesa et al., 2020). The effect exerted by the temperature was similar for 16–18 °C and 26-28 °C (different temperatures used in experimentations), confirming that these minor variations in temperature vinification's will not affect to the general results reported for the PdC.

The second tested selective factor was the ethanol addition at the PdCs. A previous study found that adding 1.5 and 3% (v/v) ethanol accelerated AF and increased the diversity and number of Sce strains during PdC fermentation (Moschetti et al., 2016). Based on these findings, we tested a lower percentage of ethanol (0.5 and 1% v/v) to allow native non-Sce to be present at the beginning of fermentation while still achieving good kinetics. We discovered that some non-Sce strains were more sensitive to ethanol than others and the fermentation kinetics was affected just at the highest concentration of ethanol. However, all strains were detectable up to the 3rd day at the highest ethanol concentration tested and the number of Sce was significantly higher. Every cellar can easily use 1% (v/v) ethanol to prepare the PdC by adding wine from the previous vintage and it does not require a significant economic investment.

Altogether, our results at laboratory experimentation with a yeast consortium of yeasts revealed that SO_2, ethanol, and temperature could be used to modulate the yeast population during PdC. Specifically, we found that 40 mg/L of SO_2 and 1% (v/v) ethanol favoured fermentative yeasts such as Sce and *S. bacillaris* while also allowing the presence of other non-Sce during the first stages.

We also evaluated the effect of time of PdC inoculation on the new must. The results showed the 48-h time point as the most appropriate to incorporate PdC to the fresh must at 2% (v/v) because at the end of the AF inoculated at 48 h with PdC, a high proportion of the yeast population was non-Sce but a higher proportion of Sce was recovered. Thus, we found the inoculation time of 48 h has a good compromise between yeast diversity and securing a good fermentation performance by the selection of fermentative yeasts. In agreement with our results, Lleixà et al. (2016) found that inoculation of the Sce strain later than 48 h led to stuck fermentations due to exhaustion of the nutrients by the non-Sce previously inoculated. Additionally, at 48 h, the PdCs had over 10^8 cells/mL and underwent a density reduction of at least 15 g/L, regardless of the treatment. These criteria would be used then as the time point of inoculation of the AFs.

Based on these results, we carried out PdCs combining the selected parameters at two different temperatures, first at the laboratory using

RCM and then, at the cellar using fresh must of Muscat of Alexandria. Four treatments were compared, and we observed differences between them. However, the AFs (AF14-17) were similar and showed comparable fermentation kinetics and maximum yeast population. Nevertheless, the total yeast population at the end of the AF was similar for AFs when compared to the inoculated AF-Sce. These results indicate that the PdC is a good strategy to achieve good fermentation kinetics and microbial control of the AF, regardless of the selective pressure we applied.

The Interdelta-PCR analysis reported a high diversity of Sce profiles for all the PdCs and different stresses selected for different strains. The intraspecific Sce diversity was similar at the beginning and the end of the fermentations, and no common profile was found to take over. On the other hand, the AF-Sce was dominated from the beginning of the fermentation by the commercial strain inoculated. A low level of cross-contamination from the DWY was detected just at the end of the AFs conducted at lower temperature. Li et al. (2012) compared the inoculation of a commercial Sce strain and the PdC method at an industrial scale. The results showed similar kinetics and yeast populations with the imposition of the commercial Sce strain in both cases. Thus, the risk of a more competitive commercial strain taking over is always present at the cellar scale. Nevertheless, in our hands, at the end of the four AFs (AF14-17), most of the yeast population consisted of Sce strains different from the commercial Sce inoculated in the AF-Sce and the routine inoculated strain at the cellar (QA23).

Regardless of the differences in diversity indexes across fermentation stages, the inoculation point of the PdC showed lower diversity values than the end of the AFs. The PdC with higher diversity was the one at 18 °C supplemented with SO_2 and ethanol. In agreement with our results, Morgan et al. (2019) described that the SO_2 addition during the PdC preparation increases the amount and diversity of autochthonous *S. uvarum* strains at the end of the main fermentation. Additionally, Moschetti et al. (2016) observed a higher diversity of Sce when the PdC was fortified with ethanol.

There was no clear clustering based on the similarity of Sce Interdelta profiles but the dendrogram allows us to confirm the right selection of profiles for diversity analysis. Some authors described that the diversity of yeast populations was not impacted by the production year or fermentation stage factors while geographic locations and ecological niches are both believed to have significant roles in Sce strain diversity (Liu et al., 2021; Peter et al., 2018).

In the study of Bordet et al. (2021) it was reported that different Sce strains showed different modulation of primary metabolism and the changes were noted at the volatilome and sensory levels. Similarly, Liu et al. (2021) described that associations between fungal microbiota diversity and wine chemicals suggest that Sce plays a primary role in determining wine aroma profiles at a sub-regional scale. To deepen the possible relation of the selected Sce strain to changes in sensorial and chemical parameters, further analysis is required, and it would be the objective of a future study. Nevertheless, most of the PdC studies just tested one modality of PdC, and studies like ours are needed to evaluate the implementation of the PdC to control the microbiology during AF as well as preserve the typicity and quality of the final wine.

5. Conclusions

Selective pressure protocols used during PdC, like SO_2 and ethanol addition and temperature, enable the modulation of yeast populations during the PdC. Laboratory's PdCs allowed us to monitor the specific effect of individual and combined parameters over a yeast consortium using SM and RCM. The addition of 40 mg/L SO_2 and 1% (v/v) ethanol favoured the takeover of Sce while allowing the presence of non-Sce at the first stages of the PdC, independently of the tested temperature. The optimal time to inoculate the PdC in the fresh must was determined as 48 h at the laboratory which correspond with a drop in must density of 15–20 g/L and 10^8 cells/mL. When these PdCs were inoculated at 2% (v/v) into new must, similar results were achieved regarding fermentation kinetics and yeast population dynamics both at the laboratory and cellar AFs. Furthermore, the technological results in terms of fermentation rate and fermentation security were comparable to those of the fermentation inoculated with a commercial strain, confirming the efficiency of the PdC method in controlling AF. However, the yeast diversity that can be a characteristic element of the typicality of the wines from a given area is favoured by the PdC. The yeast selected at the end of the AFs inoculated with PdCs were mostly autochthonous Sce different from the commercial strain used at the control. In addition, the selected Sce strains were different depending on the stress applied at the PdC preparation, with non-specific Sce taking over the fermentation. Future perspectives include the evaluation of the chemical and sensory features of the produced wines from natural must and their relation to the intraspecific diversity of Sce strains. This assessment will shed light on the ultimate influence of these autochthonous strains on the final fermentation process, leading to wines with distinct organoleptic qualities.

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CRediT authorship contribution statement

Katherine Bedoya: Writing – review & editing, Writing – original draft, Validation, Methodology. **Luis Buetas:** Writing – original draft, Methodology, Investigation. **Nicolas Rozès:** Writing – review & editing, Supervision, Funding acquisition, Formal analysis. **Albert Mas:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Project administration, Funding acquisition, Formal analysis, Conceptualization. **M. Carmen Portillo:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

None.

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Fig. 1 was created with BioRender.com under the agreement number AY25ZKSME5.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2024.104571.

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