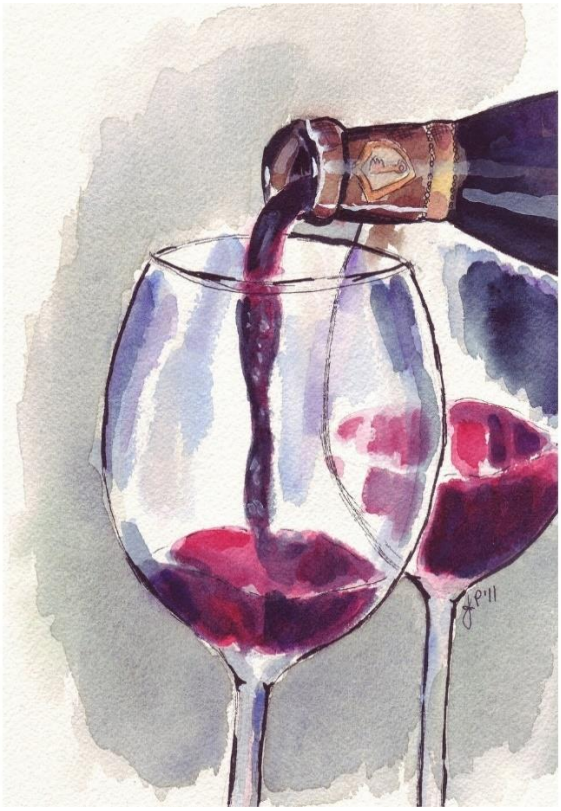
UNIVERSITAT ROVIRA i VIRGILI

POPULATION DYNAMICS OF *SACCAROMYCES CEREVISIAE* YEAST STRAINS IN ALCOHOLIC FERMENTATIONS CARRIED OUT USING THE *PIED DE CUVE* TECHNIQUE AT DIFFERENT GRAPE MATURITY POINTS.

Luis Buetas Esteban

BACHELOR'S DEGREE FINAL PROJECT



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In cooperation with: Oenological biotechnology investigation group (URV).

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CENTER DATA

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The group of Oenological biotechnology of the URV is where I have done my internship. The focus of the group is the study of microorganisms involved in oenological processes and their biotechnological applications. Yeast (be it *Saccharomyces cerevisiae* or *Non-Saccharomyces*) and lactic acid bacteria are the main microorganisms the group works with. Those are responsible for the alcoholic and malolactic fermentations of wines, respectively.

Those studies are carried out by biochemical and molecular techniques including systems biology such as proteomics, metabolomics, transcriptomics, or genomics. All the previous allows the group to identify nutritional needs, diversity of species and strains or interactions between the previously mentioned microorganisms.

The beginnings of the Wine Biotechnology Group are linked to the initial research in Oenology in the 1990s in the University Rovira i Virgili (URV) in Tarragona, while the first studies of Oenology.

GUIDE OF PLAGIARISM PREVENTION

July 2024

I, Luis Buetas Esteban, with ID number 73214574P, am aware of the guide of plagiarism prevention at the URV *Prevention, detection and treatment of plagiarism in teaching: guide for students* (approved in July 2017), (http://www.urv.cat/ca/vidacampus/serveis/crai/que-usoferim/formaciocompetencies-nuclears/plagi/) and I affirm that this bachelor's degree final project does not constitute any of the conducts considered as plagiarism by the URV.

Tarragona 29th April 2024.

1. ABSTRACT

In recent decades, winemaking has undergone significant advancements, with the refinement and implementation of various techniques that have enhanced efficiency and control over microbial populations. Practices such as the use of Active Wine Dry Yeast (ADWY), Sulphur dioxide (SO_2) and controlled fermentation temperatures have played a crucial role in these improvements. However, these practices have also introduced challenges, notably a reduction in wine complexity.

To address this issue, winemakers have begun revisiting traditional methods, including the *Pied-de-cuve* (PdC) technique. PdC is a pre-fermentation method that allows to increase the initial yeast population, thereby ensuring a more controlled and complete fermentation process.

Previous work from the group studied the effect of different tools used by winemakers to determine the appropriate conditions to provide a PdC with the

optimal population of yeasts that will later be inoculated in the must. Using this knowledge, the main objective of this study was to analyse the impact of the PdC technique on strain-level diversity of *Saccharomyces cerevisiae* populations during alcoholic fermentation (AF) using grapes with different levels of maturation.

To achieve this, must of natural grape at three different ripeness levels were fermented by three different methods: spontaneous fermentation, ADWY inoculation, and PdC under specific stress conditions identified during a previous study of the group. Samples were collected at three different stages of the fermentation: Beginning (BF), Halfway point (HF), and final or completion (FF). Diversity analysis of *S. cerevisiae* was performed by interdelta-PCR analysis at each stage.

Our findings indicate that the PdC technique significantly increased the diversity of *S. cerevisiae* strains in AFs carried out with Muscat of Alexandria grape must. Additionally, PdC was found to influence the sensory attributes of the final wines.

Keywords
Saccharomyces cerevisiae diversity; pied-de-cuve; alcoholic fermentation; Interdelta-PCR technique; Grape ripeness.

2. INTRODUCTION

Alcoholic fermentation in wine making is characterized by a succession of microorganisms interacting with each other and their environment. These interactions lead to changes in the environment that influence the kinetics of fermentation. This succession is a race between the microorganisms in which only the most adapted survive, being *S. cerevisiae* the most recognized one. Grape must be why those microorganisms need to be adapted. This medium is highly restrictive, as many microorganisms do not have the capabilities needed to proliferate in it. Low pH, high osmolarity and an imbalance between sugars

and nitrogen sources are the characteristics that makes grape must such a challenging environment (Varela et al., 2004). When the race has just started, the excess of sugars in the must results in the start of the fermentation which in turn produces a nutrient decrease and a further unbalance between sugars and nitrogen sources. Fermentation is a process that allows yeasts to produce ATP faster resulting on a faster augment of biomass thus providing it with competence against other microorganisms. Furthermore, the main product of fermentation is ethanol to which many yeasts are sensitive and consequently disappear as soon as its presence augments (Bagheri et al., 2020).

The prevalence and contribution of each species to the final wine is determined by both ecological and physicochemical parameters. Usually, the fermentation starts with the *Non-saccharomyces* yeasts originated mainly from the grape surface, but it is quickly taken over by the *Saccharomyces* species. The later ones are known to be able to carry the fermentations over until the end. Fermentations are completed when the sugars concentration on the must is 2 g/L or less. *Saccharomyces* species can be found and isolated from the grape surface. However, *Saccharomyces* species, thanks to its competitivity and adaptation mechanisms can become cellar-residents and are commonly found in the cellar and its equipment.

As previously stated, *Saccharomyces* species imposition during fermentations is important to guarantee its completion. Despite the capabilities of *Saccharomyces* species to prevail over *Non-Saccharomyces* species, in modern winemaking, this imposition is due to controlled inoculations and certain oenological practices that favor its takeover. Several practices have been applied to control the microorganisms' behaviour during the fermentations highlighting the use of sulphur dioxide (SO_2). Its antioxidant and antiseptic activities are the main reason for its widespread usage. Many microorganisms are susceptible to SO_2 toxicity and its use is very common in the actuality (Fleet, 2003). Nonetheless, in high quantities SO_2 is toxic for humans. The European Union has set a maximum of 150 g/L in dry red wines and 200 g/L in dry whites and rosés wines to allow the commercialization of wines (EU-Lex Document 32019D0207(01)). This is important because, even if the concentration of SO_2 in

wines are lower than the threshold, the popularisation of the toxic effect of SO_2 has grown a tendency between wine consumers of looking for wines that have not been treated with SO_2. This tendency is directly linked to recent changes in society with more people considering what they consume and where does it come from. All the previous means that, even if SO_2 is excellent to control microorganisms' behaviour during fermentations, alternative techniques are interesting due to customers preferences.

S. cerevisiae is known to be able to modify the environment to create a favorable niche for its own (Parapouli et al., 2020). Above 25ºC, easily reachable during alcoholic fermentation, said yeasts have clear advantages in front of the other *Non-Saccharomyces* species while at lower temperatures, *Non-Saccharomyces* species proliferate as well (Alonso-del-Real et al., 2017; Torija et al., 2003). *Non-Saccharomyces* species are key to winemaking even if they don't usually arrive to the end of the fermentation. Many Non-Saccharomyces have an important role on giving a wine its identity. Elevated diversity on yeast populations at the beginning of fermentations allows to produce certain compounds responsible for the complexity and differences between wines (Castrillo et al., 2020). Yeast diversity positively impacts de complexity of the final wines. It has been demonstrated that increasing yeast diversity leads to wines more complex and with a higher quality. This effect has been proven with increased *S. cerevisiae* strain diversity but also with increased *non-saccharomyces* species diversity (Binati et al., 2019; Castrillo et al., 2020b). Nowadays, winemakers tend to lower fermentation temperatures down to 10-16ºC to favor the growth of *Non-Saccharomyces*, to induce complexity, and differentiation of the final product. However, this practice has its own disadvantages: the proliferation of microorganisms with low fermentative capabilities risks the fermentation process (Edwards & Aplin, 2022). Low fermentative yeasts require nutrients to grow. Nutrient depletion might affect high fermentative yeasts not allowing them to properly complete the alcoholic fermentation processes.

Another approach can be addressed: The use of starter cultures which is one of the biggest achievements on the fermentation industry. Different starter cultures

can be used but the most widespread ones are the Active Dry Wine Yeasts (ADWY). Using ADWY implies an additional expense, however, the reproducibility and the microbial control compensate for this increase. The use of these ADWY produce similar and uniform products which removes the identity of different wines. Many strains are available as ADWY, but the amount is limited, and many different cellars end up producing similar wines by using the same starters. Wine market is very competitive and having similar products is not desirable.

Winemakers main interest is to keep the identity of their products, but at the same time be able to reproduce it in the following years. The identity of the wines is linked to the *terroir* concept. *Terroir* concept refers to the characteristics of climate, soil, grape variety, and microorganisms in a specific geographical area. Said area can be as small as the plot owned by one cellar (Comitini et al., 2017).

The disadvantages of all previously explained techniques has resulted on the recovery of an ancient technique known as *pied-de-cuve* (PdC). PdC is a method that allows the winemakers to keep the regional characteristics of the wines while, at the same time, accomplishing some kind of microbial control.

PdC is a technique of indirect inoculation using a small volume of must already fermenting. PdC must fermentation can be carried over spontaneously using grape berries harvested before the main harvest, or by inoculation of any AWDY.

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 kinetics and population of the fermentation (Abdo et al., 2020; Börlin et al., 2020).

Previous work from the group in which I conducted my research studied the effect of different tools used by winemakers to determine the appropriate conditions to provide a PdC with the optimal population of moderate and high

fermentative yeasts that will be later inoculated into the new fresh must (Bedoya et al., 2024). The high number of cells provided by the PdC, allow the autochthonous yeast to begin the fermentation as in spontaneous fermentations and end up with the selection of highly fermentative species and strains that ensures the correct ending of the alcoholic fermentation (Bedoya et al., 2024).

This bachelor's degree final project aimed to study the effects of the previously selected PdC on the diversity of *S. cerevisiae* strains during the fermentation of grape must from three different levels of ripeness. Additionally, we compared the effect of the PdC-inoculated fermentations with spontaneous (SF) and ADWY inoculated (QA23F) fermentations to determine if the PdC had any sensorial advantages. *S. saccharomyces* diversity across the different fermentations was performed by Interdelta-PCR analysis. The physicochemical properties and organoleptic characteristics of the produced wines were evaluated with HPLC and sensory analysis. Finally, a selection of *S. cerevisiae* strains was tested for their fermentative capacity in laboratory-scale micro fermentations. Key parameters of these micro-alcoholic fermentation were determined by HPLC analysis and were explored for potential correlations between the presence of the selected strains and the sensory analysis outcomes.

Hypothesis and objectives.

The hypothesis of the present study is that previously selected PdC increases the diversity of *S. cerevisiae* strains during alcoholic fermentation, regardless of the grape ripeness. This increased diversity is expected to enhance the complexity of the resulting wines and better preserving the unique characteristics associated with their respective *terroirs*.

Objectives:

- Isolation and typification by Interdelta-PCR analysis of *S. cerevisiae* strains from fermentations carried over by different methods and different levels of ripeness.

- Evaluate the differences in *S. cerevisiae* diversity between wines produced using different techniques and musts from grapes collected at different maturity points.
- Sensory analysis of the resulting wines under each modality.
- Identify and select of strains with higher imposition or present from the beginning to the end of each fermentation modality with the evaluation of their kinetics and main oenological parameters through laboratory scale fermentations.
- Correlation between *S. cerevisiae* strains and the main physicochemical parameters detected during laboratory fermentations.

3. MATERIALS AND METHODS

3.1. Alcoholic fermentations preparation.

3.1.1. Grape harvest and must elaboration.

The population dynamics of different *S. cerevisiae* strains was evaluated during AF using natural must from Muscat of Alexandria grapes. Grapes were harvested during the summer 2023 at the experimental cellar of the URV, Mas dels Frares, Facultad de Enología of Universidad Rovira i Virgili. Different fermentation modalities were carried out at three different maturity points of grapefruits: unripe (U) grapefruits (Probable Alcohol Content -PAC- around 10%), optimal or normal (N) grapefruits (PAC around 12%) and ripe (R) grapefruits (PAC around 13%). The PAC is a measurement used in the wine industry to calculate the possible alcoholic concentration that a certain must can achieve after fermentation process is completed. PAC value depends on the concentration of soluble sugars in the must such as glucose or fructose.

Once the grapefruits where around the desired PAC values, the harvesting process was performed. To have a representative sample of grapes, grape clusters were selected randomly from the whole Muscat of Alexandría plot and collected by different people to augment the randomness. The harvesting process was performed in two different days for each experiment. First a small

sample of grapes was harvested to produce the fermenting bat that would be used as PdC (three PdCs with a volume of 1L that would be mixed before inoculation). The following day, a bigger sampling was done to produce the must needed to perform nine different fermentations (3L each), three triplicate conditions. As previously stated, those conditions where spontaneous fermentation (SF), ADWY-inoculated fermentation with a commercial yeast strain denominated QA23 (QA23F), and PdC-inoculated fermentation using 2% (v/v) of the previously produced PdC (PdC). The process was similar in each maturity point. Once the grapes were harvested, a press was used to extract the must. After must extraction, grape skins were discarded since no maceration process was used in this experiment. The must was left for 24 hours at 8ºC to settle down.

3.1.2. PdC preparation.

After 24h settling down, for each grape maturity stage, 3 bottles of 1 litter were filled with the must and 40mg/L of SO_2 were added to each bottle (Figure 1). Additionally, 1% (v/v) of ethanol was added to each bottle using white wine from the previous vintage in the cellar. Those parameters were selected in the previous group study (Bedoya et al., 2024). The lid of each bottle had a perforation to allow the exit of CO_2 produced by fermentation and allowed to start the spontaneous fermentation at around 18 ºC. The research group have

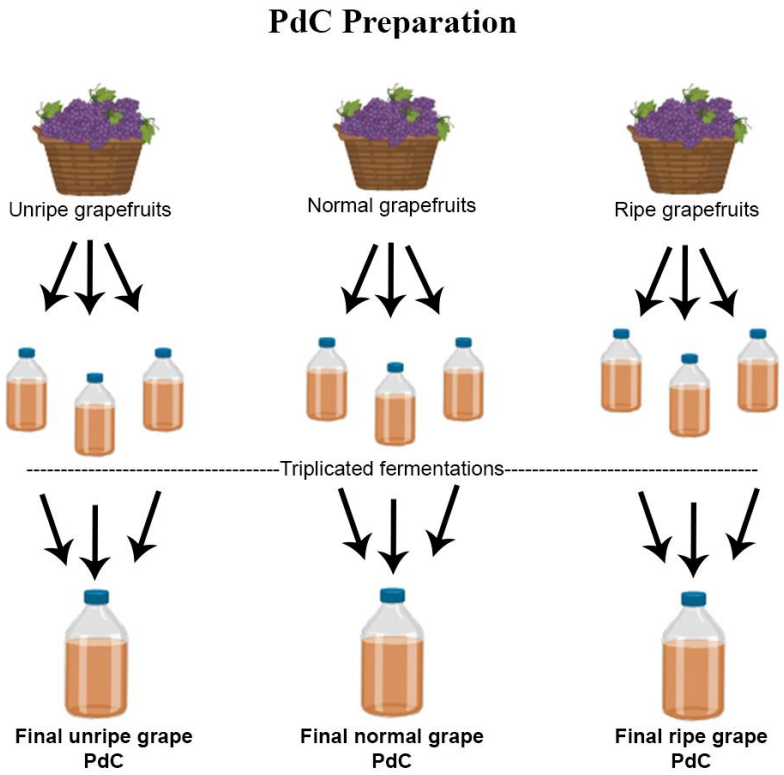


Figure 1. PdC preparation scheme for each grape maturity point.

previously stated that slight variations on temperature do not affect the general result of the PdC (Bedoya et al., 2024). After density started to decrease or cell population started to grow indicating that the fermentation had started, the final PdC inoculum was prepared by combining the fermenting must of all three bottles in equal quantities.

3.1.3. Alcoholic fermentations.

AFs were carried out by three different techniques: SF, QA23F and PdC. Every experiment was done in triplicate in 5L bottles containing 3L of must previously settled down for 24h (Figure 2). For SF samples, 40mg/L of SO_2 were added to each bottle. QA23F were prepared like SF samples but also were inoculated with $2x10^6$ cells/mL of QA23 *S. cerevisiae* strain. Finally, for PdC samples, the must was inoculated with 2% (v/v) of the combined PdC explained in the previous subsection. All the bottles were incubated at 18 ºC to start AF.

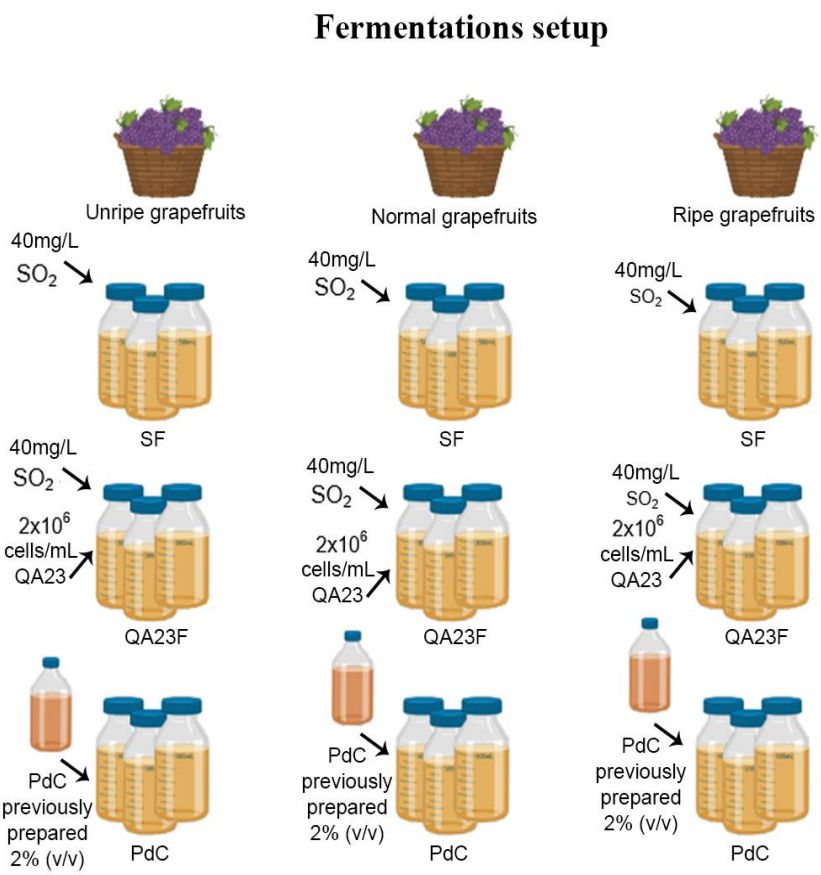


Figure 2. Representation of the Fermentations setup for the different grape maturity points. Treatment for each technique is represented.

3.2. Fermentation monitoring.

3.2.1. Fermentation kinetics.

A daily follow up was made to keep track of the AF development. This follow up was made measuring must density (electronic densitometer, Densito 30PX Portable Density Meter; Mettler Toledo, Barcelona, Spain). Fermentations were considered to be finished when must density was below 1000 g/L and the concentration of residual sugars was below 2 g/L. Residual sugars concentration was determined enzymatically using the Y15 Bioanalyzer with the corresponding enzymatic kits (BioSystems S.A, Barcelona, Spain).

3.2.2. Fermentation sampling.

Three different stages of the AF were considered in this project: Beginning of the fermentation (BF; samples of the AF taken after being put at 18ºC to start the fermentation process; must density around 1090-1080 g/L) , mid of the fermentation (HF; must density was 1050-1040 g/L) and end of the fermentation (FF; must density was below 1000 g/L and residual sugars concentration was below 2 g/L).

For each stage 1 mL was transferred to an Eppendorf tube from each AF and diluted serially to be inoculated into petri dishes containing WLN solid medium. WLN is a differential medium that allows the identification of different certain yeast species by coloration (Feng et al., 2020). *S. cerevisiae* species adopt a cream white coloration with possibility of slight light green details. Inoculated plates were left growing at 28ºC for 24-48h.

After growth in WLN, 20 differentiated *S. cerevisiae* colonies from each stage were randomly selected and transferred to new plates containing YPD medium (Table 1). YPD plates were left at 28 ºC to grow. After 24 h each colony was transferred to a different 10 mL plastic tube containing 5 mL liquid YPD medium and left for 24 h at 28ºC to grow. Finally, 1 mL of each colony was transferred into a 1,5 mL Eppendorf tube and centrifuged for 10 min at 13.000 rpm, the supernatant was then removed, and the pellet was resuspended in 1 mL of ultrapure sterile milli-Q water. The samples were centrifuged again for 10 mins

at 13.000 rpm and supernatant was then removed again. The resulting pellet was used for posterior molecular identification.

Table 1. YPD medium composition.

YPD Medium composition	
Gucose	20g/L
Peptone	20 g/L
Yeast extract	10g /L
Agar (if solid)	17 g/L

3.3. Molecular identification.

3.3.1. DNA extraction.

Pellet obtained from last subsection was used for DNA extraction. DNA extraction was carried out following the protocol previously described. (Querol et al., 1992). Briefly, pellet was resuspended in 0,5 mL of 1 M Sorbitol, 0,1 M EDTA, pH 7,5 and 0,02 mL of a solution of Zymoliase (2,5 mg/mL) was added. Tubes were incubated for 37 ºC for 30 min. The tube was then centrifuged for 1 min and supernatant was removed. Pellet was then resuspended in 0,5 mL of 50 mM Tris-HCl. 20 mM EDTA pH 7.4. 0,005 mL of 10% SDS were added, and the tubes were incubated at 65 ºC for 10 minutes. After incubation, 0,2 mL of 5M potassium acetate was added and the tubes were cooled down in ice for 10 minutes. Then they were centrifuged for 5 min at 13.000 rpm. Supernatant was transferred to a fresh 1,5 mL Eppendorf tube. DNA was precipitated adding 0,7 mL of isopropanol, incubated for 5 minutes at room temperature, and then centrifuged for 10 min at 13.000 rpm. Precipitated DNA was washed using 70% ethanol and centrifuged for 5 min at 13.000 rpm. Supernatant was removed and once the pellet was air-dried, it was resuspended in 50 µL TE pH 7,4.

3.3.2. S. cerevisiae strains characterization

Characterisation of different *S. cerevisiae* strains was performed using the Interdelta-PCR analysis with the delta 12 and 21 primers and the PCR Mix (Table 2) and program (Figure 3) previously described (Legras & Karst, 2003).

Interdelta polymorphism regions are a reliable and replicable way of *S. cerevisiae* strains typification. The PCR reactions were performed using a thermocycler 2720 Thermal Cycler (Applied Biosystems, Thermo Fischer Scientific Inc., Madrid, Spain). PCR products were separated by electrophoresis at 100V for 90 minutes on a 1,6% agarose gel stained with 0,05 µL of GreenSafe Premium (Nzytech, Lisboa, Portugal) in 1X TBE buffer. The DNA ladder marker 100-bp (Thermo Fischer Scientific Inc., Madrid, Spain) was used. Gels were visualized under UV light and the images were saved for posterior analysis.

Clustering of interdelta-PCR profiles were generated using the GelJ_v2.0 program (Department of Mathematics and Computer Science at the University of La Rioja, Logrono, Spain). To generate the dendrogram the same parameters previously selected by the group (Bedoya et al., 2024) were used: Unweighted pair group method with arithmetic mean (UPGMA) and DICE coefficients. The criterion chosen to consider two profiles of the same strain was a similarity of 90% or higher in their interdelta-PCR profiles. Duplicates were used to prove reproducibility of the process. The diversity of *S cerevisiae* strains was determined using the total number of different interdelta-PCR profiles to calculate the Shannon index (H') and Simpson's index (D) using the formulas described by (Börlin et al., 2016). Shannon index represents the biodiversity of a set population considering two factors: the number of species present and the relative abundance of each species. The higher the number, the more biodiverse the population is. Simpson's index represent the probability of two random individuals from a population to be from the same species. The value goes from 0 to 1, being 1 the higher biodiversity value and 0 the value of a population with no biodiversity at all.

Table 2. Interdelta-PCR Mix recipe.

Interdelta-PCR Mix (final volume 25 µL)								
	MiliQ	Reaction buffer NH_4	$MgCl_2$	Delta 12	Delta 21	dNTPs	BSA	TaqPol
Volume	14,75 µL	2,50 µL	1,25 µL	2,25 µL	2,25 µL	0,50 µL	0,25 µL	0,25 µL

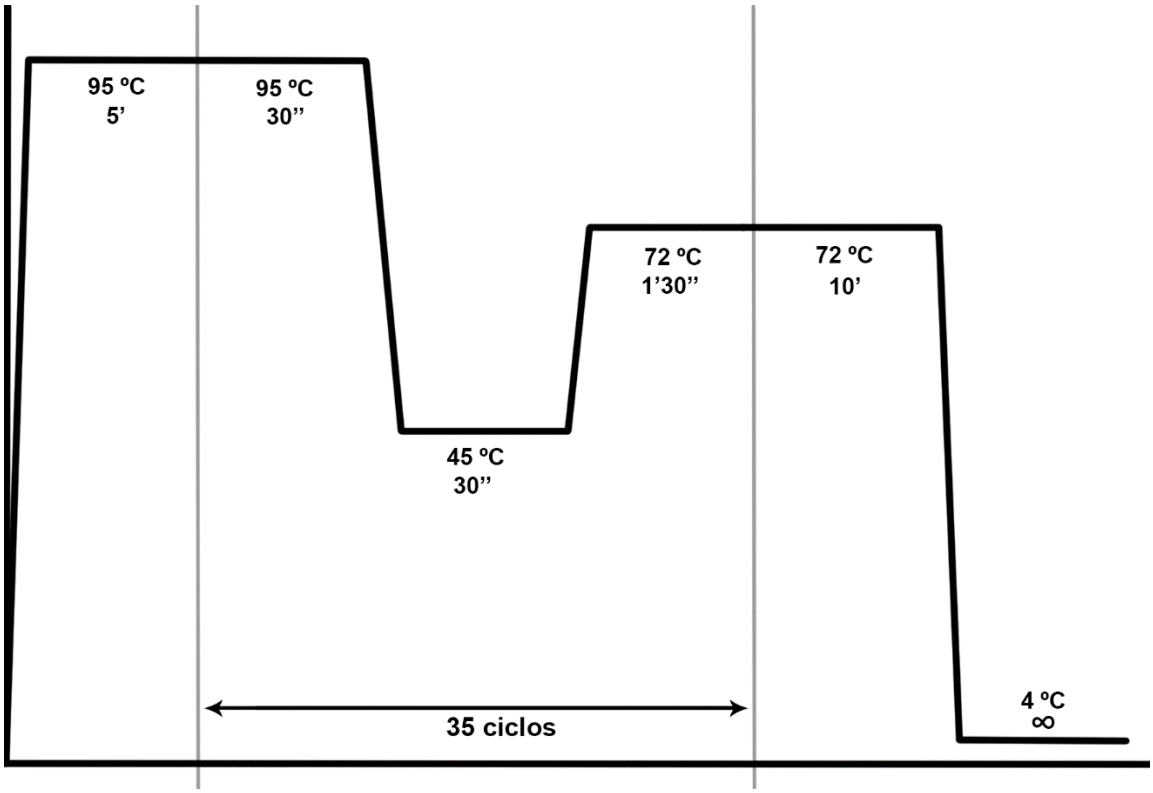


Figure 3. Interdelta-PCR program.

3.4. Sensory analysis of final wines

A sensory analysis was carried out to assess if there was any difference on the organoleptic characteristics of the final wines depending on the fermentation technique used and on the maturity point of the grapes. Fourteen experienced tasters of Oenology Faculty of URV were given 3 opaque glasses with samples of 2 different final products, and then, reported which of the glasses contained the different sample. Different sensorial attributes were evaluated by each of the assessors punctuating each of them from 0 to 5 depending on its intensity. The attributes evaluated were: terpenic, vegetal, tropical, oxidation, acidity, bitterness and quality.

3.5. Fermentative capacity evaluation of different S. cerevisiae strains.

3.5.1. Strain selection.

Interesting strains to be evaluated were selected from the analysed Interdelta-PCR profiles considering two conditions: capability of the strain to be present from the beginning to the end of each fermentation modality and those strains that represented 25% or higher relative abundance at the end of each fermentation (FF). Strains were selected for further analysis if at least one of this two conditions was accomplished.

3.5.2. Inoculum preparation.

The selected strains were refreshed into a new petri dish with YPD. This fresh culture was grown for 24h then was transferred to a plastic tube containing 5 mL liquid YPD and allowed to grow for 24h more. After cell number counting, serial dilutions of this liquid culture were used to obtain a calibration curve (Figure 4) of Optical Density at 600 nm (OD). We used the curve to calculate the volume needed to inoculate each strain at $2x10^6$ cells/mL to perform the micro fermentations.

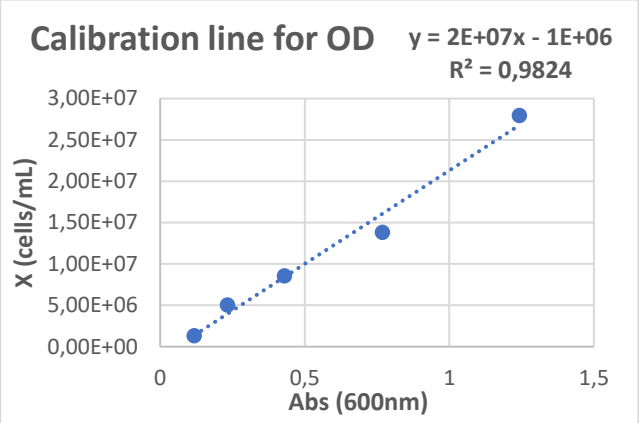


Figure 4. Calibration line used to correlate Abs obtained with the spectrophotometer and de cell concentration.

3.5.3. Micro fermentations of individual strains.

To evaluate the fermentative capacity of the selected *S. cerevisiae* strains, individual micro fermentations were carried out by duplicate. Concentrated commercial must (Concentrats Palleja S.L., Riudoms, Tarragona, España) was used to perform those fermentations since they were made on winter and no more natural must was available. Concentrated must was

resuspended in a 1:4 proportion adding three volumes of sterile distilled water under sterile conditions. The absence of microorganisms was verified by inoculating 0.1mL of reconstituted must in YPD plates in triplicate. Once confirmed that the must was sterile, 100mL of must were transferred to a 250 sterile glass bottle. Then each bottle was inoculated with one of the selected strains by duplicate with a final concentration of $2x10^6$ cells/mL. Additionally, a co-inoculation of the commercial strain QA23 with the strain Sce7 was performed in triplicate because this particular strain was present and imposed its presence in some of the QA23-inoculated fermentations. Finally, 2 bottles were inoculated with QA23 to be used as control fermentations. Fermentation monitoring was made daily measuring the density as previously described for natural must fermentations. OD was also measured daily to monitor cell growth. Temperature chosen for this experiment was 18ºC to mimic the conditions of previous experiment. Fermentations were considered to reach the end when the concentration of residual sugars was 2g/L or below. DNA extraction and Interdelta-PCR was carried out as previously described using a sample taken at the end of each AF to confirm the presence of the inoculated strains and outrule contaminations.

3.5.4. Chemical analysis and proposal for correlation between different population dynamics and organoleptic differences on produced wines from natural must.

The resulting wines from the natural must harvested during summer 2023 were subjected to a sensorial analysis to evaluate the organoleptic characteristics (similarity and wine attributes).

Chemical analysis of triplicate samples (1,5mL) from final wines produced during micro fermentations of selected strains was performed to determine the main organic compounds using High Performance Liquid Chromatography (HPLC) under the same circumstances of the previous research (Bedoya et al., 2024). The evaluated compounds were citric acid, tartaric acid, succinic acid, lactic acid, acetic acid, glucose, fructose, glycerol, and ethanol. Besides the

samples from the individual fermentations, control samples for the must without fermentation were added.

3.5.5. Statistical tests

ANOVA test was used to find differences among the sensory attributes and then a Fisher's least significant difference (LSD) was carried over to further analyse these differences and assess the impact of ripeness level and fermentation modality on the final attributes of the product. ANOVA test was carried over to assess whether there was a difference in organic compounds production between selected strains or not. Those results were then compared with the results from sensorial analysis by Pearson’s correlation to seek a relation between the presence of selected strains and the organoleptic differences. A p-value of 0.05% was used for every ANOVA test.

4. RESULTS AND DISCUSSION

4.1. Cell growth and kinetics.

Fermentation kinetics were evaluated to assess the effect of the different techniques over cell growth and performance.

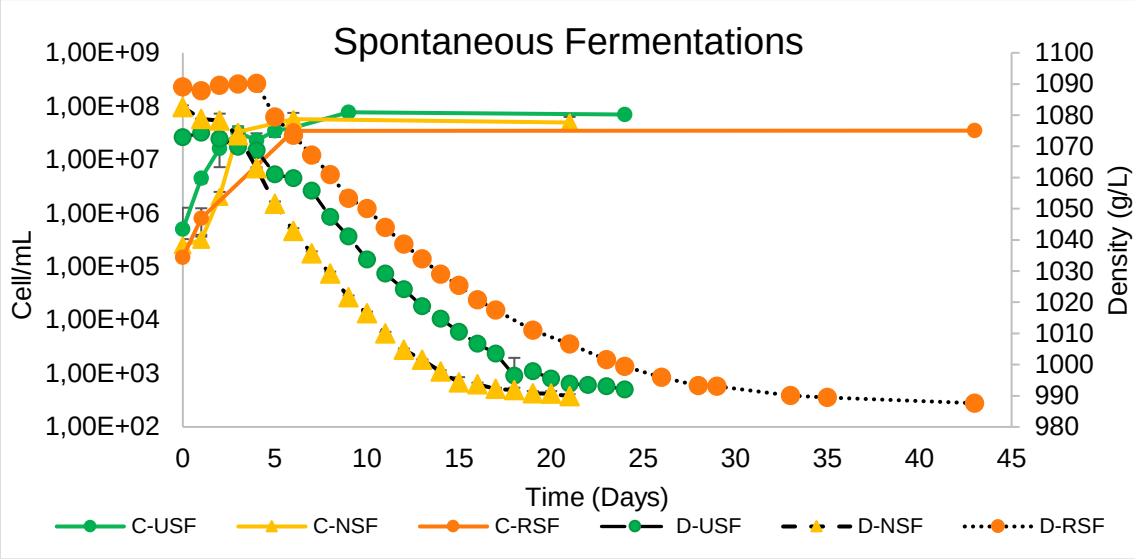


Figure 5. Effect of Spontaneous Fermentation technique over fermentation kinetics. Solid lanes represent CFU count in WLN medium. Dashed lanes represent density values. Every value in the graphs corresponds to the mean of the values from biological triplicates.

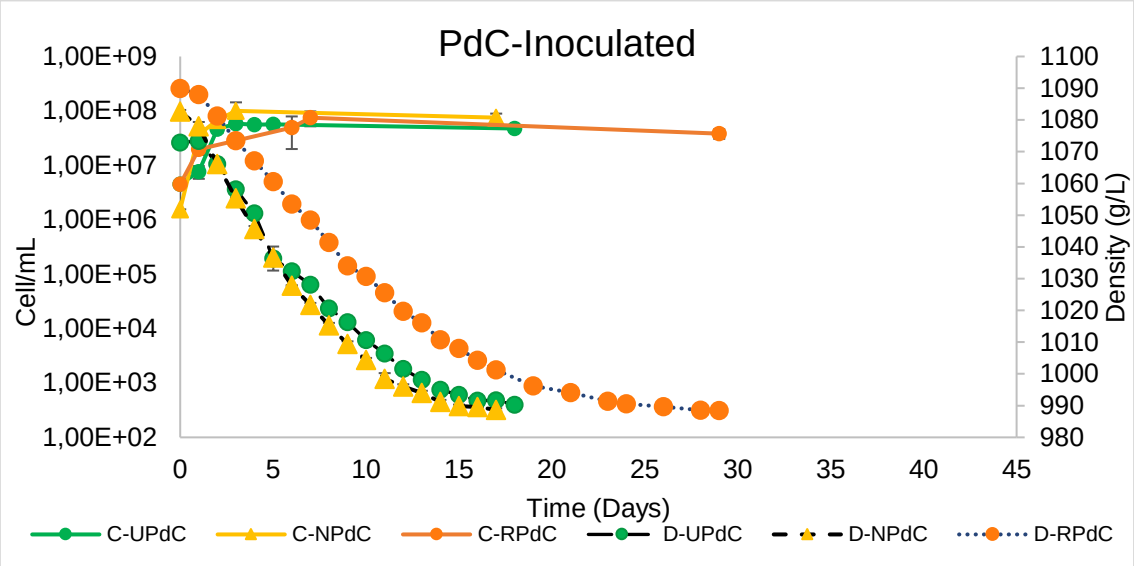


Figure 6. Effect of PdC inoculation technique over fermentation kinetics. Solid lanes represent CFU count in WLN medium. Dashed lanes represent density values. Every value in the graphs corresponds to the mean of the values from biological triplicates.

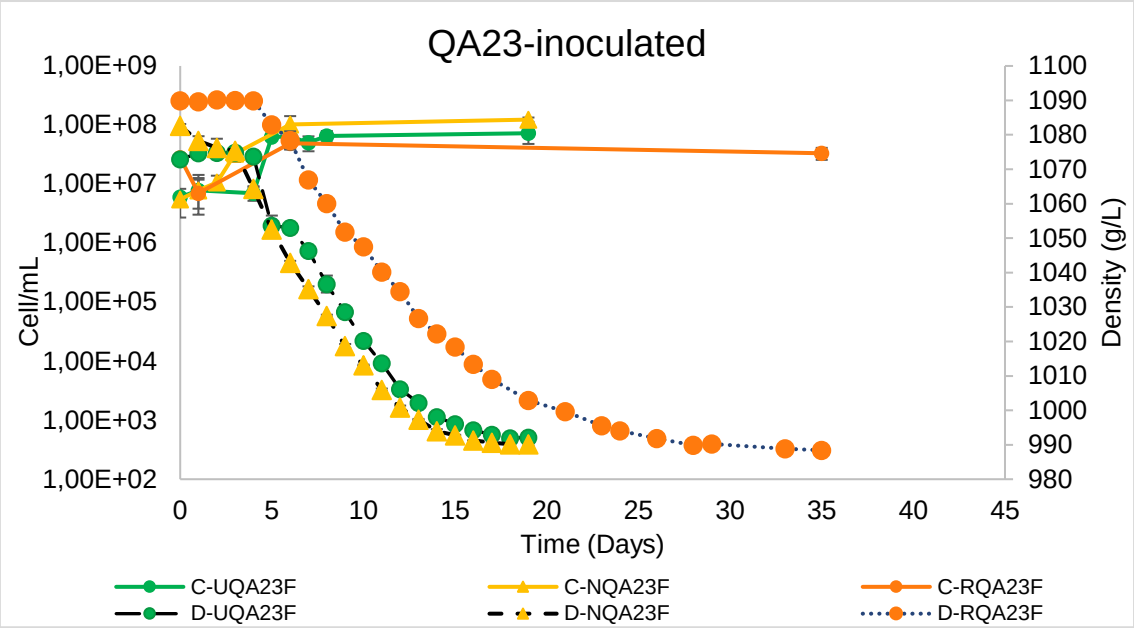


Figure 7. Effect of QA23 inoculation technique over fermentation kinetics. Solid lanes represent CFU count in WLN medium. Dashed lanes represent density values. Every value in the graphs corresponds to the mean of the values from biological triplicates.

Among all three experiments differences were observed in density evolution and cell growth.

Ripeness of the grapes seems to affect fermentation time, SF finished after 21, 24 and 43 days in U, N and R experiments respectively (Figure 5). The difference between U and N was an increase of 14% of the time following the ripeness increase which is not very big, however, ripe grapes had an increase of around 105% between U and N which is more than double the time needed with unripe grapes. PdC fermentations showed no significant increase between U and N (18 and 1 days respectively), in fact, N fermentations were 1 day faster than U, all the same, R fermentation did increase fermentation time by 11 days (61%) (Figure 6). Finally, QA23 fermentations needed 19, 19 and 35 days to reach the end of the fermentation respectively (Figure 7). There was no difference between U and N, and as with previous techniques, R fermentations needed more time, an increase of 79% in this case. Regarding differences between techniques, PdC showed the fastest fermentations for every ripeness point, even faster than QA23 which is a strain commercialised, not only for its positive contribution to organoleptic attributes but also for its fast performance in

must fermentations showing its potential, not only for controlling microbial population, but also to ensure a fast fermentation process until the end.

Regarding cell growth, QA23 showed the highest initial population, probably caused by the inoculation of $2x10^6$ cells/mL. PdC technique increased initial cell growth faster than SF. SF fermentations had the lower starting population with $1x10^5$ CFU/mL and PdC fermentations of $1x10^6$ $\mathrm{CFU/mL}$. These results are not a surprise since PdC is inoculated once the fermentation has started, which involves cell growth. However, cell growth during the fermentation was very similar with values between $1x10^7$ and $1x10^8$ $/\mathrm{mL}$ never reaching the $1x10^9$ $\mathrm{CFU/mL}$ in any case.

4.2. Isolated S. cerevisiae strains from WLN medium.

A total of 540 *S. cerevisiae* isolates were characterised via interdelta-PCR analysis from the three fermentation modalities (SF, QA23F and PdC) during the three experiments using different grade of grape ripeness (U, N and R). This revealed a total of 52 different inter delta profiles. U experiment showed a total of 16 different profiles after isolation and characterisation. Each different fermentation modality showed a different number of profiles, having PdC the lead showing 10 profiles, followed by QA23F with 5 profiles and lastly SF with 4 profiles (Fig 8.). None of the profiles of USF were present in UPdC or UQA23.

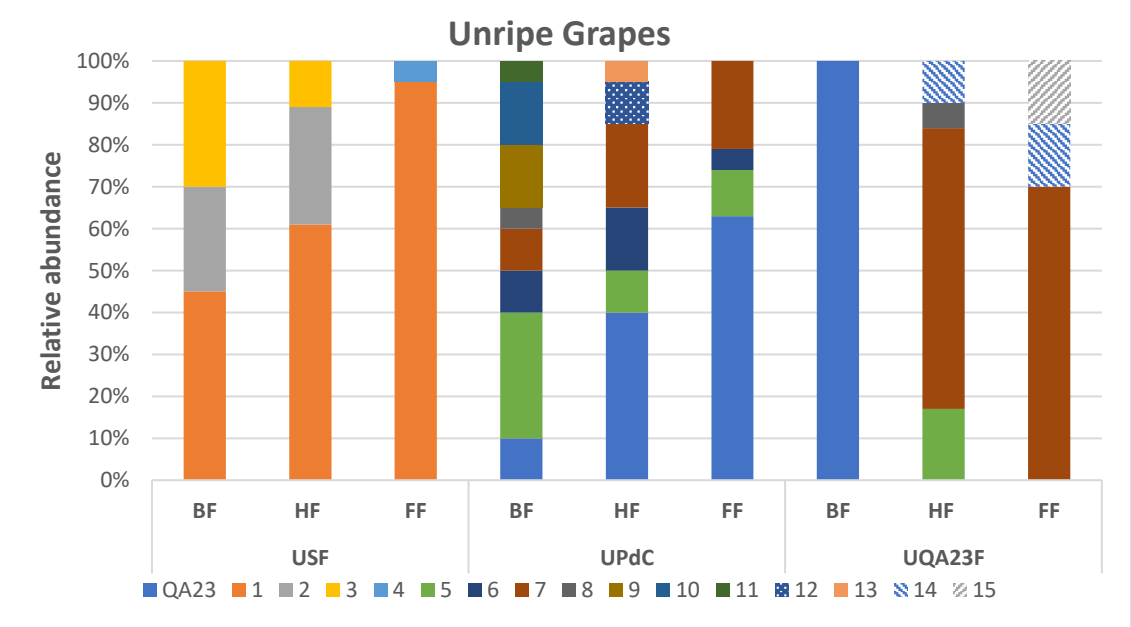


Figure 5. Interdelta profiles and its relative abundances during each fermentation phase and separated by fermentation modality using must produced with unripe grapes. The numbers refer to the different profiles (Ex: 7 stands for Sce7 profile).

These two latest fermentations shared the 5 and 7 profile, but their abundances differed significantly.

N experiment showed a total of 14 different profiles after isolation and characterisation, 11 of which were new profiles and 1 of them (Sce7) was also present in U and R experiments. In N experiment PdC and SF modalities showed a more even number of profiles being 7 different profiles in both cases while QA23F showed 3 profiles (Fig 9). Sce7 appeared in every modality but only reached the end of the fermentation in NQA23F, Sce23 appeared in NPdC and NQA23Fand stayed until the end of the fermentation in NQA23F.

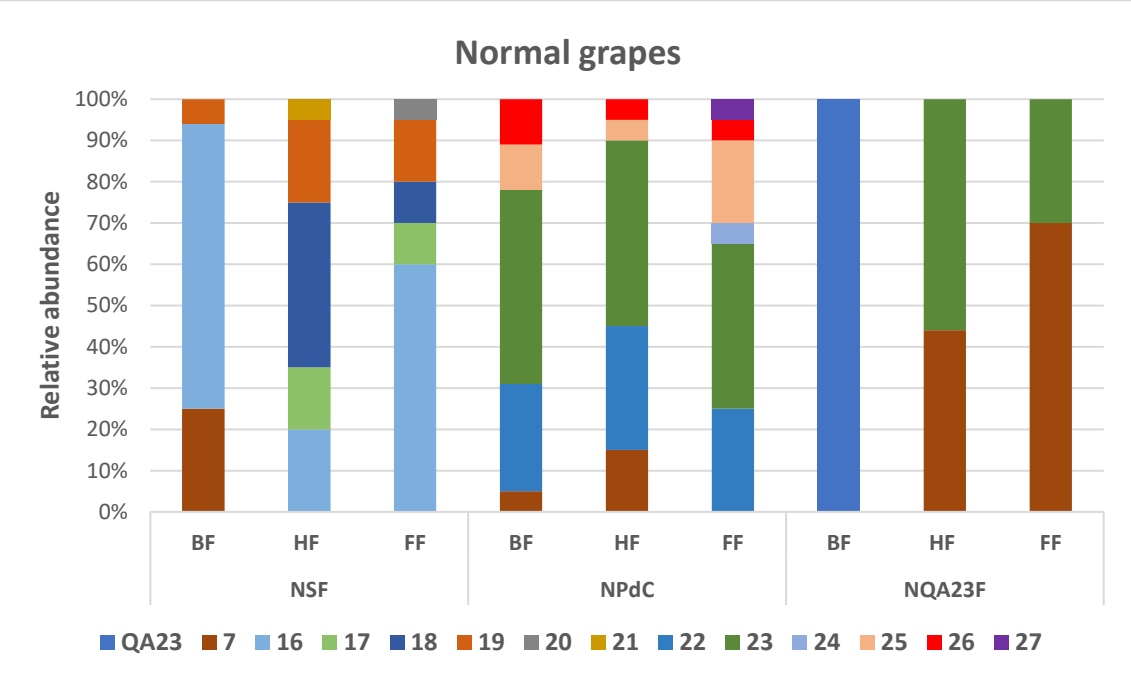


Figure 9. Interdelta profiles and its relative abundances during each fermentation phase and separated by fermentation modality using must from normal grapes. The numbers refer to the different profiles (Ex: 7 stands for Sce7 profile).

Lastly, R experiment showed 30 different profiles from which 25 were new profiles and 5 (QA23, Sce7, Sce15, Sce23 and Sce27) were present in U and N experiments. SF showed 16 different profiles, PdC showed 18 different profiles and QA23F showed 3 different profiles (Fig 10). Sce7 was shown in every modality, Sce23, Sce28, Sce29, Sce,30 and Sce33 appeared in RSF and RPdC.

Among the majority of experiments the number of profiles present on the AFs decreased as the fermentation progressed which we expected since must

become a more selective medium as the fermentation progresses. Exploring strategies that involve minimal intervention during fermentations, spontaneous fermentations has yielded positive outcomes in terms of increasing diversity of species and strains associated with the terroir (Börlin et al., 2020). Nonspecific strain was detected alone at the end of any of AF, but some represented 70% or higher of abundance at the FF stage (profile 7 in the three QA23F, profile 1 in the USF). Even if NSF and NPdC had the same number of strain profiles, SF presented the same number of profiles at HF and FF stages while PdC presented higher number of profiles at FF stage. We also expected QA23F fermentations to show QA23 strain as the dominant profile at the end (FF) of the AF as it is an ADWY known to be very dominant in AFs thanks to its low nitrogen requirement, alcohol resistance and killer phenotype, nevertheless, QA23 was not detected at HF and FF of any of the AF carried over(Beltran et al., 2005; Vendramini et al., 2017) . The common characteristic of every QA23F, regardless of the ripeness grade of the grapes, was the presence of the profile Sce7, which takeover AFs even when QA23 was inoculated. These results make Sce7 a very interesting profile for further experimentation, hence we decided to include micro fermentation with co-inoculation of QA23 and Sce7 by triplicate.

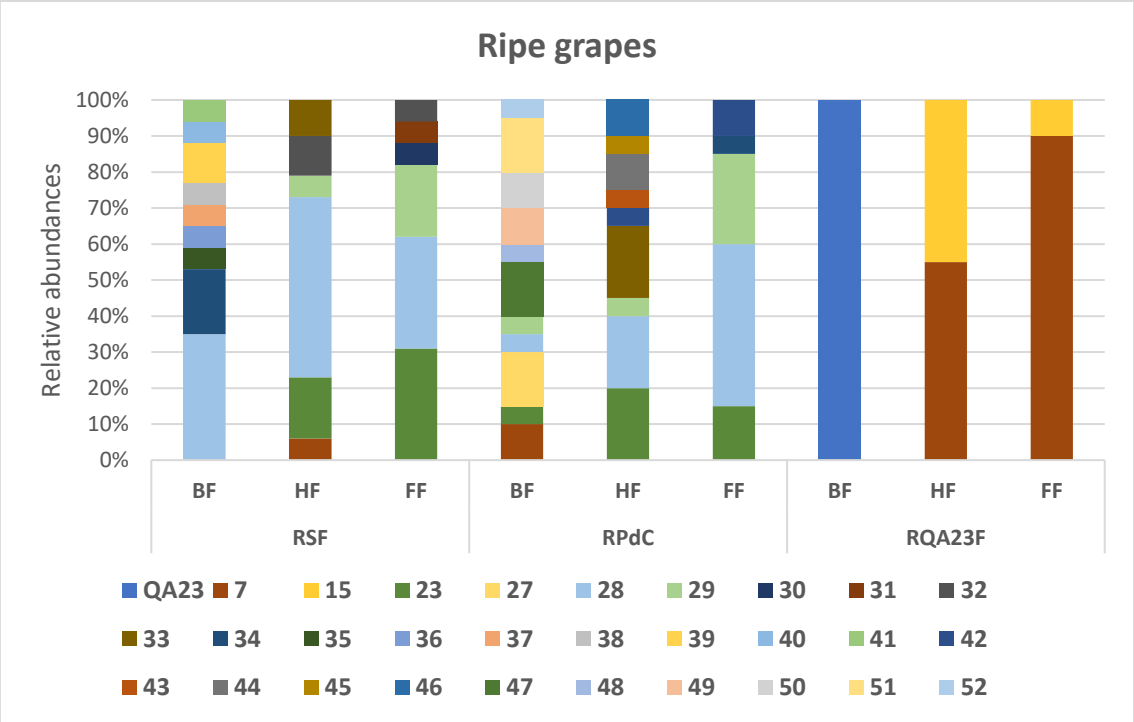


Figure 6. Interdelta profiles and its relative abundances during each fermentation phase and separated by fermentation modality using must from ripe grapes. The numbers refer to the different profiles (Ex: 7 stands for Sce7 profile).

4.3. Strain diversity.

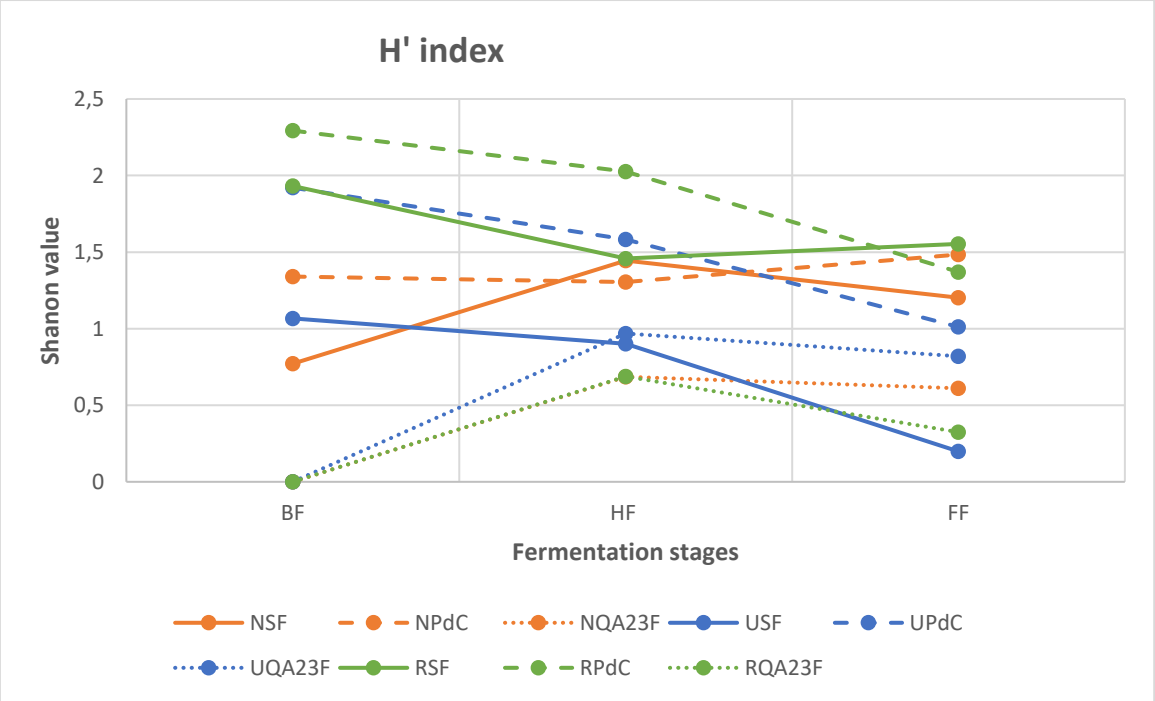


Figure 7. Shannon indexes representation for each fermentation phase, technique, and grape maturity point.

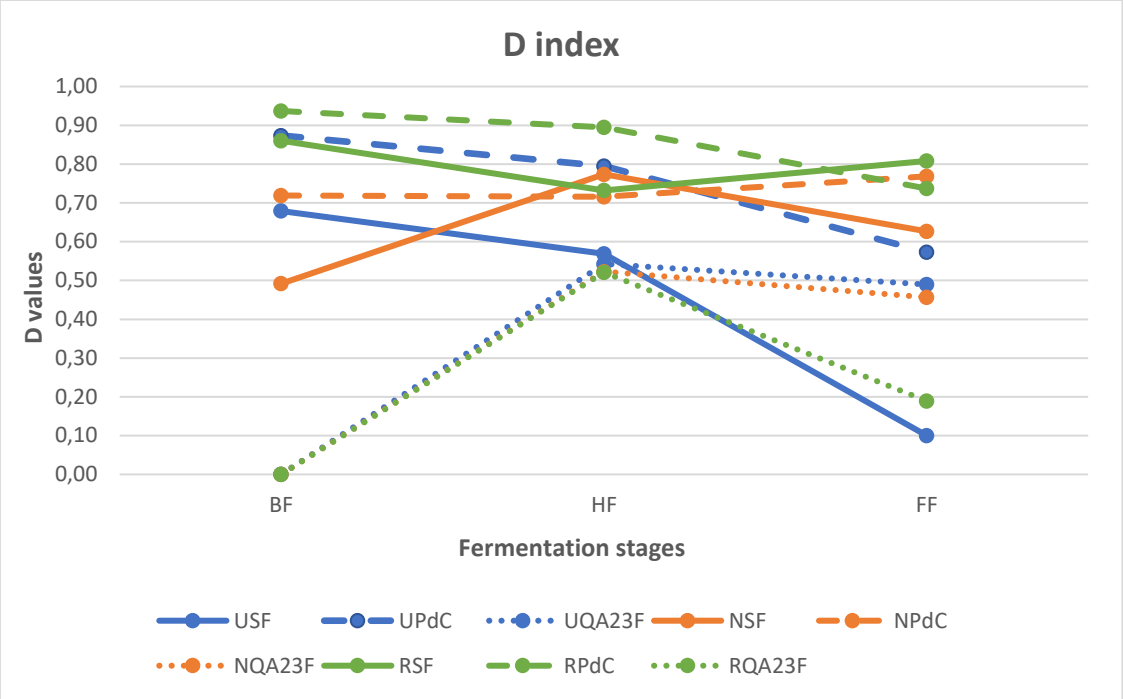


Figure 12. Simpson's indexes representation.

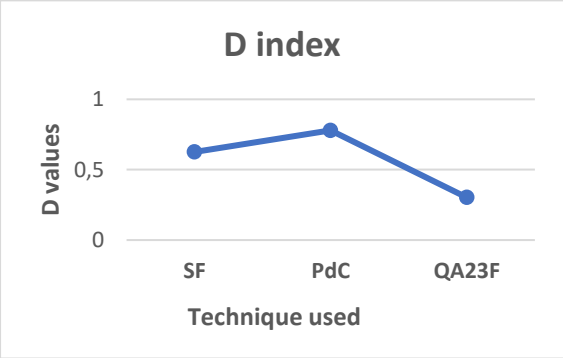


Figure 13. Simpson's index calculated to evaluate the effect of different techniques on the diversity of S. cerevisiae strains

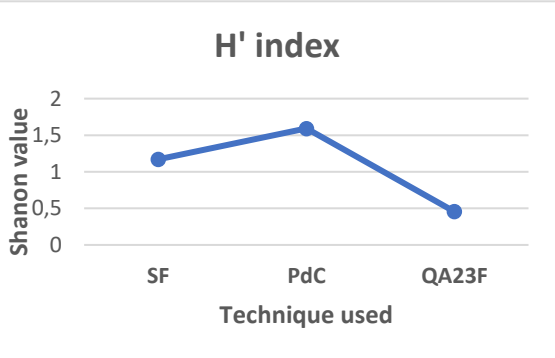


Figure 14. Shannon index calculated to evaluate the effect of different techniques on the diversity of S. cerevisiae strains.

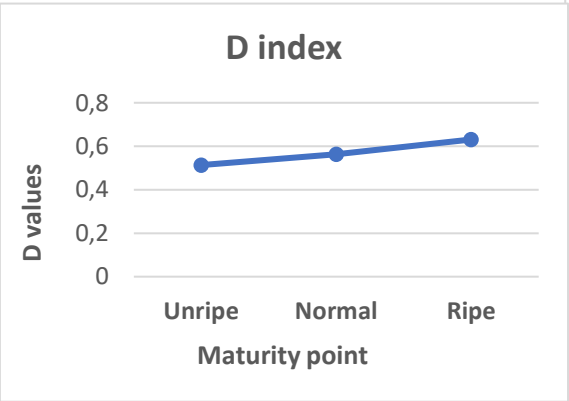


Figure 15. Simpson's indexes calculated to evaluate the effect of different maturity points on the diversity of S. cerevisiae strains.

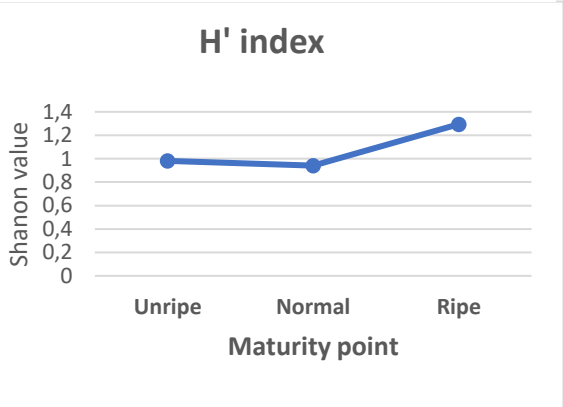


Figure 16. Shannon index calculated to evaluate the effect of different maturity points on the diversity of S. cerevisiae strains.

To assess the diversity of *S. cerevisiae* strains under different conditions Shannon index and Simpson's index were used. Both were calculated to then compare the reliability of the results. After representation of both indexes, the graphics generated were very similar confirming the reliability of the results (Figure 11 and 12). Analysing the graphics and the values, H' index and D index the higher diversity was observed for PdC fermentations. Usually, At the final stage of the fermentation, the diversity decreased, except in RSF and RPdC (Figures 11 and 12).

S. cerevisiae diversity was also assessed by grouping samples by methodology without separating them by maturity points (Figures 13 and 14). Values showed that PdC fermentations had higher biodiversity, followed by SF and lastly QA23F showed the lower biodiversity. Additionally, the effect of different grape maturity points was evaluated grouping the samples by grape ripeness level and not separating by the technique used (Figures 15 and 16). Results showed that biodiversity increased with grape ripeness.

Fermentation stage, SO_2 and grape variety have been described to impact *S. cerevisiae* diversity (Chen et al., 2022). The results obtained showed that both, technique used, and grape maturity point affects to the diversity of *S. cerevisiae* strains during AF. Nevertheless, regardless of the grape ripeness level, the higher diversity was observed for the PdC fermentations. These results confirmed the first part of our hypothesis presenting the potential of the PdC technique as a substitute to commercial ADWY starters to maintain the microbiological terroir while assuring the completion of the fermentation.

4.4. Clustering of the different profiles to assess grouping depending on the maturity point of the grapes or the phase of the AF.

A dendrogram was obtained after clustering the 52 different profiles some of them, repeated to confirm a reliable grouping using GelJ_v2.0 (Figure 14).

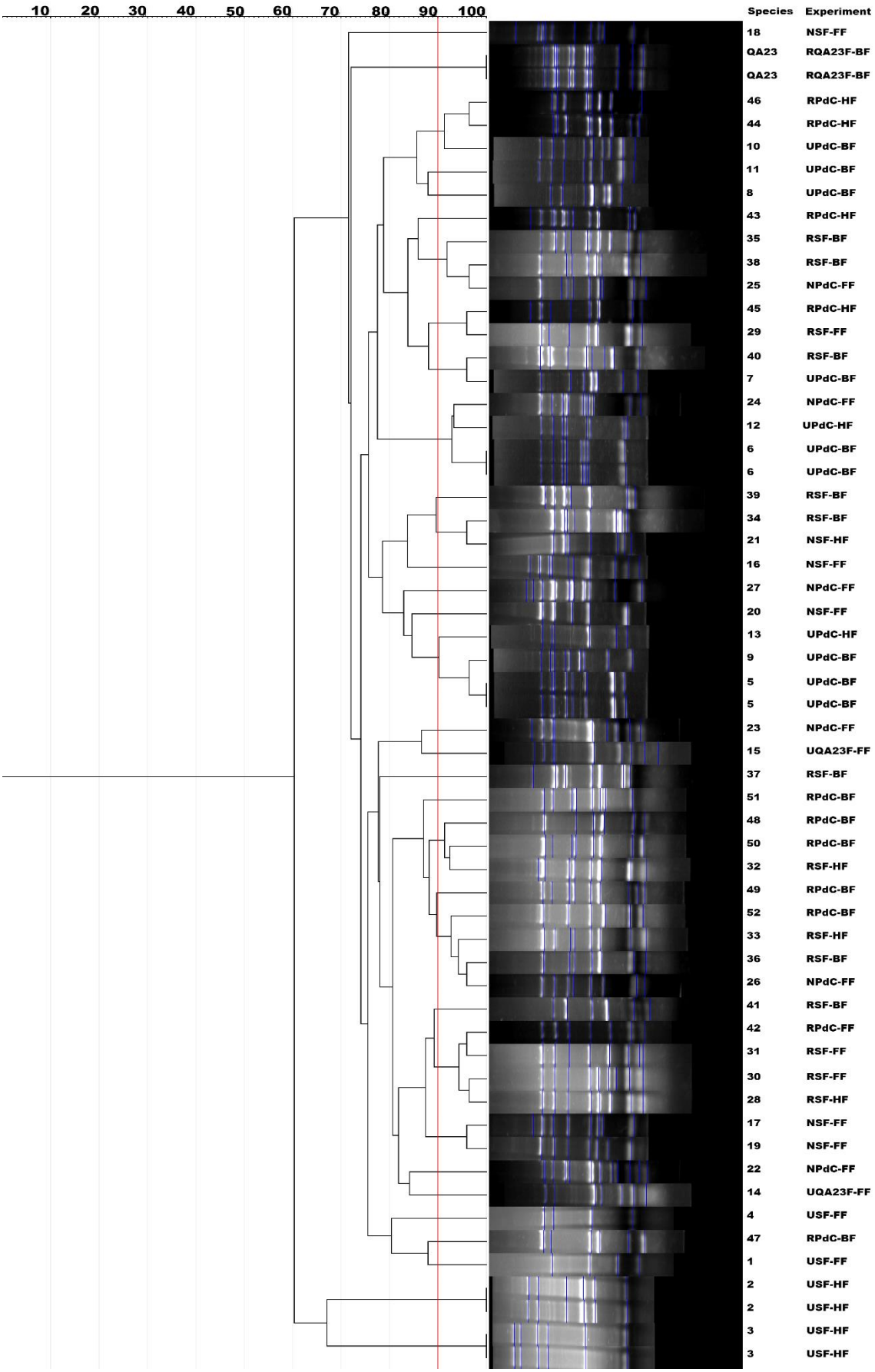


Figure 17. Dendrogram from the 52 different profiles, red line indicates 90% similarity.

The interdelta-PCR profiles were grouped with at 90% similarity by a dendrogram into 33 clusters (Figure 17), however this clustering did not follow either the different fermentation modality used, the stages of the fermentation, the maturity point of the grape. On the other side, the dendrogram confirmed the right selection of profiles for the diversity analysis. Clustering was described previously to not be successful to group strains depending on physicochemical parameters or to distinguish between phenotypes and be successful to cluster depending on grape variety (Bedoya et al., 2024; Chen et al., 2022; Reis et al., 2017).

4.5. Sensory analysis of the final wines.

Unripe grape final wines were significantly different for each of the techniques used (Table 3). Normal grapes showed significant differences in SF vs QA23F and PdC vs QA23F but the difference between SF and PdC was not significant. Lastly, Ripe grapes showed a significant difference only between SF and QA23 meaning that SF vs PdC and PdC vs QA23 were not significantly different. PdC inoculated wines did not result in a consistent variation of the sensory perception. These results were interesting because part of our hypothesis was that PdC technique helped preserve the characteristics linked to the respective *terroir.* PdC-inoculated fermentations not being significantly different from SF reveal the potential of PdC technique related to *terroir* linked properties preservation. ADWYs inoculated fermentations (QA23F) were significantly different from SF on every maturity point which means a loss of *terroir* identity on the final wines. These two results reveal the superiority of PdC-inoculation over ADWY-inoculation for *terroir* properties conservation.

Table 3. Effect of the maturity index and inoculation conditions on the significant differences of the triangle tests. Unripe grapes (1), Normal grapes (2) and Ripe grapes (3). S means significant differences and NS means not significant differences for a p value<0.05.

Maturity level	Triangle 1 SF vs PdC	Triangle 2 SF vs QA23F	Triangle 3 PdC vs QA23F
1	< 0.001 S	< 0.001 S	< 0.001 S
2	0.327 NS	0.016 S	0.003 S
3	0.541 NS	0.016 S	0.327 NS

Different sensorial attributes were evaluated by each of the assessors punctuating each of them from 0 to 5 depending on its intensity. The attributes evaluated were: terpenic, vegetal, tropical, oxidation, acidity, bitterness, and quality. A spider plot was generated to ease the visualization of the results (Figure 18).

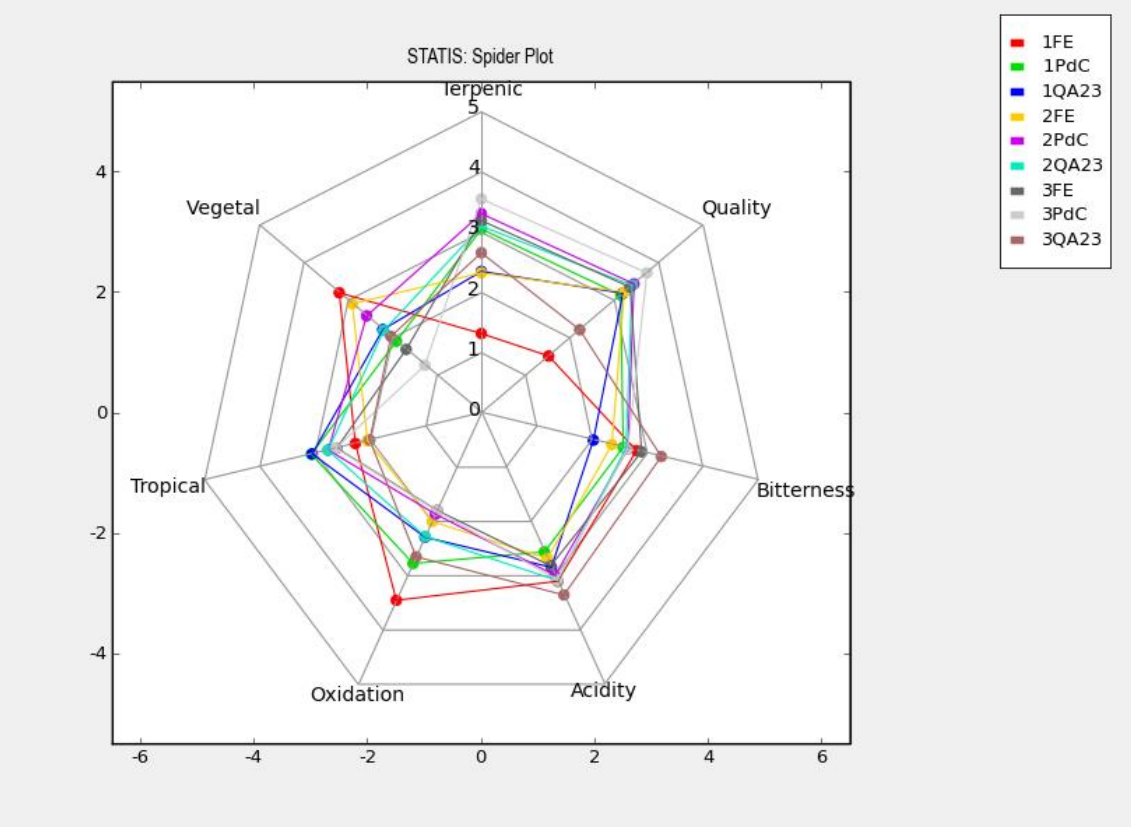


Figure 18 Spider plot of descriptive sensory analysis. Assessors = 14, one repetition for each assessor and wine. Unripe grapes (1), Normal grapes (2) and Ripe grapes (3).

To assess the difference of each sensorial attribute between the different AFs confidence intervals were calculated for each at attribute by Fisher's least significative difference (LSD) procedure (Figure 19). This concluded a significative difference between AFs on five attributes: Terpenic, Vegetal, Tropical, Oxidation and Quality. Acidity and bitterness did not show significant differences. We will discuss each of the significant attributes independently.

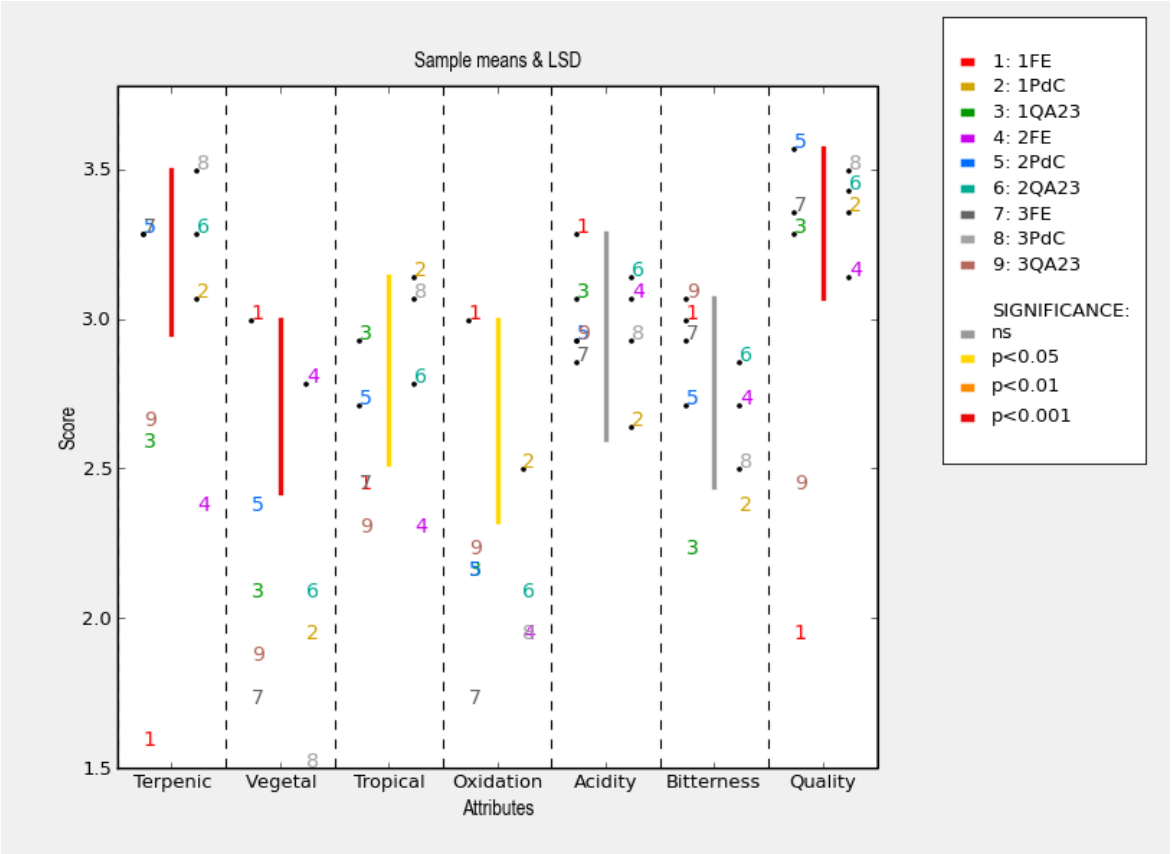


Figure 19 Confidence interval of assessor's judgments for each attribute by Fisher's least significant difference (LSD) procedure. Assessors = 14, one repetition for each assessor and wine. Unripe grapes (1), Normal grapes (2) and Ripe grapes (3).

Terpenic attribute

Samples showed a significant difference in terpenic attribute. USF, NSF UQA23F and RQA23 showed the lower values and were different from UPdC, NPdC, RSF, NQA23F and RPdC which had the higher values. Terpenes are a characteristic feature of *Muscat of Alexandria* and the responsible for fruity aromas and flavors in wines (Black et al., 2015). Fruity aromas and flavors are one of the qualities that define the quality of a wine, higher values of terpenic attributes are desirable and usually imply a higher quality of the wine. Results showed that PdC-Inoculated wines had relatively higher terpenic values highlighting a positive effect of PdC technique over final wines quality.

Vegetal attribute

Only two final wines were significantly different USF and NSF both of which showed the higher values of this attribute. Vegetal attribute is usually perceived

as negative when it is predominant and can be linked to a non-optimal grape ripeness. The three samples that showed the lower values were these from AFs carried over with ripe grapes. Technique used seems to not affect vegetal attribute since the values were randomly spread among the value range.

Tropical attribute

Tropical attribute was significantly different among the different AFs. UPdC, NPdC, RPdC, UQA23F and NQA23F showed higher values of tropical attribute and were different from USF, NSF, RSF and RQA23F. It had been previously stated by the research group on previous experiments that QA23 technique positively affects tropical attribute in wines (Bedoya et al., 2024) The results reasserted this statement. However, RQA23F was interesting, QA23 strain is known to contribute to tropical notes in finished which is the opposite of what was observed, having these samples the lower value on tropical attribute. Additional experimentation could be interesting to assess if excess grape ripeness negatively affects QA23 strain capacity of contributing with tropical notes.

Oxidation attribute

Oxidation is usually a bad attribute acquired by wines, mainly, when in contact with air. During aging, chemical transformations occur in wines, leading to colour and flavor changes. A range of off-flavors can be formed from wine oxidation. At low concentrations these flavors may add to the complexity of a wine, but as these increase they begin to detract from wine quality (Oliveira et al., 2011). When exposed to air too long, a wine can become oxidized to the point that the acetaldehyde converts to acetic acid which is an undesired compound in wines. USF and UPdC where significantly different from the other AFs with higher values of oxidation. Neither grape ripeness nor technique used seem to affect the oxidation attribute, oxidation values for AFs are randomly spread over the graphic.

Acidity and bitterness

Neither of these attributes showed a significative difference and values where very similar for the different AFs.

Quality attribute

Quality is the attribute that defines how good a wine is. Quality is defined by the combination of every other attribute of the wine. The preferences and perceptions of wine quality and attributes as tropical can vary greatly between nationalities, limiting definitive conclusions. Including individuals from diverse nationalities and backgrounds in tasting panels is beneficial to achieve more reliable results. Significant differences were observed on the quality of final wines. USF and RQA23F showed a significantly lower quality which was expected from the results of the rest of the attributes since both AFs scored low and high values on desirable and undesirable attributes respectively. AFs performed by PdC technique scored 3 of the 4 highest values of quality, this means that PdC might positively affect overall quality of wines, but the difference was not big enough, it could be the result of the varied preferences of the individuals participating on the tasting.

4.6. Strain selection for laboratory scale AFs

From the 52 different characterised trains, the most interesting according to our described criteria were selected.

As previously stated, we consider 2 criteria for this selection: the capability of a strain to be present from the beginning to the end of the fermentations or a relative abundance higher than 25% at the end of the fermentation. Accomplishing one of these conditions was enough to consider the strain as candidate for the evaluation of its fermentative capacity.

From fermentations using must from unripe grapes (U), 4 isolates were selected: Sce1, Sce5, Sce6 and Sce7. Sce1 was present from the beginning to the end of the SF and at FF it had dominated the FF of SF with a total relative abundance of 95%. Sce5, Sce6 and Sce7 were present from the beginning to the end of the PdC fermentation. Additionally, Sce7 was also present at the end of QA23F fermentations with a total relative abundance over 60% (Figure 5).

From fermentations using must obtained from normal grapes (N) 5 new isolates were selected: Sce16, Sce19, Sce22, Sce23 and Sce25. Sce16 and Sce19

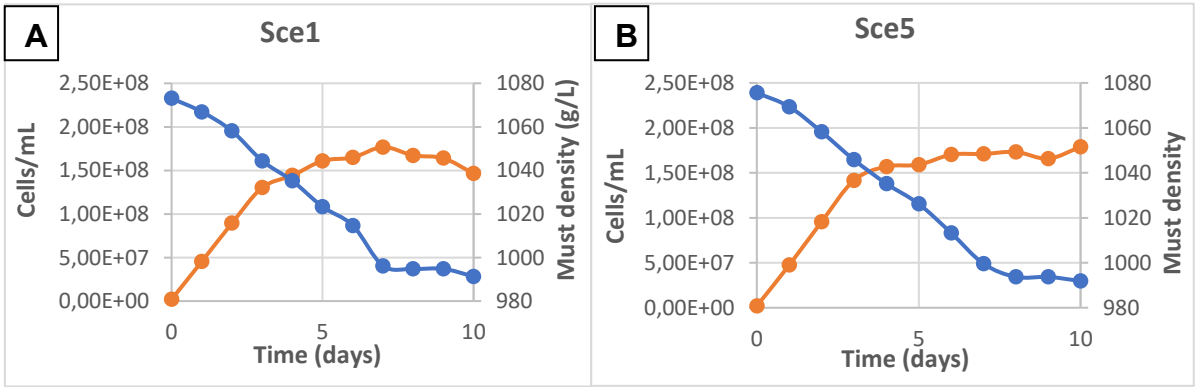
were present during SF, from the beginning to the end, Sce16 dominated SF with a total abundance of 60% at FF point. Sce22, Sce23 and Sce25 appeared during every phase of the PdC fermentation. Sce22 and Sce23 presented a relative abundance higher than 25%, 25% and 40% respectively. Finally, from QA23F fermentation no new profile was selected. Only 2 profiles were present at HF and FF points, and they were Sce7 and Sce23 which were already selected (Figure 6).

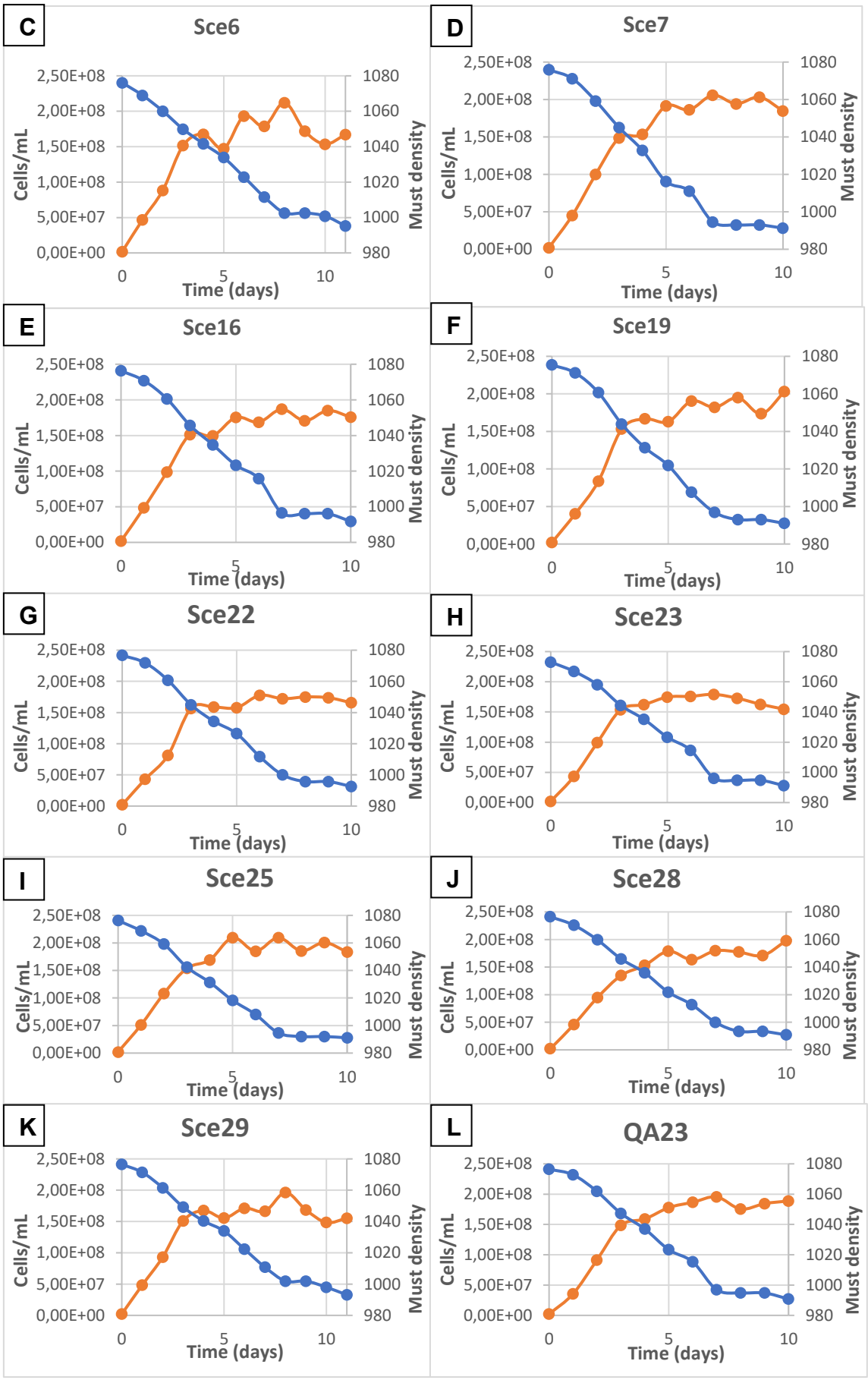
When using ripe grapes for the must production many new profiles appeared. 2 of the new profiles were selected for further analysis: Sce28 and Sce29 were both present in every phase of the fermentation for SF and PdC fermentation. However, in SF Sce29 had a lower abundance than 25% while in PdC fermentation it had a total abundance of 25%. Sce28 had an abundance of 31% and 45% for SF and PdC fermentation respectively (Figure 7).

With these results, a total of 11 isolates were selected for the evaluation of their fermentative capacity.

4.7. Evaluation of fermentative capacity of individual selected strains.

The results of the individual micro fermentations performed in laboratory scale are shown in the Figure 20. The fermentations were tracked until they reached a concentration of residual sugars of 2 g/L or less.




C
Sce6
D
Sce7
E
Sce16
F
Sce19
G
Sce22
H
Sce23
I
Sce25
J
Sce28
K
Sce29
L
QA23
Cells/mL
Must density
Time (days)
2,50E+08
2,00E+08
1,50E+08
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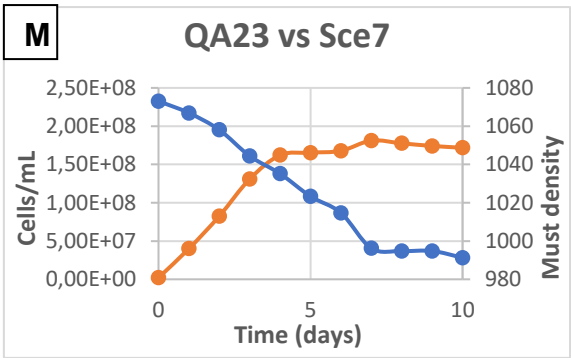

Figure 20. Graphic representation of all the individual fermentations performed in laboratory scale and the co-inoculation of QA23 and Sce7. The orange line represents the cell growth during the AF. The blue line represents the evolution of the density during the AF process.

The evaluation of the fermentative capacity of the selected strains showed very similar fermentation kinetics with slight variations on cell concentrations during the process. 11 of the total 13 fermentations set up ended after 10 days of AF. Sce6 and Sce29 fermentations were slower and ended in day 11 which was not a significative difference in time. The evolution of the density did not show significative differences and almost every strain arrived at a density under 1000 g/L during the 7th day. Sce6 and Sce29 got stuck around 1000 g/L for a few days (Sce6 until day 10 and Sce29 until day 9) but lowered their density at day 11. Analysis with the enzymatic kit for residual sugars on days 9 and 10 were carried out with Sce6 and Sce29 strains even if the density was higher to confirm that the fermentation was not ended regardless of the higher density. The density on the must get lower during AF because the yeasts are actively producing CO_2 and ethanol which means that yeasts with lower ethanol production result in wines with higher density at the end of the AF. The analysis was performed with the Y15 bioanalyzer and showed that the concentration of residual sugars was between 3 and 2 g/L, analysis on day 11 showed that the concentration had become lower than 2 g/L and the AF was considered finished. Residual sugars analysis was performed since day 7 on all the other AFs to check if they had already finished, as previously stated, all of them finished on day 10. Interderdelta-PCR analysis confirmed the presence of each inoculated strain at the end of their respective inoculated micro fermentations and the absence of contamination with other *S. cerevisiae* strains or microorganisms.

Interdelta-PCR analysis was also used to assess the imposition of Sce7 strain over QA23 commercial ADWY, out of the 15 random samples took at the end of the co-inoculated fermentation 13 belonged to Sce7 and 2 to QA23 thus confirming the imposition of Sce7 over QA23.

The co-inoculation of QA23 and Sce7 is represented in (Figure 20M). The kinetic is highly similar to the individual fermentations of each of the strains. However, during the co-inoculation, the number of total cells is lower at the end of the fermentation, probably due to competition. Interdelta-PCR analysis was also used to assess the imposition of Sce7 strain over QA23 commercial ADWY during the co-inoculated fermentations. Out of the 15 random samples took at the end of fermentation resulted in 13 profiles belonging to Sce7 and 2 to QA23 thus confirming the imposition of Sce7 over QA23.

4.8. Chemical analysis of micro fermentations using individual strains

Each individual strain AF was analysed by triplicate by HPLC. First ANOVA test was carried over to evaluate the presence of significant differences in the concentration of each compound among strains (Table 4).

Significant differences were found for five of the eight parameters evaluated. citric acid, tartaric acid, succinic acid, lactic acid, acetic acid concentration and pH were found to be significantly different. Glycerol and ethanol concentrations were similar.

Table 4. Statistical ANOVA test to evaluate the significance of differences in the organic compounds at the end of each fermentation. A p value of 0,05 or below was considered inside the significance threshold.

ANOVA	Citric	Tartaric	Succinic	Lactic	Acetic	Glycerol	Ethanol	pH
F	173,3480	3,1316	3,2918	1,2795	4,5652	0,5957	2,5728	3,9728
Crit F	2,6037	2,6037	2,6037	2,6037	2,6037	2,6037	2,6037	2,6037
p	2,68E-12	0,0257	0,0212	0,3320	0,0054	0,8110	0,0521	0,0099
Ho	p > 0,05	p > 0,05	p > 0,05	p > 0,05	p > 0,05	p > 0,05	p > 0,05	p > 0,05
Ha	p < 0,05	p < 0,05	p < 0,05	p < 0,05	p < 0,05	p < 0,05	p < 0,05	p < 0,05
Hypothesis	Ha	Ha	Ha	Ho	Ha	Ho	Ho	Ha
Significance	Yes	Yes	Yes	No	Yes	No	No	Yes

Table 5. Final analysis of the wines product of the micro fermentations carried out with each selected S. cerevisiae strain. Units are g/L. The table represents the average value of each compound and the standard deviation related to it. Lower values are highlighted in red and higher with blue for each compound.

Sample	Citric	σ Cit	Tartaric	σ Tar	Succinic	σ Suc	Lactic	σ Lac	Acetic	σ Ace	Glycerol	σ Gli	Ethanol	σ EtOH	pH
Sce1	0,64	0,12	1,15	0,02	2,22	0,04	0,22	0,04	0,81	0,05	5,62	1,42	10,59	0,06	3,309
Sce5	0,57	0,11	1,15	0,01	2,14	0,02	0,24	0,01	0,61	0,03	5,32	0,97	10,67	0,05	3,305
Sce6	1,78	0,85	1,21	0,01	2,93	0,02	0,27	0,02	0,92	0,07	6,30	0,88	10,42	0,01	3,12
Sce7	0,69	0,74	1,15	0,01	2,52	0,15	0,14	0,10	0,68	0,03	5,51	1,09	10,52	0,03	3,316
Sce16	0,74	0,06	1,15	0,01	2,41	0,06	0,24	0,01	0,63	0,01	4,64	0,03	10,45	0,07	3,302
Sce19	0,70	0,02	1,16	0,00	2,58	0,05	0,17	0,07	0,59	0,03	4,59	0,08	10,52	0,01	3,314
Sce22	1,41	0,52	1,18	0,01	2,40	0,05	0,27	0,01	0,56	0,03	5,75	1,20	10,65	0,06	3,26
Sce23	1,11	0,22	1,15	0,01	2,18	0,08	0,27	0,02	0,69	0,09	5,23	0,73	10,63	0,07	3,306
Sce25	0,67	0,33	1,12	0,02	2,16	0,11	0,21	0,03	0,76	0,08	5,41	0,63	10,34	0,30	3,335
Sce28	0,73	0,02	1,15	0,00	2,30	0,21	0,17	0,11	0,60	0,03	5,44	0,96	10,48	0,05	3,304
Sce29	0,56	0,13	1,11	0,06	2,16	0,63	0,47	0,32	0,75	0,20	5,06	0,49	10,50	0,05	3,235
QA23	0,63	0,04	1,13	0,02	1,95	0,03	0,28	0,01	0,59	0,02	5,25	0,99	10,76	0,10	3,375
QA23 vs Sce7	0,69	0,06	1,15	0,00	2,34	0,06	0,23	0,01	0,61	0,07	4,79	0,11	10,64	0,11	3,353

To assess the relation between each strain and the differences on the sensorial analysis comparation. The significantly different compounds were evaluated. To ease visualization higher values were highlighted in blue, and lower values in red (Table 5). Organic acids were the main compounds analysed. Total quantity of organic acids is what gives final wines its final acidity (Payan et al., 2023). Sce6 showed the highest values for every acid evaluated besides lactic acid, however production of lactic acid by *S. cerevisiae* strains is very limited and has a negligible impact on overall total acidity (Dequin et al., 1999). Sce6 strain was isolated from UPdC AF. Surprisingly, sensorial analysis concluded one of the lowest acidity values for said fermentation which differs from HPLC results. Opposite to Sce6, QA23 strain had lower than the average values for every organic acid besides lactic acid, however QA23-inoculated AFs scored relatively high scores on the sensorial analysis for acidity attribute. On this side a corelation between the organic acids concentration of each single-strain fermentation and the total acidity attribute found in sensorial analysis was not clear, on the other side, total pH values followed sensory analysis results: Sce6 had the lowest final pH while QA23 had the highest. A possibility for these results is that the main driver of acidity attribute in both cases were an unanalysed organic acid such as malic acid. Sensory analysis did not find significant differences in acidity between wines and that is not in accordance

with the HPLC results of individual strains that predominated in each fermentation. Additionally, comparing sensory analysis results with compounds found in final wines from each modality and experiment, we observe relatively high acetic acid levels in QA23-inoculated fermentations, and a relatively lower one in UPdC fermentation, where Sce6 was isolated from (Table 6). This explains the results of volatile acidity found in sensorial analysis, also reveals that even if these isolated strains were present from high to end of the fermentation or in high abundances, the result of the final wines depended on the combination of every strain and microorganism participating.

Table 6. Final compounds of the wines from each modality and experiment.

	PAC 10%			PAC 12%			PAC 13%		
	USF	**UPdC**	**UQA23F**	**NSF**	**NPdC**	**NQA23**	**RSF**	**RPdC**	**RQA23F**
Ethanol (%(v/v))	11.29 ± 0.02	11.75 ± 0.02	11.62 ± 0.05	12.09 ± 0.11	12.12 ± 0.04	12.01 ± 0.09	13.35 ± 0.08	12.85 ± 0.07	13.35 ± 0.04
Citric acid (g/L)	0.36 ± 0.03	0.55 ± 0.50	0.73 ± 0.04	0.79 ± 0.02	0.98 ± 0.03	1.08 ± 0.01	0.69 ± 0.09	0.37 ± 0.02	0.41 ± 0.07
Acetic acid (g/L)	0.38 ± 0.04	0.47 ± 0.03	0.62 ± 0.01	0.86 ± 0.03	0.46 ± 0.05	0.69 ± 0.02	1.03 ± 0.04	0.84 ± 0.05	0.80 ± 0.03

Another key aspect that we wanted to evaluate with the single-strain fermentations was the ethanol production. Reducing the alcohol levels on wines is an interest nowadays for wine industry. The impact of climate change and the seek for new wine styles, often requiring increased grape maturity has been raising the ethanol content in wines for the last 30 years (Ciani et al., 2016). High concentrations of ethanol can be lethal for microorganisms and led to stuck fermentations and unbalanced wines unpleasant for customers. This increase in ethanol content affects wine industry, as well as social and public safety problems related to alcohol consumption. In addition, lower concentrations of ethanol are economically interesting in countries with high taxes linked to ethanol.

Results did not show any significant difference in alcohol production among the selected strains. Neither of the selected strains were interesting to lower ethanol concentration in wines.

5. CONCLUSSIONS

Nowadays wine industry has many ways to control microbial populations in wine fermentations to ensure final products with desirable quality. Wine production efficiency has also increased over years. This has been possible thanks to the implementation of new techniques but also to the improvement of old techniques.

The objective was to study the PdC technique, an ancient and widespread technique used to kickstart the fermentation process by adding a small volume from previously fermenting batch to grape must. The interest on PdC lies on its supposed capability of maintaining the identity and complexity of wines linked to *terroir* and the capability to reliably drive AFs to the end. This presumption was tested in different ways.

First, the effect of PdC over biodiversity of *S. cerevisiae* strains was studied for three different grape maturity grades. PdC was found to positively increase the diversity of *S. cerevisiae* strains for every maturity point showing its superiority over ADWY-inoculation technique on maintaining diversity of strains during the AF thus contributing to the identity of the wines. Additionally, grape maturity point seemed to also impact diversity of *S. cerevisiae* strains positively resulting in a higher diversity for PdC regardless of maturity point.

Secondly, clustering of the different S. cerevisiae strain interdelta-PCR profiles was performed to seek a correlation between profile similarity and technique used, grape ripeness or AF phase from the samples. Clustering did not reveal any correlation.

Then, a sensory analysis was performed to assess organoleptic differences among final wines. PdC proved to lead to final wines different from ADWY-inoculation (QA23 in this case) but not different from SF. Different attributes showed significant differences: terpenic, vegetal, tropical, oxidation and quality. Further analysis revealed the positive effect of PdC technique over the final values of these sensory attributes.

With these results, a selection of *S. cerevisiae* strains was made and then single-strain inoculated AFs with concentrated must were carried over to evaluate the fermentative capacity of these strains. The objective was to try to find new and interesting *S. cerevisiae* strains and to relate results from the sensory analysis with the presence of these strains. Selected strains showed very similar fermentative profiles not revealing any strain with distinctive good performance. However, the strain Sce7 was able to take over QA23-inoculated fermentations, regardless of the ripeness of the grapes and this was confirmed during co-inoculated micro fermentations. A lower ethanol production in *S. cerevisiae* is interesting, however, ethanol production of selected strains was very even and neither of the strains were interesting in this aspect. Results did not reveal any correlation between organoleptic attributes and the selected strains.

Future research should assess the effect of PdC at cellar scale and evaluate its capabilities at industrial scale. Additionally, it could be interesting the evaluation of more oenological properties of the selected strains, such as SO_2 resistance and production, or total terpenic acidity to deeper assess correlation between strains and the observed organoleptic attributes. Evaluating PdC effects with must from different grape varieties might also be interesting to assess its effect and potential in wine industry.

6. REFERENCES

Abdo, H., Catacchio, C. R., Ventura, M., D'addabbo, P., Calabrese, F. M., Laurent, J., David-Vaizant, V., Alexandre, H., Guilloux-Bénatier, M., & Rousseaux, S. (2020). Colonization of Wild Saccharomyces cerevisiae Strains in a New Winery. *Beverages 2020, Vol. 6, Page 9*, *6*(1), 9. https://doi.org/10.3390/BEVERAGES6010009

Alonso-del-Real, J., Lairón-Peris, M., Barrio, E., & Querol, A. (2017). Effect of temperature on the prevalence of Saccharomyces non cerevisiae species against a S. cerevisiae Wine strain in wine fermentation: Competition, physiological fitness, and influence in final wine composition. *Frontiers in Microbiology*, *8*(FEB), 246426. https://doi.org/10.3389/FMICB.2017.00150/BIBTEX

Bagheri, B., Bauer, F. F., Cardinali, G., & Setati, M. E. (2020). Ecological interactions are a primary driver of population dynamics in wine yeast microbiota during fermentation. *Scientific Reports*, *10*(1). https://doi.org/10.1038/S41598-020-61690-Z

Bedoya, K., Buetas, L., Rozès, N., Mas, A., & Portillo, M. C. (2024). Influence of different stress factors during the elaboration of grape must's pied de cuve on the dynamics of yeast populations during alcoholic fermentation. *Food Microbiology*, *123*, 104571. https://doi.org/10.1016/J.FM.2024.104571

Bedoya, K., Mas, A., Rozès, N., Jara, C., Del, M., & Portillo, C. (2024). The Impact of the Inoculation of Different Pied de Cuve on the Chemical and Organoleptic Profiles of Wines. *Microorganisms 2024, Vol. 12, Page 1655*, *12*(8), 1655. https://doi.org/10.3390/MICROORGANISMS12081655

Beltran, G., Esteve-Zarzoso, B., Rozès, N., Mas, A., & Guillamón, J. M. (2005). Influence of the Timing of Nitrogen Additions during Synthetic Grape Must Fermentations on Fermentation Kinetics and Nitrogen

Consumption. *Journal of Agricultural and Food Chemistry*, *53*(4), 996–1002. https://doi.org/10.1021/JF0487001

Binati, R. L., Innocente, G., Gatto, V., Celebrin, A., Polo, M., Felis, G. E., & Torriani, S. (2019). Exploring the diversity of a collection of native non-Saccharomyces yeasts to develop co-starter cultures for winemaking. *Food Research International (Ottawa, Ont.)*, *122*, 432–442. https://doi.org/10.1016/J.FOODRES.2019.04.043

Black, C. A., Parker, M., Siebert, T. E., Capone, D. L., & Francis, I. L. (2015). Terpenoids and their role in wine flavour: recent advances. *Australian Journal of Grape and Wine Research*, *21*, 582–600. https://doi.org/10.1111/AJGW.12186

Börlin, M., Miot-Sertier, C., Vinsonneau, E., Becquet, S., Salin, F., Bely, M., Lucas, P., Albertin, W., Legras, J. L., & Masneuf-Pomarède, I. (2020). The “pied de cuve” as an alternative way to manage indigenous fermentation: impact on the fermentative process and *Saccharomyces cerevisiae* diversity. *OENO One*, *54*(3), 335–342. https://doi.org/10.20870/OENO-ONE.2020.54.3.3105

Börlin, M., Venet, P., Claisse, O., Salin, F., Legras, J. L., & Masneuf-Pomarede, I. (2016). Cellar-associated Saccharomyces cerevisiae population structure revealed high-level diversity and perennial persistence at Sauternes wine estates. *Applied and Environmental Microbiology*, *82*(10), 2909–2918. https://doi.org/10.1128/AEM.03627-15/SUPPL_FILE/ZAM999117130SO1.PDF

Castrillo, D., Neira, N., & Blanco, P. (2020a). Saccharomyces cerevisiae Strain Diversity Associated with Spontaneous Fermentations in Organic Wineries from Galicia (NW Spain). *Fermentation 2020, Vol. 6, Page 89*, *6*(3), 89. https://doi.org/10.3390/FERMENTATION6030089

Castrillo, D., Neira, N., & Blanco, P. (2020b). Saccharomyces cerevisiae Strain Diversity Associated with Spontaneous Fermentations in Organic Wineries from Galicia (NW Spain). *Fermentation 2020, Vol. 6, Page 89*, *6*(3), 89. https://doi.org/10.3390/FERMENTATION6030089

Chen, Y., Jiang, J., Song, Y., Zang, X., Wang, G., Pei, Y., Song, Y., Qin, Y., & Liu, Y. (2022). Yeast Diversity during Spontaneous Fermentations and Oenological Characterisation of Indigenous Saccharomyces cerevisiae for Potential as Wine Starter Cultures. *Microorganisms*, *10*(7), 1455. https://doi.org/10.3390/MICROORGANISMS10071455/S1

Ciani, M., Morales, P., Comitini, F., Tronchoni, J., Canonico, L., Curiel, J. A., Oro, L., Rodrigues, A. J., & Gonzalez, R. (2016). Non-conventional yeast species for lowering ethanol content of wines. *Frontiers in Microbiology*, *7*(MAY), 194514. https://doi.org/10.3389/FMICB.2016.00642/BIBTEX

Comitini, F., Capece, A., Ciani, M., & Romano, P. (2017). New insights on the use of wine yeasts. *Current Opinion in Food Science*, *13*, 44–49. https://doi.org/10.1016/J.COFS.2017.02.005

Dequin, S., Baptista, E., & Barre, P. (1999). Acidification of Grape Musts by Saccharomyces cerevisiae Wine Yeast Strains Genetically Engineered to Produce Lactic Acid. *American Journal of Enology and Viticulture*, *50*(1), 45–50. https://doi.org/10.5344/AJEV.1999.50.1.45

Edwards, C. G., & Aplin, J. J. (2022). Application of Cool Fermentation Temperatures to Encourage Non-Saccharomyces Yeasts to Yield Lower Ethanol Concentrations in Wines. *Fermentation 2022, Vol. 8, Page 421*, *8*(9), 421. https://doi.org/10.3390/FERMENTATION8090421

Feng, L., Wang, J., Ye, D., Song, Y., Qin, Y., & Liu, Y. (2020). Yeast population dynamics during spontaneous fermentation of icewine and selection of indigenous Saccharomyces cerevisiae strains for the winemaking in Qilian, China. *Journal of the Science of Food and Agriculture*, *100*(15), 5385–5394. https://doi.org/10.1002/JSFA.10588

Legras, J. L., & Karst, F. (2003). Optimisation of interdelta analysis for Saccharomyces cerevisiae strain characterisation. *FEMS Microbiology Letters*, *221*(2), 249–255. https://doi.org/10.1016/S0378-1097(03)00205-2

Oliveira, C. M., Ferreira, A. C. S., De Freitas, V., & Silva, A. M. S. (2011). Oxidation mechanisms occurring in wines. *Food Research International*, *44*(5), 1115–1126. https://doi.org/10.1016/J.FOODRES.2011.03.050

Parapouli, M., Vasileiadis, A., Afendra, A. S., & Hatziloukas, E. (2020). Saccharomyces cerevisiae and its industrial applications. *AIMS Microbiology*, *6*(1), 1. https://doi.org/10.3934/MICROBIOL.2020001

Payan, C., Gancel, A. L., Jourdes, M., Christmann, M., & Teissedre, P. L. (2023). Wine acidification methods: a review. *OENO One*, *57*(3), 113–126. https://doi.org/10.20870/OENO-ONE.2023.57.3.7476

Querol, A., Barrio, E., & Ramón, D. (1992). A Comparative Study of Different Methods of Yeast Strain Characterization. *Systematic and Applied Microbiology*, *15*(3), 439–446. https://doi.org/10.1016/S0723-2020(11)80219-5

Reis, V. R., Antonangelo, A. T. B. F., Bassi, A. P. G., Colombi, D., & Ceccato-Antonini, S. R. (2017). Bioethanol strains of Saccharomyces cerevisiae characterised by microsatellite and stress resistance. *Brazilian Journal of Microbiology*, *48*(2), 268–274. https://doi.org/10.1016/J.BJM.2016.09.017

Torija, M. J., Rozès, N., Poblet, M., Guillamón, J. M., & Mas, A. (2003). Effects of fermentation temperature on the strain population of Saccharomyces cerevisiae. *International Journal of Food Microbiology*, *80*(1), 47–53. https://doi.org/10.1016/S0168-1605(02)00144-7

Varela, C., Pizarro, F., & Agosin, E. (2004). Biomass Content Governs Fermentation Rate in Nitrogen-Deficient Wine Musts. *Applied and Environmental Microbiology*, *70*(6), 3392. https://doi.org/10.1128/AEM.70.6.3392-3400.2004

Vendramini, C., Beltran, G., Nadai, C., Giacomini, A., Mas, A., & Corich, V. (2017). The role of nitrogen uptake on the competition ability of three vineyard Saccharomyces cerevisiae strains. *International Journal of*

Food Microbiology, *258*, 1–11. https://doi.org/10.1016/J.IJFOODMICRO.2017.07.006

7. AUTOEVALUATION

Food technology has always been one of the biotechnology fields that interested me the most. Winemaking is something that has always been in my family and doing my bachelor's degree final project about a project related to food biotechnology and wine seemed an interesting idea. I first reached Oenological biotechnology group from the URV and got in contact with María del Carmen Portillo who told me that they had a study on the process that I could join and do my bachelor's degree final project about. When she explained the project to me, I thought it was very interesting since it was something that could have an immediate impact and application to winemaking once finished.

Something that finally made me decide to take this opportunity was the wide range of activities I should do, such as harvesting the grapes, laboratory work, or writing. It was a perfect opportunity to learn about every activity an Oenological biotechnologist needs to do and develop some skills that would be very useful in my future.

Another detail that was very convenient is that, after I suggested doing also my extracurricular internship with them they agreed which allowed me to stay for almost 9 months with the group learning.

When I first started my laboratory work, I had to complete a two-weeks training where I was taught every technique I would be doing for the following months, also to start familiarising myself with the laboratory and its equipment. I found this training very convenient and useful, first because once you started working on the project, you already knew how to do your tasks, even if practise was still needed to improve.

At first I was helping a PhD with her tasks to improve my skills, but after some time she decided that I was ready and started to give me my own tasks, giving

me a lot of freedom to maybe commit a mistake, but most importantly, to learn. By the end of the second month, I was already completely independent.

This independence would not have been viable without the help of the other experienced researchers that were part of the research group and were always willing to help me if I had any doubts or questions. I am grateful with the group I have worked with, the help they gave me Is what made me learn the most.

This was my first time having to do my own research of information while also doing laboratory work which has been a challenge that has taught me a lot.

Finally, being able to put in practise all what I had been learning for years really inspired me to stay motivated everyday and helped me to keep a mile when any experiment failed or needed to be repeated.

In general, I am very satisfied with this experience, from the work I have done to the people I have met. I have learnt a lot of things and conducted research that is partially published already and will be completely published soon. I would repeat this experience anytime.

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Lastly, but most important, I thank my family for giving me the opportunity of studying the bachelor's degree I dreamed with and for their unconditional support among the years.

And wine can of their wits the wise beguile, make the sage frolic, and the serious smile. – Alexander Pope.

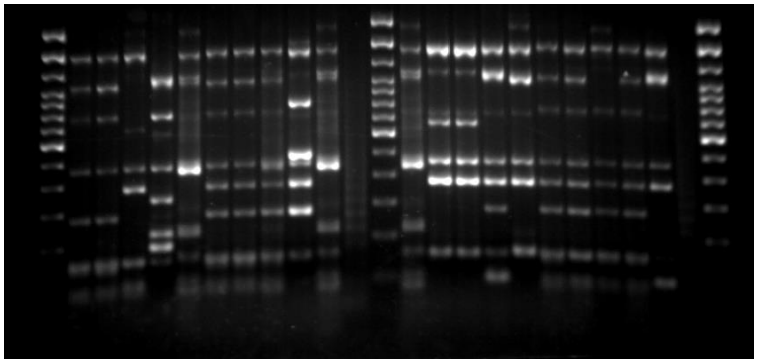
9. ANNEXES

Annex 1. Table containing Shannon index values.

H'index								
USF			UPdC			UQA23F		
BF	HF	FF	BF	HF	FF	BF	HF	FF
1,07	0,90	0,20	1,92	1,58	1,01	0,00	0,97	0,82
NSF			NPdC			NQA23F		
BF	HF	FF	BF	HF	FF	BF	HF	FF
0,77	1,44	1,20	1,34	1,30	1,48	0,00	0,69	0,61
RSF			RPdC			RQA23F		
BF	HF	FF	BF	HF	FF	BF	HF	FF
1,93	1,46	1,55	2,29	2,03	1,37	0,00	0,69	0,33

Annex 2. Table containing Simpson's index values.

D index								
USF			UPdC			UQA23F		
BF	HF	FF	BF	HF	FF	BF	HF	FF
0,68	0,57	0,10	0,87	0,79	0,57	0,00	0,54	0,49
NSF			NPdC			NQA23F		
BF	HF	FF	BF	HF	FF	BF	HF	FF
0,49	0,77	0,63	0,72	0,72	0,77	0,00	0,52	0,46
RSF			RPdC			RQA23F		
BF	HF	FF	BF	HF	FF	BF	HF	FF
0,86	0,73	0,81	0,94	0,89	0,74	0,00	0,52	0,19



Annex 3. Figure example of interdelta-PCR producs visualized using UV, SYBR-Green gel stain and 100kb molecular marker.