Microbiological and organoleptic influence of different stresses during the pied de cuve applied to white wine vinification

KATHERINE BEDOYA ALVIRA

UNIVERSITAT ROVIRA i VIRGILI

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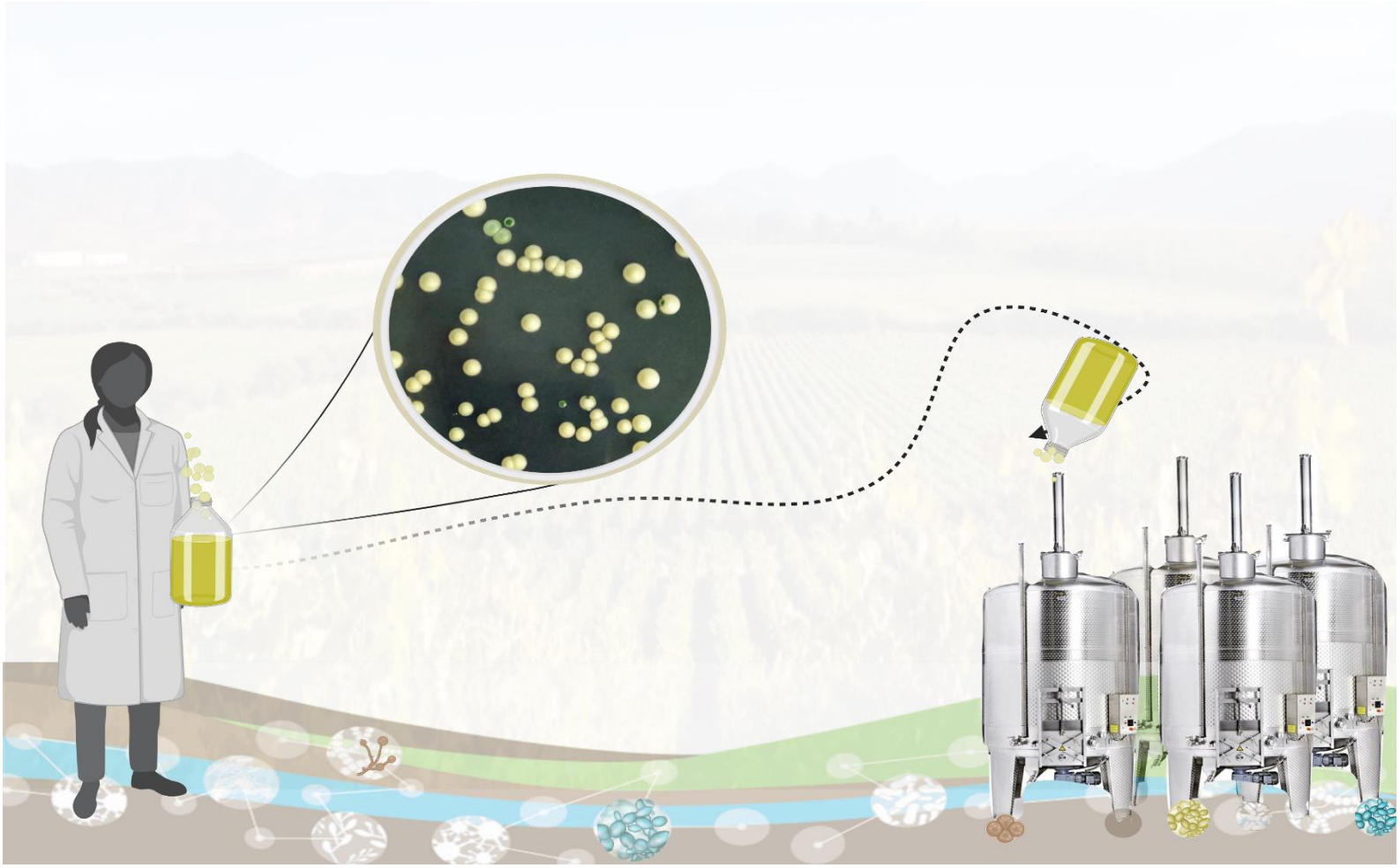

Doctoral Thesis

2024

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Doctoral Thesis

Supervised by Dra. Maria del Carmen Portillo, Dr. Albert Mas, and

Dr. Nicolás Rozès

Department of Biochemistry and Biotechnology

Universitat Rovira i Virgili

UNIVERSITAT ROVIRA i VIRGILI

Tarragona, 2024

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Dra. Maria del Carmen Portillo, Dr. Albert Mas and Dr. Nicolas Rozès CERTIFY,

That the present study, entitled “Microbiological and organoleptic influence of different stresses during the *pied de cuve* applied to white wine vinification”, presented by Katherine Bedoya Alvira for the award of the degree of Doctor, has been carried out under our supervision at the Department Department of Biochemistry and Biotechnology of Universitat Rovira i Virgili.

This thesis is eligible to apply for the Degree of Doctor with International Mention.

Tarragona, 04th October 2024

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"Cuando nos comprometemos con la educación como la práctica de la libertad, participamos en la construcción de una comunidad académica en la que podemos ser y convertirnos en intelectuales en el sentido más amplio y más profundo de la palabra. Participamos en una manera de aprender y de ser que hace que el mundo sea más real, no menos, una manera que nos permite vivir con plenitud y libertad.

Esa es la alegría de nuestra búsqueda."

bell hooks

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A mi mami Lucy,

A mis hemanos Xoxo y Monchi

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Summary

This thesis studies the use of *pied de cuve* (PdC) as alternative of the inoculation of commercial yeasts that have been related to the uniformization of the organoleptic profile of wine. The PdC studied was generated through spontaneous fermentation using several oenological practices as stress factors to guarantee an autochthonous microbiota with good fermentation performance. The stress factors used in PdC were the sulphur dioxide (SO_2), ethanol and temperature. The dynamic of yeasts population was monitored by microscopy, plating, quantitative polymerase chain reaction (q-PCR). Chemical analysis was done by reflectometry, densitometry, high-performance liquid chromatography (HPLC), and enzymatic kids. Wines inoculated with different PdC were submitted at tasting sessions with trained oenologists in all harvest.

At laboratory level, a consortium of yeast was used to establish the parameters under which a PdC, under different stressors reaches higher population and diversity of yeasts population to then applied this strategy in cellar. Inoculation with a PdC with addition of 40 mg/L of SO_2, 1% (v/v) ethanol and fermented at 18 ºC resulted in wines high diversity of *Saccharomyces* strains with good chemical and organoleptic quality. These outcomes were consistent across several harvests, grape varieties, geographical location and different maturity levels.

Resumen

Esta tesis analiza el uso de *pie de cuba* (PdC) como alternativa a la inoculación con levaduras comerciales, lo que está relacionado con una cierta uniformización del perfil aromático del vino. El PdC analizado se ha generado mediante fermentación espontánea usando diferentes prácticas enológicas como factores de estrés para garantizar una microbiota completamente autóctona y con plena capacidad fermentativa. Los factores de estrés evaluados durante la preparación del PdC incluyen el dióxido de azufre (SO_2), el etanol y la temperatura. El seguimiento microbiológico se hizo mediante el conteo por microscopio, siembra de placa, y reacción en cadena de la polimerasa cuantitativa (q-PCR). A nivel químico se evaluaron los principales parámetros por reflectometría, densitometría, cromatografía líquida de alta eficacia (HPLC) y kits enzimáticos. A nivel sensorial se realizaron catas por parte de profesionales en todas las vendimias.

En laboratorio, se usó un consorcio de levaduras para establecer los parámetros en los que el PdC alcanza una mayor diversidad y población de levaduras. Los factores de estrés seleccionados para su aplicación en bodega fueron 40 mg/L de SO_2, 1% (v/v) etanol y 18 ºC, resultando en vinos con elevada diversidad de cepas de *Saccharomyces* y una buena calidad química y organoléptica. Estos resultados fueron reproducibles a través de diferentes vendimias, variedades de uva, locación geográfica y grados de maduración de las uvas.

Resum

Aquesta tesi analitza l'ús de *peu de cuba* (PdC) com a alternativa a la inoculació amb llevats comercials, que produeix una certa uniformització del perfil aromàtic del vi. El PdC analitzat es genera a partir de fermentació espontània emprant diferents pràctiques enològiques com a factors d'estrès per garantir una microbiota completament autòctona i amb plena capacitat fermentativa. Els factors d'estrès avaluats durant la preparació del PdC inclouen el diòxid de sofre (SO_2), l'etanol i la temperatura. El seguiment microbiològic es va fer mitjançant recompte per microscopi, sembra en placa, i reacció en cadena de la polimerasa quantitativa (qPCR). A nivell químic, es van avaluar els principals paràmetres per reflectometria, densitometria, cromatografia líquida d'alta eficàcia (HPLC) i kits enzimàtics. A nivell sensorial, es van realitzar tasts per part de professionals a totes les veremes.

Al laboratori, es va emprar un consorci de llevats per establir els paràmetres en què el PdC assoleix una major diversitat i població de llevats. Els factors d'estrès seleccionats per a la seva aplicació al celler van ser 40 mg/L de SO_2, 1% (v/v) d'etanol i 18 ºC, resultant en vins amb elevada diversitat de soques de *Saccharomyces* i una bona qualitat química i organolèptica. Aquests resultats van ser reproduïbles a través de diferents veremes, varietats de raïm, localització geogràfica i graus de maduració del raïm.

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Introduction

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Wine has been an integral part of humanity. Since ancient times it has been used in medicine, religious ceremonies, celebrations, and daily life across various cultures worldwide. Wine is a product obtained from a partial or complete alcoholic fermentation of grapes and it has been registered from southern Caucasia (Azerbaijan, Armenia, and Georgia) and includes parts of present-day north-western Turkey and northern Iraq (Jackson, 2008; Mas et al., 2021).

1. Ripening and microbiota of grapes

The grape maturity consists in achieving the optimal rate of sugar and acidity in the fruit (Ribéreau-Gayon et al., 2006). This is a continuous process, which goes through various ripening stages, as reviewed by Ribéreau-Gayon et al. (2006):

- **Stage** I**:** it consists of a rapid berry growth that comes after bloom. During this stage, the sugar content of the berries remains low and the organic acids such as tartaric and malic acid begin to accumulate.
- **Stage II:** it is commonly known as *véraison* and corresponds to the lag phase of berry growth. In this phase, the organic acids increase at the highest level and the synthesis of growth substances decreases in the berries.
- **Stage III:** it starts the ripening or maturation process. This stage is characterized by rapid growth where the berries start to accumulate sugars. Additionally, the berries become softer, lose chlorophyll, and experience a reduction in organic acids, mainly L-malic acid. The phenolic compounds and aromatic substances reach their maximum concentration

in the grape skin. At this stage, the mature berries can be harvested for winemaking.

Therefore, the grape skin undergoes multiple transformations during maturation after fruit sets. The residing microbiota on the surface also changes in response to nutrient availability, the developmental stage and macroclimatic conditions (Barata et al., 2012; Wei et al., 2022b). The International Code of Oenological Practices (Resolution OIV-VITI 655-2021; (2021a)) and studies by Wei et al. (2022b) confirm a significant correlation between epidermal microbial communities and grape maturity stage. Additionally, the microbial community's composition and quantity are regulated by soil, vineyard practices, and the relationship between rainfall, temperature, and the phyllosphere microbiota (Perazzolli et al., 2014).

Yeast and filamentous fungi are one of the main eukaryotic communities found on grape surfaces (Barata et al., 2012). They also reside in diverse ecological habitats, including water, soil, air, and the surfaces of plants and fruits (Estela-Escalante, 2018; Maicas, 2020; Wang et al., 2012). Mostly, yeasts are frequently present during the decomposition of ripe fruits where they find essential nutrients and substrates for their metabolism and fermentative functions. These vital compounds include fermentable sugars, amino acids, vitamins, minerals, and oxygen (Clavijo et al., 2010; Estela-Escalante, 2018; Mercado et al., 2007).

Extensive research has explored the microbiota inhabiting ripened grape surfaces using culturable-dependent and molecular techniques. On healthy berries, the colony-forming unit (CFU) of yeast population increases from 10^2-10^3 CFU/g in immature stages and goes until 10^3-10^6 CFU/g in mature berries

(Prakitchaiwattana et al., 2004; Setati et al., 2012). This increase is attributed to the high sugar content in ripe grapes. In damaged berries, where many microorganisms can proliferate, yeasts are present at 10^6-10^8 CFU/berry (Barata et al., 2012). Figure 1 shows the three major groups of yeasts found on the grape surface, with the main genera identified within each group, as reported in the cited articles.

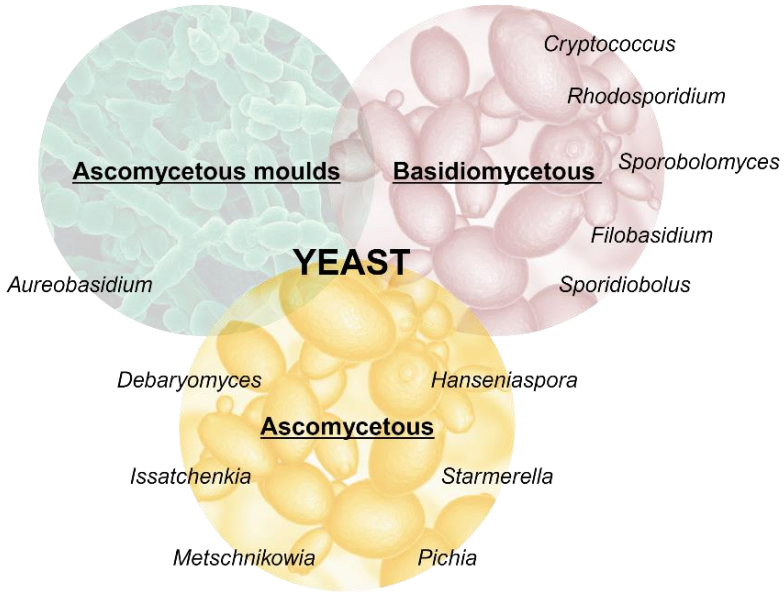

Figure 1. Main yeast genera found in healthy grapes (Barata et al., 2008; Bokulich et al., 2014; Kántor et al., 2017; Mas et al., 2022, 2020; Mayo et al., 2014; Prakitchaiwattana et al., 2004; Renouf et al., 2005; Subden et al., 2003).

Firstly, the Ascomycetous yeasts harbour the genus *Aureobasidium*, where *Aureobasidium pullulans* is the most representative species (Mas et al., 2020; Prakitchaiwattana et al., 2004; Subden et al., 2003). Secondly, the Basidiomycetous yeasts comprise genera such as *Cryptococcus*, *Sporobolomyces*, *Sporidiobolus*, *Filobasidium* and *Rhodosporidium* (Barata et

al., 2008; Kántor et al., 2017; Mas et al., 2022, 2020; Prakitchaiwattana et al., 2004; Renouf et al., 2005; Subden et al., 2003).

Finally, the Ascomycetous yeasts are the third group found in ripe grapes (Mas et al., 2020). This group includes genera such as *Hanseniaspora*, *Starmerella*, *Issatchenkia*, *Debaryomyces*, *Metschnikowia*, and *Pichia*. Other studies have identified additional genera such as *Dothideomycetes* and *Saccharomycetes* (David et al., 2014; Taylor et al., 2014). Nonetheless, *Hanseniaspora uvarum* is the most common species found in ripe grapes (Barata et al., 2008).

Despite Ascomycetous yeasts being in abundance on ripe berries, their isolation by culture-dependent techniques represents a challenge (Barata et al., 2012; Mas et al., 2020; Mayo et al., 2014). In recent years, next-generation sequencing (NGS) has emerged as an invaluable tool for understanding microbial communities involved in complex biological processes, regardless of culture-dependent techniques (Mayo et al., 2014). NGS involves several technologies that massively sequence heterogeneous DNA fragments to generate large, genome-scale datasets (Bokulich et al., 2014).

In wine microbiology, some studies have uncovered more complexity in grape microbiota during the grape ripening process. For example, Wei et al. (2022a) analysed the grape by amplicon sequencing using the Illumina Novaseq platform microbiome during the ripeness of Cabernet Sauvignon grapes (Figure 2). They collected grapes at different stages based on the numbers of the E-L system: 31, 35, 36 and 38. Number 31 corresponds to fruit settling (A), when the berry size is about 7 mm in diameter. Number 35 corresponds to early *veraison* (B), when berries begin to colour and enlarge. Number 36 corresponds to the end *veraison*

(C), when the berries have intermediate sugar values. Number 37 corresponds to mid-maturity (D), when berries are not quite ripe. Number 38 corresponds to harvest (E), when berries are ripe. The relative abundance of Operational Taxonomic Units (OTUs) at the fungal phylum level, showed that *Alternaria* was the dominant genus in the grape epidermis and increased as the development stage proceeded (Figure 2).

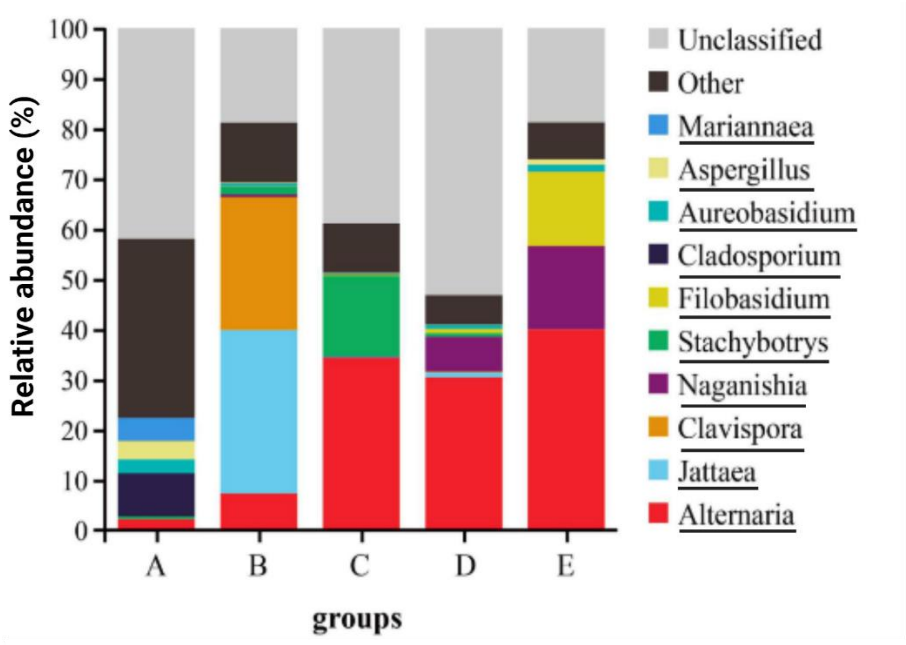

Figure 2. Genera diversity of yeasts of Cabernet Sauvignon grapes identified by next-generation sequencing (NGS) at different growth stages: (A), fruit setting; (B), early *veraison*; (C), end *veraison*; (D), mid maturity; (E), harvest (Wei et al., 2022a).

Liu et al. (2021) evaluated the fungal community in the different parts of the Pinot Noir grapevine at three points: fruit set, *veraison* and harvest. They also showed the different microbiota composition during the ripe process. On one hand, some genera were more abundant in the fruit set and decreased at harvest time (Figure 3). For example, the abundance of *Aureobasidium* decreased from 41.15% in the fruit set to 14.09 % at harvest. Similarly, *Cryptococcus* went from 12.78% to

0.43%, *Mycosphaerella* from 6.95% to 1.17%, and *Saccharomyces* from 3.43% to 0.29%. Other genera showed lower abundance in the fruit set but increased before harvest. *Cladosporium* increased from 11.07% to 32.74%, *Epicoccum* from 1.90% to 13.27%, *Alternaria* from 2.20% to 9.55 %, and *Dydimella* from 0.90% to 4.95%.

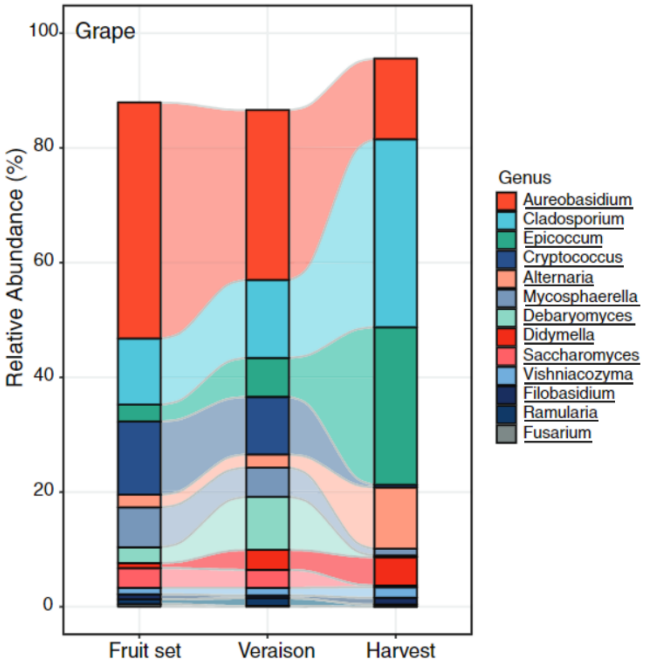

Figure 3. Genera diversity of yeast in Pinot Noir variety grapes identified by next-generation sequencing (NGS) at different growth stages (Liu and Howell, 2021).

Other authors have reported other fungi at the harvest point. These include *Mycosphaerella*, *Aspergillus*, *Rhodotorula*, *Sporobolomyces*, *Colletotrichum*, *Penicillium, Saccharomycopsis* and *Fusarium* (Bettenfeld et al., 2022; Costantini et al., 2022; Kioroglou et al., 2019; Liu and Howell, 2021; Lleixà et al., 2018; Tronchoni et al., 2022).

The bacterial community is also abundant at the beginning of the fermentation; however, they disappear under extreme conditions during the fermentation process. These conditions are related to the osmotic stress due to high sugar concentrations in must, low oxygen, temperature changes, low pH, high ethanol levels, and the presence of sulphur dioxide (SO_2) that is used as a preservative during the process (Barata et al., 2012; Bauer and Pretorius, 2000). As yeasts are the protagonists in alcoholic fermentation (AF); therefore, subsequent sections will be focused exclusively on them.

2. Pre-fermentation process

Different practices are employed before beginning the AF. They vary depending on the type of wine to be elaborated (white, red, rosé, or others). Below is a summary of general pre-fermentation practices in white winemaking:

2.1. Harvest berries

It involves the collection of grape berries maintaining the integrity of the skin tissue to prevent the entry of undesirable yeast and bacteria through wounds (Ribéreau-Gayon et al., 2006).

2.2. Crushing and destemming

Crushing involves bursting the berries to release pulp and some of the juice by using one or two pairs of rollers rotating in opposite directions. On the other hand, destemming separates the berries from their stems to extract efficiently the must

during pressing. Destemming typically precedes roller crushing in commercial winemaking to maximise press capacity. However, crushing could occur before destemming in some small devices (Nordestgaard, 2011).

2.3. Draining and pressing

It involves collecting the free juice expelled from the berry. It is usually performed in the press devices (Ribéreau-Gayon et al., 2006). Diverse types of press are used depending on the type of wine to elaborate and the volume of production. Variables such as pressure levels, and the number of press cycles can be adjusted according to the winemaker's preferences.

2.4. Clarification

The elimination of insoluble solids can come into the fresh must collected. These solids come from the vineyard (soil, skin and stem fragments, cellular debris from grape pulp). Clarification improves the aroma of dry wines, prevents unpleasant vegetal odours, eliminates part of the wild yeasts, and favours more pleasant aromas in the wine (Ribéreau-Gayon et al., 2006). The standard procedure in spontaneous settling occurs at low temperatures between 8 ºC and 10 ºC (Williams et al., 1978). This step usually lasts between 16 and 24 h (Vernhet, 2021).

Clarification is a natural settling of suspended solids followed by careful racking (Ribéreau-Gayon et al., 2006). Other methods include centrifugation, filtration, tangential microfiltration, carbon dioxide (CO_2), or nitrogen flotations. However, enzymatic treatment is the most employed method (Ridge et al., 2021).

3. Fermentation Process

The conversion of grape must into wine involves a complex process spanning a multistage and a succession of diverse microorganisms (Bisson et al., 2017). These microorganisms come from various parts of the vine, including roots, stems, branches, leaves, and the surface of the grape berries (Vitulo et al., 2019).

Microbial populations can migrate from the grape skin into the juice after crushing and join resident yeasts in the winery to initiate spontaneous fermentation (SF) (Fleet, 1999).

Transformation of grape must into wine involves the sequential development of microbial species, yeasts, lactic acid bacteria (LAB), and acetic acid bacteria (AAB). *Saccharomyces cerevisiae* (Sce) is the yeast primarily responsible for AF, while *Oenococcus oeni* is the common bacteria known for conducting malolactic fermentation (MLF), a process used mainly in red wines. However, molecular techniques and NGS have evidence that other non-conventional species of yeasts and bacteria also participate in the fermentation process.

4. Yeasts in winemaking

Wine production involves a complex ecosystem where different yeast species and strains interact and carry out biochemical activities (Englezos et al., 2022). These yeasts come from three main sources: (i) the natural microbiota of the grapes, (ii) the microorganisms present on winery equipment and the winery environment (such as air and insects), and (iii) if used, the addition of starter

cultures either selected from the same winery or purchased commercially (Fleet, 2003).

Yeasts are divided into two main generic groups without any taxonomic category: non-*Saccharomyces* (non-Sce) and *Saccharomyces* genus, particularly Sce. Non-conventional yeast species, including non-Sce and *Saccharomyces* non-*cerevisiae*, are now recognized for enhancing wine quality and are being used by the wine industry (Ciani et al., 2010; Goddard, 2008; González and Morales, 2021; Howell et al., 2006; Pérez-Torrado et al., 2018). However, Sce remains the most widely used yeast and has received significant research attention for its role in this process. The following sections provide detailed information on the most relevant species in each group.

4.1. Non-*Saccharomyces*

Non-Sce yeasts represent a genetically diverse group with specific metabolic characteristics and high potential in fermentation processes (Estela-Escalante, 2018). The most prevalent non-Sce yeasts genus found in musts are *Hanseniaspora*, *Issatchenkia*, *Pichia*, *Starmerella*, and *Zygosaccharomyces* (Lleixà et al., 2018; Wang and Liu, 2013). Indeed, *H. uvarum* and *Starmerella bacillaris* (previously classified as *Candida stellata*) are the most abundant species in grape must after crushing (Fleet, 2003; Torija et al., 2001; Wang et al., 2015b).

♦ ***Negative considerations.*** In winemaking, non-Sce yeasts were reconsidered undesirable because they were associated with a negative sensory profile or indicative of spoilage in the final wine (Amerine et al., 1972; Ribéreau-Gayon and

Peynaud, 1960). For example, laboratory fermentations inoculated with pure cultures of non-Sce yeasts, often led to the production of undesirable metabolites such as acetic acid, acetoin, ethyl acetate, and acetaldehyde (Ciani et al., 2010; Ciani and Comitini, 2011). These yeasts used exclusively resulted in a risk of stuck fermentations (Heard, 1999; Jolly et al., 2017; Romano et al., 2003a).

♦ ***Positive considerations*.** Nowadays, several studies have shown the non-Sce yeasts can improve **chemical wine quality** (Fazio et al., 2023). For example, *S*. *bacillaris* has shown positive impact on the reduction of ethanol concentration and the increase of glycerol. *Lachancea thermotolerans* has been associated with glycerol increase and L-lactic acid production (Gobbi et al., 2013; Mas et al., 2021). Non-Sce yeasts influence the sensory characteristics of wine by producing various metabolites and interacting enzymatically with aromatic compound precursors such as β-glucosidases. These enzymes can hydrolyse the glycosylated aromatic precursors into free volatile compounds, to enhance the wine's flavour profile (Jolly et al., 2014; Swangkeaw et al., 2011). Additionally, specific studies have shown that the co-inoculation of *Torulaspora delbrueckii* or *L*. *thermotolerans* with Sce aims to **enhance the organoleptic profile of wine**s and even can **impact positively on MLF** (Balmaseda et al., 2021; Ciani et al., 2010; Roca-Mesa, 2022; Ruiz-de-Villa et al., 2023; Vejarano and Gil-Calderón, 2021).

Non-Sce are useful as **bioprotective and biocontrol agents**. Simonin et al. (2018) found a *T*. *delbrueckii* strain offers a bioprotective agent against oxidation by analysing the colour and phenolic compounds of the wine. Additionally, the

fermentations have no impact on their kinetics and showed high yeast biodiversity during the pre-fermentation stages compared to sulphite controls. As a biocontrol agent, the non-Sce yeasts release pectinases which are used in wine clarification and produce proteolytic enzymes that can improve protein stability in wine (Maturano et al., 2012). Other non-Sce species can produce **antimicrobial** substances or killer toxins to control spontaneous and spoilage microbiota (Chalutz and Droby, 1998; Ciani and Comitini, 2011; Kuchen et al., 2019). For example, *T. delbrueckii* limited the development of spoilage microorganisms, comparable to the action of sulphites (Simonin et al., 2018). Also, some yeasts can liberate 1,3-β-glucanases that destroy fungal walls or killer toxins of yeasts that may also inhibit filamentous fungi as described in Schaffrath et al. (2018).

4.2. *Saccharomyces*

Saccharomyces genus comprises eight species, namely: *S. cerevisiae* (Sce), *S. paradoxus*, *S. mikatae*, *S. jurei*, *S. kudriavzevii*, *S. arboricola*, *S. eubayanus* and *S. uvarum*. Some of these species are parents of natural hybrids that either formed spontaneously in the wild without human intervention or in habitats created by humans, i.e., industrial environments (Alsammar and Delneri, 2020).

Saccharomyces genus, a member of the Ascomycetes group, is hard to detect or isolate on grape skin or in must. Some studies have reported that *Saccharomyces* represented less than 2% of the yeast found, and sometimes is completely absent (Abdo et al., 2020; Barata et al., 2012; Wang et al., 2015b). However, Sce population is higher in the cellar environment that in natural habitat (Beltran et al., 2002; Bokulich et al., 2014).

Sce is the main yeast that drives the AF in winemaking, but other *Saccharomyces* non-*cerevisiae* also participate in or can conduct the AF independently. These *Saccharomyces* non-*cerevisiae* species are now considered relevant as innovative starters to mitigate climate change and obtain high-quality wines (Ciani et al., 2016b; Maturano et al., 2019; Varela and Borneman, 2017). Climate variations include earlier harvest times and rising temperatures which result in elevated sugar levels in grapes, consequently in higher ethanol content, reduced acidity, and alterations in varietal aroma compounds (Mira de Orduña, 2010). For instance, *S. kudriavzevii* and *S. bayanus* var. *uvarum* have been used to produce wines with lower ethanol yield and higher glycerol concentrations (Alonso-del-Real et al., 2017a).

The use of *Saccharomyces* non-*cerevisiae* species in AF has both advantages and disadvantages. They have good fermentative capabilities, show slight differences in metabolic activity than Sce and contribute to good aromatic profiles (Alonso-del-Real et al., 2017a; González et al., 2007). Nonetheless, *Saccharomyces* non-*cerevisiae* may encounter challenges when competing with Sce on an industrial scale. This is because Sce perform better at higher temperatures and it exhibits superior ethanol resistance (Arroyo-López et al., 2009; Oliveira et al., 2014; Salvadó et al., 2011a). Additionally, Sce has established a well-characterized profile for its fermentation performance and has delivered reliable and predictable outcomes in winemaking (Pretorius, 2000).

- ***Saccharomyces cerevisiae***

Saccharomyces cerevisiae (Sce) is considered the ideal microbe for cell factories due to its brief replication cycle, easy domestication process, high sporulation

efficiency, ubiquity, and small genome size (Gallone et al., 2016). The advantages of using Sce in AF over other species are outlined in Figure 4, based on the article of Alonso-del-Real et al. (2017b).

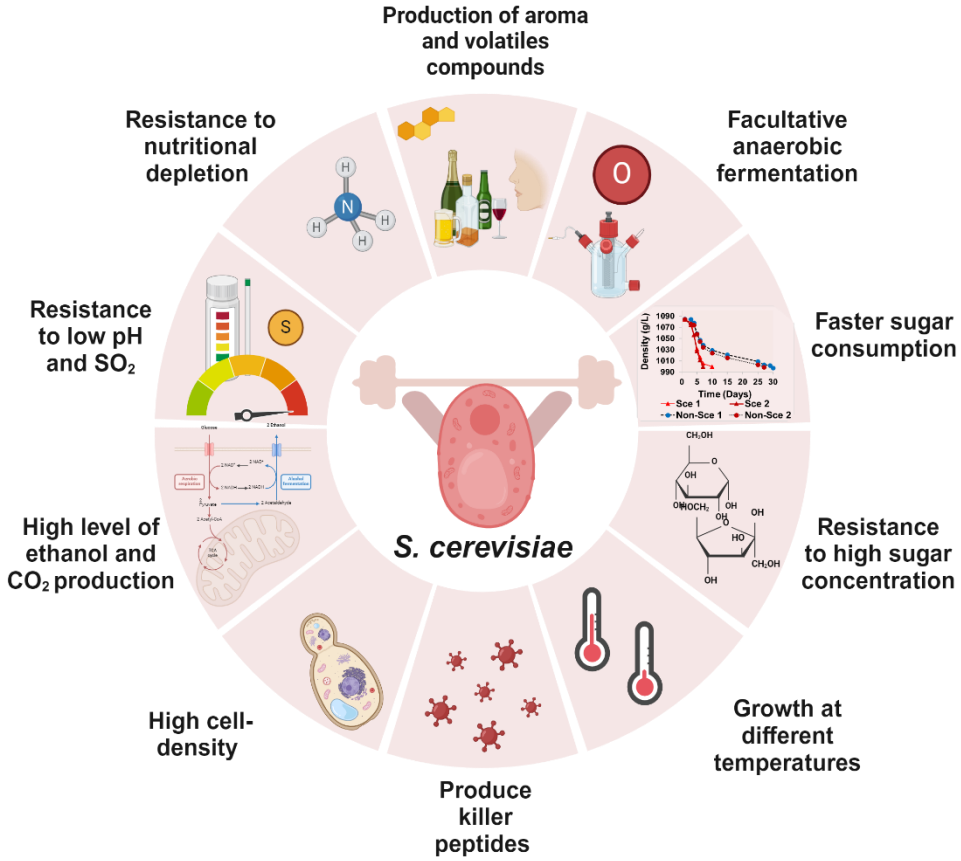

Figure 4. Main advantages of using *Saccharomyces cerevisiae* (Sce) in alcoholic fermentation (AF).

Sce can produce ATP from sugars through two pathways: **respiration and fermentation**. Despite fermentation being less energy-efficient than respiration, it means a competitive advantage over other microorganisms due Sce can **consume faster the nutrients** with the production of ethanol, SO_2, and heat (Fleet, 2008, 2003; Morard et al., 2019). However, these by-products are harmful

or less tolerated by competing organisms (Alonso-del-Real et al., 2017b; Hagman and Piškur, 2015; Morard et al., 2019).

Additionally, Sce has other advantages over microorganisms. It has efficient growth at a **wide range of temperatures**, especially at higher temperatures (32 °C). It has been demonstrated Sce creates a special niche by increasing the temperature during vigorous fermentation, inclusively can outcompete *S. kudriavzevii* even at temperatures that are more suitable to it (Arroyo-López et al., 2009; Goddard, 2008; Salvadó et al., 2011b).

Also, many Sce strains present the **phenotype killer**, producing proteins known as killer toxins or killer factors. Those substances can eliminate or inhibit the growth of bacteria or other yeast strains sensitive to these toxins (Albergaria et al., 2010; Crabtree et al., 2023; Pérez-Nevado et al., 2006; Woods and Bevan, 1968). Additionally, Sce produces antimicrobial substances like toxic glycolipids, which also confer a killer-like phenotype to the producer strain (Schaffrath et al., 2018).

Sce can surround other non-Sce yeasts and **impose confinement stress** due to its higher cell density (Arneborg et al., 2005). Also, Sce can use ethanol as a substrate for aerobic respiration in the presence of oxygen (Piškur et al., 2006; Thomson et al., 2005).

Sce is **highly resistant to various stresses**, like low pH, high ethanol concentration, endogenous CO_2, osmotic stress, high SO_2 doses, and nutrient stress depletion (Albergaria and Arneborg, 2016; Bisson, 1999; Bokulich et al., 2015). Finally, Sce produces a **large quantity of active-aroma** secondary

metabolites and releases various aroma compounds from inactive precursors in grape juice (Chambers and Pretorius, 2010; Swiegers and Pretorius, 2007).

5. Yeast inoculation strategies

AF can be initiated using various methods, each offering distinct advantages and considerations. SF is a traditional method where fermentation is conducted by natural or wild yeast microbiota present in the vineyard and the cellar environment (Mas et al., 2021).

Wild yeasts have offered novel sensory profiles through enzymatic activities that release volatile compounds from their precursors. Over time, specific native strains have been isolated and propagated to obtain pure cultures for subsequent harvests (Belda et al., 2017d; Mira de Orduña, 2010; Tilloy et al., 2015). This practice has contributed significantly to large-scale production and the development of commercial yeast starters as dry wine yeast (DWY) (Mas et al., 2021). The first production of DWY strains began with the commercialization of two strains at a California winery in 1965 (Pretorius, 2000). This practice became very popular among winemakers and was considered *cellar-friendly*, and now the industry offers a wide variety of DWY products (Portillo and Mas, 2022).

Currently, the use of DWY strains is the main inoculation practice in winemaking, although some wineries use SF. SF is valued for its less interventionist approach (*low-input*), favoured by consumers who perceive such practices as more environmentally friendly. SF also preserves the regional *microbiota footprint* and enhances differentiation in the wine's aromatic profile, which DWY inoculation

may not achieve (Belda et al., 2017d; Fleet, 2003; Moschetti et al., 2016). However, SF presents risks such as off-flavour production and stuck fermentation (Bagheri et al., 2015; Tello et al., 2012). Consequently, winemakers are exploring various strategies to leverage the benefits of SF while achieving microbial control of AF. The use of the *pied de cuve* (PdC) technique is one of the inoculation strategies that have emerged recently, outlined by Mas and Portillo (2022). In the following sections, these inoculation strategies are described in detail.

5.1. Commercial strains

Commercial yeast strains are isolates that come from fermentation-related environments or are derived from breeding programs, selected for certain phenotypic traits (Molinet and Cubillos, 2020). These traits include optimal conversion of grape sugar into ethanol and CO_2, efficient nitrogen consumption, and minimizing off-flavours development (Bauer and Pretorius, 2000). The wine industry continues searching for yeast strains with additional desirable characteristics. These include low fermentation foam, good adaptation to the stressful wine environment (i.e., SO_2 resistance and higher concentrations of sugars), large cell size, and nutrient storage within the cell (Bauer and Pretorius, 2000; Mas et al., 2022; Spor et al., 2009, 2008). Additional desirable features include low formation of sulphur compounds and volatile acidity, effective action on organic acids, and synthesis of pleasant aromatic compounds (Fleet and Heard, 1993; Jolly et al., 2014). Commercial yeast strains exhibit high ethanol and low pH tolerance (Duan et al., 2018; Kang et al., 2019).

Sce strains are the most commercialized as DWY, however, non-Sce species such as *L. thermotolerans*, *Metschnikowia pulcherrima*, *T. delbrueckii*, and *Pichia kluyveri* are also available commercially due to several research has demonstrated multiple benefits in fermenting musts with them (Vejarano and Gil-Calderón, 2021). For example, the co-inoculation with non-Sce and Sce yeasts enhances the sensory complexity of wines compared to using Sce alone (Fleet, 2003; Mas and Portillo, 2022). Also, some non-Sce yeast starters are interesting by their biocontrol effects (Comitini et al., 2017; Kuchen et al., 2019), their influence on colour composition especially in rosé wines and carbonic maceration (Balmaseda et al., 2021; Ruiz-de-Villa et al., 2023), their lower production of volatile acidity (Belda et al., 2017b; Benito, 2018; Jolly et al., 2014), and their ethanol reduction (Contreras et al., 2014; García et al., 2020; Zhu et al., 2021). Recently, some studies have shown their positive interactions with bacteria of MLF (Balmaseda et al., 2021; Ruiz-de-Villa et al., 2023).

Commercial yeast is inoculated into grape must in a recommended dosage 10-20 g/hL using the supplier's protocol. This protocol involves brief rehydration of the DWY in warm water at 35-40 °C for 30 min (Bauer and Pretorius, 2000; Mas et al., 2022). Following, the yeast can either be added directly to the must, named as direct inoculation, or can be pre-adapted by mixing it with a small portion of the must before introducing it to the main batch intended for AF, named indirect inoculation.

The use of DWY is an effective way to control the AF and can produce wines with predictable and reproducible outcomes (Fleet and Heard, 1993; Mas et al., 2021). However, new approaches in winemaking are searching for strategies to obtain

wines with aromatic profiles complex that protect the authenticity of wines. To achieve these goals are recommended to exploiting wild yeast diversity, with an emphasis on the diversity of Sce (Mas et al., 2021).

5.2. Wild *S. cerevisiae* strains

The autochthonous or wild Sce variability has been investigated in grapes and vineyards since the 90s' (Versavaud et al., 1995). Many reports have observed an extensive polymorphism in Sce strains in the vineyard linked to the same location over several consecutive years (Frezier and Dubourdieu, 1992; Henschke, 1997a; Pretorius, 2000; Romano et al., 2003a; Vezinhet et al., 1992). This microbial biodiversity is still confirmed by actual studies (Gayevskiy and Goddard, 2012; González et al., 2023; Kang et al., 2019; Knight et al., 2015; Vigentini et al., 2015).

The exploration and preservation of native Sce strain has garnered attention in recent years to face climate change and current consumer preferences. In Figure 5 based on Molinet and Cubillos, 2020 is a summary of the advantages:

- The autochthonous microbiota is more **adapted** to the musts of the **different viticultural areas and substrates** (Capece et al., 2012; Esteve-Zarzoso et al., 2000; Molinet and Cubillos, 2020).
- Wild strains have demonstrated comparatively **higher extracellular concentrations of diverse secondary metabolites** such as fatty acids. These acids are associated with a more robust response to different stresses (Kang et al., 2019).

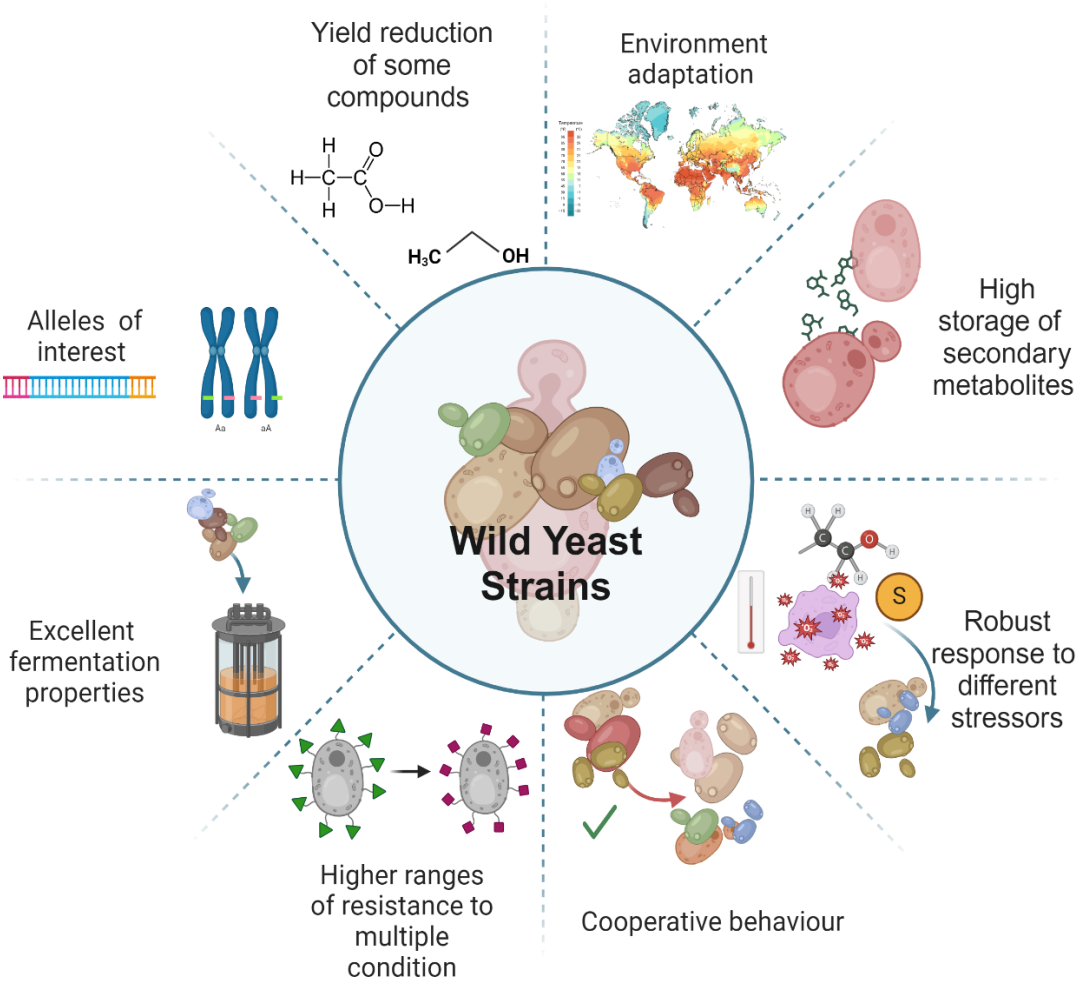

Figure 5. Main advantages of the use of wild *Saccharomyces cerevisiae* (Sce) in alcoholic fermentation (AF) (Molinet and Cubillos, 2020).

- Wild strains display **cooperative behaviour** and save resources for the whole group at the expense of their reproduction. They are supposed to exploit resources slowly but efficiently (Spor et al., 2009).
- Wild strains store cell resources rather than **secreting secondary products to cross-feed or poison competitors** (Spor et al., 2009, 2008).

- Wild strains tend to exhibit **higher ranges of resistance conditions** such as high temperature, inhibitory compounds, and acidic pH (Kang et al., 2019).
- Wild strains tend to exhibit **excellent fermentation propierties** with a controlled and precise performance to avoid stuck or sluggish fermentations (Albergaria and Arneborg, 2016).
- Wild Sce strain **alleles** play a decisive role in the adaptation to low nitrogen concentrations, and volatile compound production (Kessi-Pérez et al., 2020; Molinet and Cubillos, 2020). Some of them yield low ethanol without an increase in volatile acidity content during aerobic AF (Tronchoni et al., 2022) .
- Wild Sce strains can release various aroma compounds. They are an essential source for strain selection to obtain desirable oenological characteristics (Capece et al., 2012; Romano et al., 2008).

In summary, wild strains offer several advantages in winemaking. They offer good adaptation to the cellar environment, enhance the production of secondary metabolites for stress resistance, show enzymatic activities of great interest (esterases, β-glucosidase, pectinolytic, etc) and can produce variable aromatic compounds, thus contributing to the regional character and complexity of wines (Fleet and Heard, 1993; Jolly et al., 2014; Schuller and Casal, 2005; Settanni et al., 2012; Valero et al., 2005).

However, fermentations using wild yeast strains through SF are also challenging. This is due to the microbiota composition changes according to the climate conditions and depends on the aseptic conditions of wineries (Griggs et al., 2021;

Mas et al., 2020). Also, the lack of control in AF can result in off-flavours or undesirable by-products that affect the wine sensory profile.

Several strategies can be applied to incorporate wild yeast strains into AF to enhance desirable traits while minimizing undesirable ones. These strategies include selective breeding, genetic modification, hybridization, and adaptative evolution techniques. Cellar practices can also facilitate their implementation (Agarbati et al., 2020; Steensels and Verstrepen, 2014). However, winemakers' acceptance of these innovative strategies may be limited due to the strong tradition of winemaking. Additionally, marketing wine requires a balance of innovation, novelty, and tradition; and some of these techniques may not align with the winery's marketing plan or consumer expectations (Barber et al., 2021; Gardner et al., 2023). Moreover, implementing certain strategies in small or medium-sized cellars could entail high costs.

Terroir. The term *terroir* refers to the unique confluence of local and regional environments, soil properties, grapevine cultivars, surrounding plants and animals, and human interventions such as crop handling (Griggs et al., 2021; Mas et al., 2022). This concept is associated with a socio-environmental construction, the legacy of centuries or millennia of local-regional interactions between a human community with distinctive cultural traits, knowledge, and practices, and the natural environment, including geological characteristics of land (Castelló, 2021; Rigobello and Evans, 2024).

The concept of *microbial terroir* emerged based on studies that used NGS to demonstrate that microbial communities of the grape must are linked to the soil and the geography (Alexandre, 2020; Belda et al., 2017d; Bokulich et al., 2014).

This concept is also related to the interaction of indigenous grape microbiota with the resident microbiota in the cellar present in the equipment such as presses, pumps, and tanks (Mas and Portillo, 2022). For these reasons, the winery microbiota also contributes to the unique *terroir* of each cellar, influenced by cellar-specific practices. These practices include pellicular maceration, temperature, clarification processes that involve the natural settling of vegetal sediment, pellicular maceration, temperature conditions, and the addition of SO_2 to the must (Ribéreau-Gayon et al., 2006; Versavaud et al., 1995). These factors collectively shape microbial diversity, measurable in molecules that each microorganism releases, which influences the individual character of wines produced from various locations (Fleet and Heard, 1993; Griggs et al., 2021).

Different approaches have been studied to preserve the *terroir* concept in wines. SF is used for this purpose; despite its risks due to the microbiological control against spoilage microorganisms and its unpredictable outcomes that linked to the initial variability of microbiota composition (Mas and Portillo, 2022). Another strategy is the creation of multi-strain and multi-species yeast consortia isolated from the same geographical area (Padilla et al., 2016). Finally, a spontaneous PdC method has gained attention for its ability to provide microbiological control and rapid kinetic of sugar consumption during AF compared to SF and DWY inoculation. A spontaneous PdC has shown promise in preserving the typicity and *terroir* of the final wine (Moschetti et al., 2016).

5.3. *Pied de cuve*

Pied de cuve (PdC) involves creating an activated must, which is then inoculated into a fresh must to start the AF. The PdC is made by taking a small volume (3-10% (v/v)) of must that undergoes a partial fermentation (Mas and Portillo, 2022; Togores, 2011). The volume of the PdC should guarantee an inoculation of 10^6-10^7 million of active yeast cells per millilitre; to take over the entire deposit where be conducted the AF with these active and growing yeasts (Portillo and Mas, 2022). Next, the prepared PdC is inoculated and thoroughly mixed into a fresh must to conduct AF, either by pumping or placement at the bottom of the deposit before filling (Mas and Portillo, 2022; Togores, 2011).

There are three different methods to prepare a PdC:

- The first method involves reserving a specific quantity of grapes from 2-5 days before the harvest to obtain the must needed to create a PdC (Figure 6). The must is partially fermented by its natural microbiota, along with the resident microbiota present on the winery equipment that enters in contact with it (Sturm et al., 2006).
- Concerning the second method, the PdC can be prepared by taking a small proportion of a fermenting must from the main AFs in the winery, with a density between 1030 to 1020 g/L. This form facilitates the increasing of yeast population by successive additions of musts and promotes the selection of yeasts from the final stages of fermentations with good performance (Li et al., 2012).

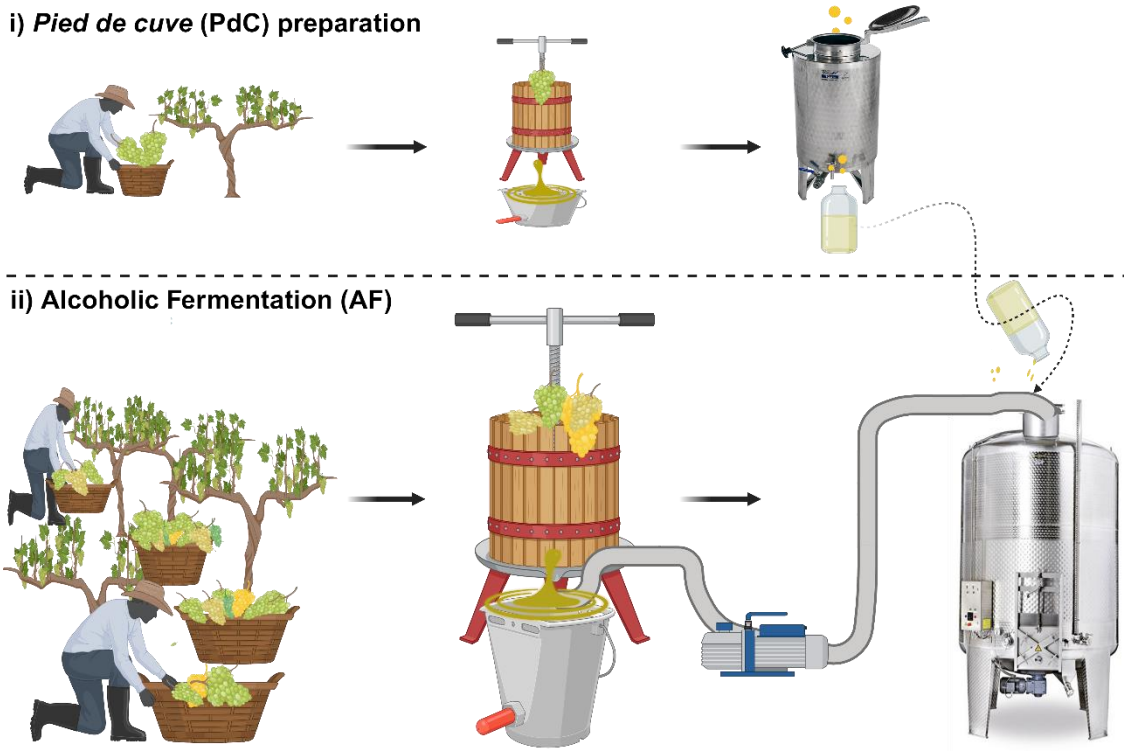

Figure 6. Steps for start alcoholic fermentation (AF) using the *pied de cuve* (PdC) from a reservoir harvest (Graph created with Biorender).

- Finally, the third method to prepare the PdC is by inoculation of a single isolated yeast (from a laboratory-selected strain or commercial strain) and then propagated in an appropriate matrix and volume (Mas and Portillo, 2022).

Different PdC prepared without the inoculation of any strain using both first or the second method mentioned before, are known as spontaneous or uninoculated PdC (Ubeda Iranzo et al., 2000).

Since the last 20 years, the influence of PdC over the AF has been regarded in a few studies. For example, Morgan et al. (2019) evaluated AF of Chardonnay grape must inoculated with different spontaneous PdC. These PdC were inoculated to conduct the AF in must with the addition of two concentrations of SO_2 (0 and 40 mg/L). They found that all fermentations were genetically diverse

in the indigenous population of *S. uvarum* with more than 150 strains of them. Other studies have found the use of PdCs guarantees a quick fermentation start and a favourable rate on their fermentation kinetics (Li et al., 2012; Moschetti et al., 2016).

PdC appears a good alternative for non-conventional or natural wines related to organic, biodynamic, *low-input* practices. These practices are related to the *low-input* approach to preserve native yeast and limiting the diversity loss that occurs when the musts are inoculated with a single DWY (Chalvantzi et al., 2020; Portillo and Mas, 2022). In this way, PdC is useful in wineries that aim to preserve the *terroir* in their wines and meet consumer preferences at a low cost (Portillo and Mas, 2022).

6. Alcoholic fermentation

The epiphytic microbiota on grape berries and the microbiota present in the winery use the nutrients released after crushing or pressing the grapes, but its population changes during the AF (González and Morales, 2021).

Many microorganisms proliferate at the onset of AF; however, they cannot survive the extreme conditions during AF. These conditions include high sugar concentrations, low pH, temperature variation, and transition to ethanolic and anaerobic environments (Bokulich et al., 2014). However, most yeasts can thrive in this environment and are the primary microorganisms responsible for conducting AF. Table 1 describes the most common genera found in AF, where

Aureobasidium, *Candida*, *Hanseniaspora*, *Issatchenkia*, *Lachancea*, *Metschnikowia*, *Starmerella* are the most abundant at the beginning of the SF.

Table 1. The main genera of yeasts found at the beginning of spontaneous fermentations (SF) detected by different methods of plating, q-PCR, RFLP-PCR or NGS. Percentages are calculated as relative abundance (%). Values > 5% are in bold.

Genus	Relative abundance of (%)	Reference
Apiotricum	< 1	(Abdo et al., 2020)
Aureobasidium	< 1	(Abdo et al., 2020)
	20	(Bagheri et al., 2015)
Candida	< 1.6	(Abdo et al., 2020)
	2	(Bagheri et al., 2015)
	10	(Portillo and Mas, 2016)
Brettanomyces (*Dekkera*)	< 0.28	(Abdo et al., 2020)
Debaryomyces	< 1	(Abdo et al., 2020)
Filobasidium	< 1	(Abdo et al., 2020)
Hanseniaspora	**94**	(Abdo et al., 2020)
	20	(Bagheri et al., 2015)
	38	(Lleixà et al., 2018)
	58	(Portillo and Mas, 2016)
	72	(Torija et al., 2001)
	24	(Hierro et al., 2006b)
Issatchenkia	4	(Bagheri et al., 2015)
	28	(Portillo and Mas, 2016)
	32	(Wang et al., 2015b)
Lachancea	**15**	(Bagheri et al., 2015)
Metschnikowia	**20**	(Bagheri et al., 2015)
	1-**5**	(Torija et al., 2001)
Pichia	1.5	(Wang and Liu, 2013)
Saccharomyces	< 0.1	(Abdo et al., 2020)

	1.5	(Lleixà et al., 2018)
	14-27	(Torija et al., 2001)
	1	(Wang et al., 2015b)
Saccharomycodes	3	(Portillo and Mas, 2016)
Schizosaccharomyces	**9.6**	(Torija et al., 2001)
Starmerella	< 0.1	(Abdo et al., 2020)
	16	(Bagheri et al., 2015)
	49.5	(Hierro et al., 2006b)
	1.5	(Lleixà et al., 2018)
	60-75	(Torija et al., 2001)
Torulaspora	1.3	(Lleixà et al., 2018)
Wickerhamomyces	<1	(Abdo et al., 2020)
Zygosaccharomyces	**5**	(Lleixà et al., 2018)
	1-**5**	(Torija et al., 2001)

As mentioned before, yeast population dynamic changes during AF. First, it starts with an abundance of non-Sce yeasts limited to the first 2 or 3 days and then they are substituted by Sce (Mas et al., 2022). The most prevalent non-Sce yeasts found in must are *Hanseniaspora*, *Issatchenkia*, *Lachancea*, *Metschnikowia*, *Pichia*, *Starmerella*, and *Zygosaccharomyces* (Table 1). Indeed, *H. uvarum* and *S. bacillaris* are the most abundant species found (Fleet, 2003; Torija et al., 2001; Wang et al., 2015b). Nonetheless, *H. uvarum* disappears soon due to its sensitivity to ethanol, SO_2, and the presence of Sce (Divol et al., 2012; Padilla et al., 2017; Wang et al., 2015b). Conversely, *S. bacillaris* remains viable longer at temperatures lower than 25 °C but above 10 °C than Sce (Erten, 2002; Gao and Fleet, 1988; Heard and Fleet, 1988). Subsequently, *Saccharomyces* genus takes

over during the AF due to their tolerance to ethanol, SO_2 and heat stress (González and Morales, 2021; Vigentini et al., 2014).

The dynamic yeast population expressed as relative abundance (%) during AF is illustrated in Figure 7, based on the study of Bagheri et al. (2015) from 2013 campaign. They evaluated the influence of different vineyard practices on the succession of microorganisms in SF using Cabernet Sauvignon grapes. This figure represents data obtained during AF with must from conventional vineyard practice. Most non-Sce species struggle to survive as ethanol production increases as sugars are consumed. However, *Saccharomyces* genus, particularly Sce, overtakes the fermentation, from the middle until the final stages.

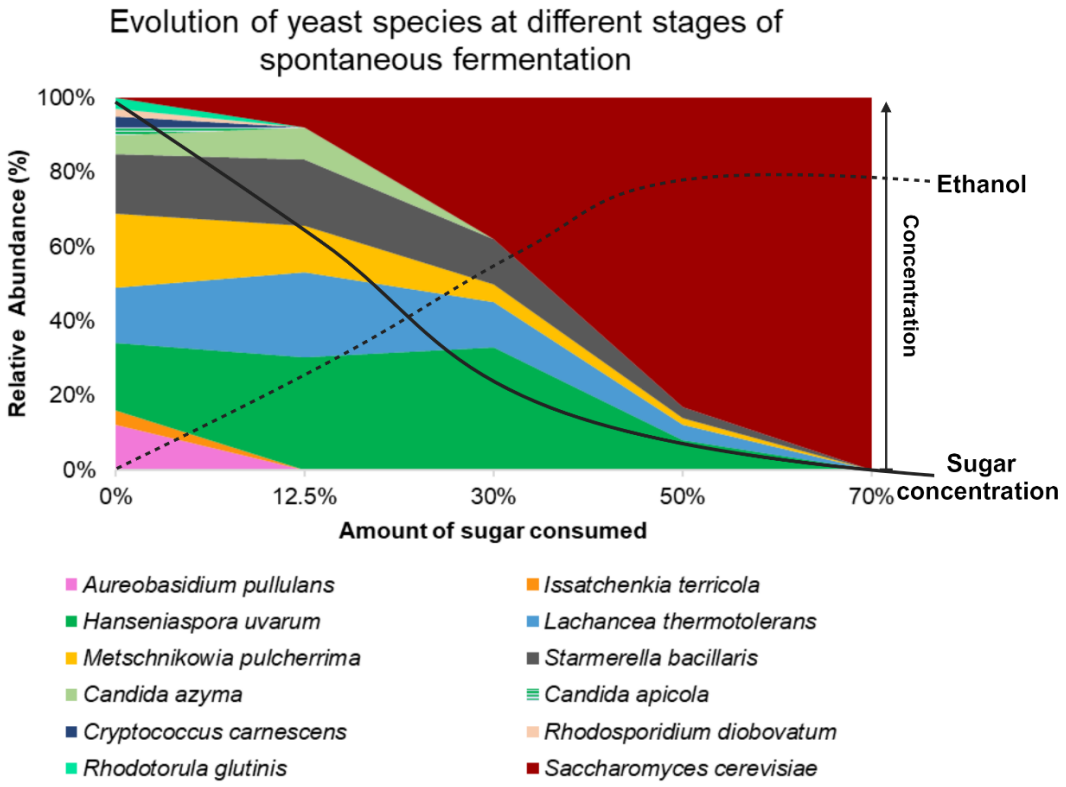

Figure 7. Yeast population dynamic on spontaneous fermentation (SF) of Cabernet Sauvignon must from conventional vineyard agriculture (Adapted from Bagheri et al. (2015).

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Nonetheless, in other studies, some species have been identified until the end of AF. These species belong to the genera *Starmerella*, *Torulaspora*, and *Saccharomyces* non-*cerevisiae* such as *S*. *uvarum*, *S*. *paradoxus*, *S*. *kudriavzevii*, and *S*. *bayanus* that also can be present until the final of the fermentation (Bagheri et al., 2015; González et al., 2006; Morgan et al., 2019; Naumov et al., 2000; Redžepović et al., 2002; Wang et al., 2015b).

After AF, LAB can perform MLF. These bacteria can originate from the spontaneous microbiota or be introduced through inoculation. This process consists of conversion of L-malic acid into L-lactic acid and CO_2 production, thereby reducing total acidity, enhancing biological stability, and altering the aroma profile of the wine (Brizuela et al., 2019; Styger et al., 2011). MLF is desirable for most red wines and some white wines (Ribéreau-Gayon et al., 2006).

Wine, as a final product from the AF, consists of several compounds at different concentrations. The main compounds are water, ethanol, and glycerol which represent 98-99% of the wine composition (Amerine et al., 1972; Mas et al., 2022). Other compounds are higher alcohols, residual sugars, organic acids, and phenolic compounds, various ions in high concentrations such as potassium, calcium, magnesium, and sodium (Ribéreau-Gayon et al., 2006). Other aliphatic and aromatic alcohols, amino acids mainly proline, and esters such as acetate esters (isoamyl acetate and phenylethyl acetate) and ethyl esters (hexanoate and ethyl decanoate) are present at much lower concentrations (Ferreira et al., 1999; Kidrič and Košir, 2008; Ribéreau-Gayon et al., 2006). The main organic acids such as citric, tartaric, and malic acids originate from the grapes. Contrarily,

acetic, succinic, D- and L-lactic acids are produced from the fermentation process (Ribéreau-Gayon et al., 2006).

Wine has more than 800 volatile compounds identified, with a concentration range from hundreds of mg/L to µg/L or ng/L (Ferreira et al., 1999; Li, 2006). Some production of fermentative aromas such as esters, either acetate esters or ethyl esters of fatty acids depends on the vinification type than on the species and strains. For example, Sce strains have been studied to their contribution for the varietal aroma of Sauvignon Blanc wines releasing volatile thiols, in particular 4-mercapto-4-methylpentan-2-one, 3-mercaptohexan-1-ol, and 3-mercaptohexyl acetate (Swiegers et al., 2006). Non-Sce also has been studied, González-Royo et al. (2015) demonstrated that *H. uvarum* produces aromatic and flowery wines related to the 2-phenyl acetate (rose aroma) and benzyl acetate (jasmine aroma). Also, *T. delbrueckii* is known for reducing volatile acidity and producing fruity notes, *M. pulcherrima* can increase the thiols and terpenes.

The presence and quantity of the aromas are also affected by grape varieties, viticultural and winemaking practices, and climate conditions (Mira de Orduña, 2010). The combination between them, the relative and absolute concentration of these compounds, and the odour detection threshold distinguish one grape variety from another (Jackson, 2008). For example, Muscat varieties are characterized by the prevalence of monoterpene alcohols, C_{13}-norisoprenoids and thiols in minor concentrations (Jackson, 2008; Tominaga et al., 2000). The thiols also contribute to the varietal fragrance mainly in Sauvignon Blanc and are measured in less concentration in other varieties such as Chardonnay,

Gewürztraminer, Colombard, Riesling, and others (Capone et al., 2018; Tominaga et al., 2000).

The green pepper attribute is associated with Sauvignon Blanc and Cabernet varieties, Merlot, etc. Also, it is associated with wines elaborated with unripe grapes (Allen and Lacey, 1993; Belancic and Agosin, 2007; Lacey et al., 1991; Van Leeuwen et al., 2020). This attribute is due primarily to the presence of 2-methoxy-3-isobutylpyrazine, isopropyl, and a minor concentration of sec-butyl methoxypyrazines (Jackson, 2008).

7. Identification and yeast population following during alcoholic fermentation

In oenology, different methods are used to enumerate, isolate and identify yeast in grapes, must, wine, and cellar (Manzanares and Vallés, 2005). Estimations of yeast populations during AF have been done by direct counting in microscopy using a Neubauer chamber and CFU in agar-solidified plates and flow cytometry. Other methods include membrane filters and several physiological tests (Manzanares and Vallés, 2005).

The most affordable methods in the cellar to enumerate and identify yeast morphology are microscopy and plate cultures. However, they can be time-consuming and lead to mistakes. For instance, these methods require a minimum population to be detected or in a large population, they can be agglomerated making difficult the counting. Additionally, the morphology of each species varies according to the age or the physiological state of the yeasts (Mas et al., 2022).

Nonetheless, plating has served to enumerate, isolate, and identify yeasts and is recognized as a culture-dependent method. Alternatively, other techniques can enumerate and identify yeasts without the necessity of culture; they are known as culture-independent methods.

7.1. Culture-dependent techniques

Plating is a culture-dependent technique that requires a nutrient-rich medium. This medium includes a carbon source (such as glucose, fructose, or sucrose), a protein hydrolysate (peptone, tryptone, or casitone), a vitamin complex, amino acids and growth factors (yeast or malt extract). Use of antibiotics is recommended to prevent bacterial growth (oxytetracycline, chlortetracycline, or chloramphenicol), and compounds to inhibit moulds (Dichloran Rose Bengal or biphenyl) (Beuchat, 1998; OIV, 2024).

YPD is the medium commonly used to estimate the CFU. This medium allows the growth of the total culturable yeasts, but higher dilutions are often required to count the CFU accurately. Consequently, isolation of minor species is difficult because most predominant populations dominate the plates. To overcome this problem, Lysine (Lys) medium has been used in oenology to distinguish Sce from non-Sce. This medium allows the fast growth of non-Sce that use lysine as a nitrogen source, for example, yeasts from the *Pichia* and *Metschnikowia* genera. Some strains of Sce cannot synthesize other amino acids with lysine as the sole nitrogen source and others can use it but grow slowly (Bisson and Walker, 2015; Walters and Thiselton, 1953).

Wallerstein Laboratory Nutrient (WLN) is another selective medium to distinguish colour and morphology among a mixture of yeast colonies in fermentation (Figure 8). However, it is necessary to characterize previously each strain because colour and morphology vary during the culture. Additionally, different species could have the same morphology or vice versa. Therefore, molecular identification is required (Pallmann et al., 2001; Torija et al., 2021).

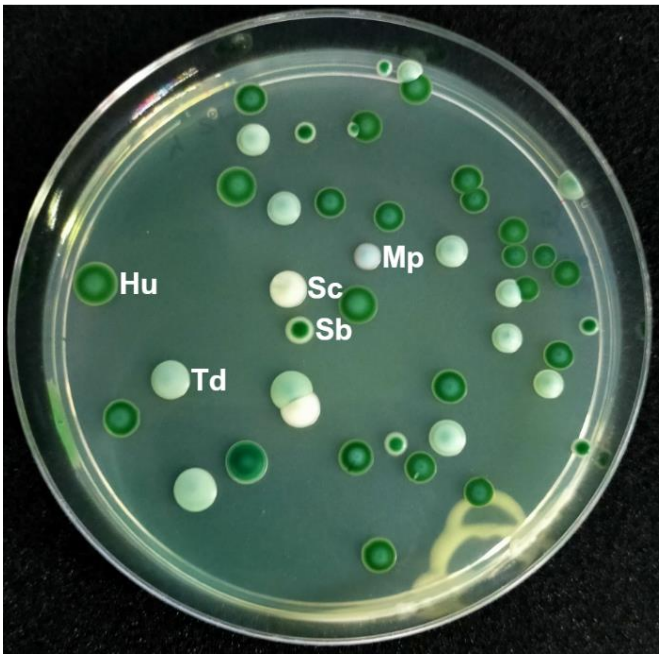

Figure 8. Colony-forming unit (CFU/mL) of a consortium of yeast growth 72 h in WLN media. The identification name is on the right side of each colony. (**Hu**), *Hanseniaspora uvarum*, (**Sb**), *Starmerella bacillaris*; (**Mp**), *Metschnikowia pulcherrima;* (**Td**), *Torulaspora delbrueckii* and (**Sc**), *Saccharomyces cerevisiae*.

Identification methods are required after isolation to have reliable results. Molecular biology techniques offer a good alternative to identify species from their genomes independently of their physiologic status (Manzanares and Vallés, 2005). These methods are based mainly on PCR (polymerase chain reaction) of different ribosomal regions, these regions are useful to establish phylogenetic relationships and identify organisms. The ribosomal genes of organisms are

grouped in tandem, forming transcription units that are repeated in the genome. Between each transcription unit are the internal transcribed spacers (ITS), the external transcriber spacers (ETS), and rRNA-codifying genes. ITS regions are commonly used to identify closely related species by comparing the sequences in a database (Esteve-Zarzoso et al., 1999; Mas et al., 2021).

In oenology, the most used identification technique is restriction fragment length polymorphism (RFLP) of mitochondrial DNA for routine analysis of many samples. Other techniques are chromosomal DNA electrophoresis, ribosomal DNA restriction analysis, and randomly amplified polymorphic DNA (RAPD) (Esteve-Zarzoso et al., 1998). Sce strains are genotyped using the region comprised between the delta elements. Delta elements are conserved sequences that flank the transposable Ty elements. The distance between these elements is between 1-2 kb; and allows differentiation among Sce strains (Mas et al., 2021).

Despite the usefulness of culture-dependent techniques in enumerating, isolating, and identifying yeasts have several disadvantages. Some yeasts could be present in the media but may not grow in culture, a state known as viable but non-culturable (VBNC). The VBNC is considered a survival strategy inherited from the species or influenced by their life-cycle phase in the fermentation. Also, some species enter the VBNC state by contact with other non-Sce or by the increasing ethanol levels or the absence of nutrients in the medium (Mas et al., 2022). For example, Navarro et al. (2020) found VBNC yeast population was more than 85% to 94% from the middle till the end of AF.

Also, external factors can induce VBNC state in yeasts. These include heat shock stress, thermosonication, and hypoxic growth conditions (Hommel et al., 2019; Križanović et al., 2020). For example, Sce, *Z. bailii*, and *Brettanomyces bruxellensis* are induced to the VBNC by the addition of SO_2 and can survive for more than a month depending on the pH of the environment (Capozzi et al., 2016; Divol and Lonvaud-Funel, 2005; du Toit and Pretorius, 2019; Padilla et al., 2016). However, industrial Sce is SO_2-resistant and can release peptides that inhibit the growth of non-Sce. For instance, Sce releases some antimicrobial peptides to induce the VBNC state in *H. uvarum* (Branco et al., 2015). Both strategies are suitable when the dominance of Sce is desired in AF (Navarro et al., 2020). For these reasons, alternative methods that can identify and quantify yeasts even in VBNC state without the need to culture strains on solid media, are referred to as independent techniques.

7.2. Culture-independent techniques

Independent techniques serve to detect microorganisms taxonomically of a defined group using universal primers to amplify heterogeneous DNA sequences. These methods include single-strand conformational polymorphisms (SSCP), denaturing gradient gel electrophoresis (DGGE), (T-RFLP), automated ribosomal intergenic spacer analysis, quantitative-PCR (qPCR), DNA binding dye coupled to prevent the amplification of DNA on qPCR (EMA-qPCR or PMA-qPCR), and NGS (reviewed by Morgan et al., 2017). This thesis will focus specifically on qPCR and NGS.

qPCR. This is a quick and sensitive technique for detecting and quantifying microorganisms. This technique is essential for winemakers to control and prevent product spoilage in the wine industry, particularly, in low-intervention winemaking, where fermentation is driven by native rather than starter yeasts (Hierro et al., 2006a; Wang et al., 2020). qPCR consists of amplification of the DNA monitored in real-time using fluorescence techniques. The fluorescence dyes of dsDNA-specific dyes (i.e., SYBR Green I) or sequence-specific probes produce a signal directly proportional to the amount of amplified DNA (Figure 9).

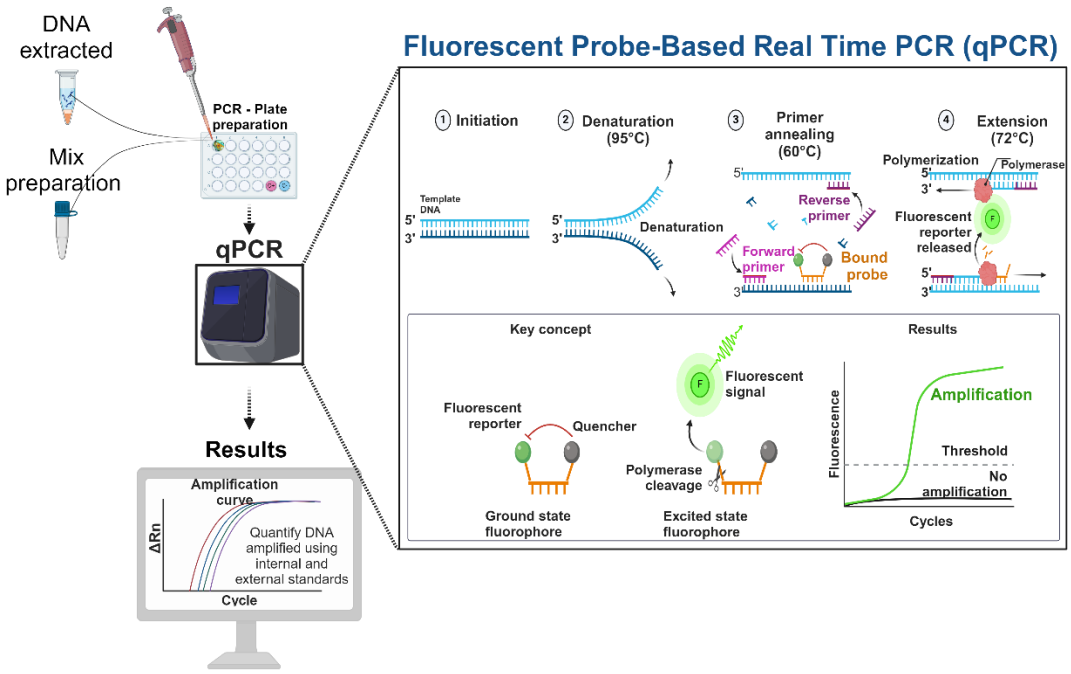

Figure 9. Main steps of quantitative polymerase chain reaction (q-PCR) and its key concept. Created with BioRender.com.

Each amplification curve comprises three distinct phases: **the initial lag phase** with no measurable product accumulation, **the exponential phase**, and the **plateau phase**. Although extrapolation of the exponential phase to Cycle 0 can theoretically estimate the template copy number is considerable to error. The

determining of the cycle threshold (Ct), quantification cycle (Cq) or crossing point (Cp) is more precise for estimation of template copy number. Cq, is the most recently used term, and is defined as the cycle number at which the fluorescence signal reaches an arbitrary threshold set within the exponential phase. This threshold must intersect the exponential phase to ensure a correlation with product accumulation. These signals start to increase while the product is generated until depletion of deoxynucleotide triphosphates (dNPTs) or the fluorescent dye.

The amplification reaction's efficiency must be consistent between standards (samples with known template concentrations) and unknown samples. The amplification efficiency can be calculated from the Cq values of the standards. This ensures identical efficiencies across samples, which is crucial for precise quantification (Wilhelm et al., 2003). The detection limits depend on the specific conditions, the specificity of the primer pair used, and the sample type (Hierro et al., 2006a; Wang et al., 2020). For example, Hierro et al. (2006a) found the lowest detection limit from 10^2 to 10^3 cells/mL from samples cultured in YPD medium and wine samples, respectively.

Using qPCR has both pros and cons. One advantage is that qPCR allows quantifying under-represented populations, or those in the VBNC state (Andorrà et al., 2010; Cocolin et al., 2013). Also, qPCR detects species in transient states such as old cells that retain the capacity to grow under optimal conditions, old cells that have lost the capacity to grow but it is still functional, or cells that are entering an autolysis (Mas et al., 2022). However, this technique only detects target microorganisms and could lead to population overestimation due to not

distinguishing live cells from dead (Table 2). Also, sometimes the amplification efficiencies of most primer pairs are lower than desired, due to the presence of PCR inhibitors in the matrix (Mas et al., 2022; Okubara et al., 2013).

Table 2. Main differences among culturable independent techniques qPCR and NGS (Next Generation Sequency) tools (Chaudhari et al., 2024; Illumina, 2024; Sternes et al., 2017; Stoler and Nekrutenko, 2021).

Tool	**qPCR**	**NGS (Illumina sequencing MiSeq System)**
Target Size or lectures	100 – 150 bp	2 x 150 bp
Sensitivity	10-10^3 cells/mL	10^7-10^9 cells/mL
Run time	45 min-1 h	4h-24 h
Advantages	• Sensitivity, quantitative, specificity • Differentiate accurately at species level • Fast • Cost-effective • Ease of use	• High resolution • Targeting the entire community • Long fragments length read • Analyse unknown samples
Disadvantages	• Targeted technique, knowing the species previously • Population overestimation • Effective primer designed • High labour and material	• Short reads and high error rates in homopolymer repeats • Expensive • It can differentiate until genus level • Population overestimation • Complex • Data management is time consuming • Significant computational resources for storage/ analysis data

NGS. Toghether omics, such as metabarcoding, meta-transcriptomics, meta-proteomics, and metabolomics, NGS has emerged as powerful method for understanding the genomes of entire microbial communities, with application in winemaking (Portillo and Mas, 2022). NGS allows the analysis of both culturable and unculturable microorganisms (bacteria, filamentous fungi and yeasts) across various environmental niches of different samples (soils, wood, grapes, musts, wine, etc.), facilitating comprehensive monitoring and diagnostics (Belda et al., 2017a; García-Izquierdo et al., 2024). This approach has been important in revealing the relationship between the microbiota and wine *terroir*, the provenience of some microbial groups, monitoring the AF under different conditions, making relations between the microbial communities and production of chemical compounds, etc (reviewed by Portillo and Mas, 2022).

NGS technologies depend on a parallel process where individual DNA fragments are sequenced and analysed independently (Diaz-Sanchez et al., 2013; Wooley et al., 2010). It enables high-throughput sequencing, capable of processing tens of thousands of sequences fixed to a specific location (Schadt et al., 2010). The length of the fragments obtained from the analyses depends on the sequencing method employed (Bokulich et al., 2016; Wooley et al., 2010).

The Illumina and 454 pyrosequencing platforms have been the most common platforms used for grapevine ecology surveys. At least 48% of the published data on the vineyard, grapevine and wine microbiome is derived from 454 pyrosequencing while the remaining 52% is derived from Illumina sequencing (Morgan et al., 2017). The target marker genes are universally present across all assessed species and comprise highly conserved segments that facilitate the

design of PCR primers capable of targeting the entire community. In both fungi and bacteria, ribosomal RNA genes serve as suitable targets. Specifically, in bacteria, the 16S rRNA gene is commonly targeted, whereas in fungi, the ITS1-5.8S and rRNA-ITS2 region and the 26S rRNA gene are targeted for high-throughput amplicon sequencing and microbiome analyses. After that, a large amount of data should be processed using to complete analysis procedure for NGS data following the procedure described by Morgan et al. (2017). However, this tool is powerful to differentiate groups generally until taxa or genus level but cannot achieve the identification at species or strain level (Mas et al., 2022).

8. Factors that impact yeast dynamics in alcoholic fermentation

The presence of yeasts during different steps of AF can be influenced by several factors. These include grape variety, vineyard practices as pesticide use, sugar concentration (which is directly related with ethanol yield), nutrient availability, and fermentation conditions such as SO_2 addition, temperature, pH, aeration (Bagheri et al., 2015). In the following section, it will be described in detail some of these factors directly related to the present thesis.

8.1. Sulfur or sulphur dioxide

Sulfur or sulphur dioxide (SO_2) is one of the most used additives throughout the winemaking process. It is used during the pre-fermentative steps, during the AF, in barrels ageing and before bottling (see Table 3).

Table 3. Different timing and uses of sulphur dioxide (SO_2) in winemaking processes.

	Function
Settling process	• ***Antimicrobial.*** It acts especially against the lactic acid bacteria (LAB), acetic acid bacteria (AAB), moulds, and spoilage yeasts. SO_2 delays the start of the alcoholic fermentation (AF), which is useful during the clarification process of must (Portillo and Mas, 2022; Wibowo et al., 1988) • ***Dissolving power.*** It enhances the degradation of the skin to optimize or maintain the maceration process, especially in red wine. Sulfiting favours the dissolution of minerals, organic acids and especially phenolic compounds (anthocyanins and tannins) (Ribéreau-Gayon et al., 2006) • ***Antioxidant protective effect in white wine.*** SO_2 retards the Maillard process and regulates oxidative activities, such as polyphenol oxidase, that affect the nutritional and sensory qualities of wine (Oliveira et al., 2011; Torija et al., 2021) • ***Antioxidasic effect***. It inhibits the oxidation enzymes function such as tyrosinase and laccase. SO_2 can reduce their activity over time (Boulton et al., 1999)
Alcoholic Fermentation	• SO_2 reduces the growth of undesirable yeast during the initial stages of fermentation (Capece et al., 2020; Van Wyk and Silva, 2019) • It inhibits the growth of spoilage bacteria throughout fermentation, minimizing undesirable secondary fermentations (Avramova et al., 2018)
Malolactic Fermentation and ageing process (generally for red wines)	• Disinfection of barrels/tanks. SO_2 prevents the spoilage of yeast such as *Brettanomyces*, and bacteria such as LAB and AAB (Lisanti et al., 2019; Stadler and Fischer, 2020)
Bottling	• SO_2 addition before bottling, prolongs the shelf life of wine and reduces the chance of undesirable aroma production (du Toit and Pretorius, 2019) • It could refine the wine and enhance its aromatic profile if is applied in correct doses (Ribéreau-Gayon et al., 2006)

SO_2 is the active molecule of different compounds used as food additives for their preservation. They are available in the form of sulphur dioxide, sodium sulphite, sodium hydrogen sulphite, sodium metabisulphite, potassium metabisulphite, calcium sulphite, calcium hydrogen sulphite, and potassium hydrogen sulphite reviewed by du Toit and Pretorius (2019).

Sulphur dioxide (SO_2), sulphur anhydride or sulphurous gas are words used equally in oenology. SO_2 can be applied as either sulphur dioxide gas or liquid solution, or in forms such as potassium bisulfite ($KHSO_3$) or potassium metabisulfite ($K_2S_2O_5$). In wine, SO_2 is always expressed in the anhydrous form, in mg/L or g/hL. The equilibrium established between these forms is identical and depends on the pH and the presence of molecules that bind to SO_2 (Ribéreau-Gayon et al., 2006).

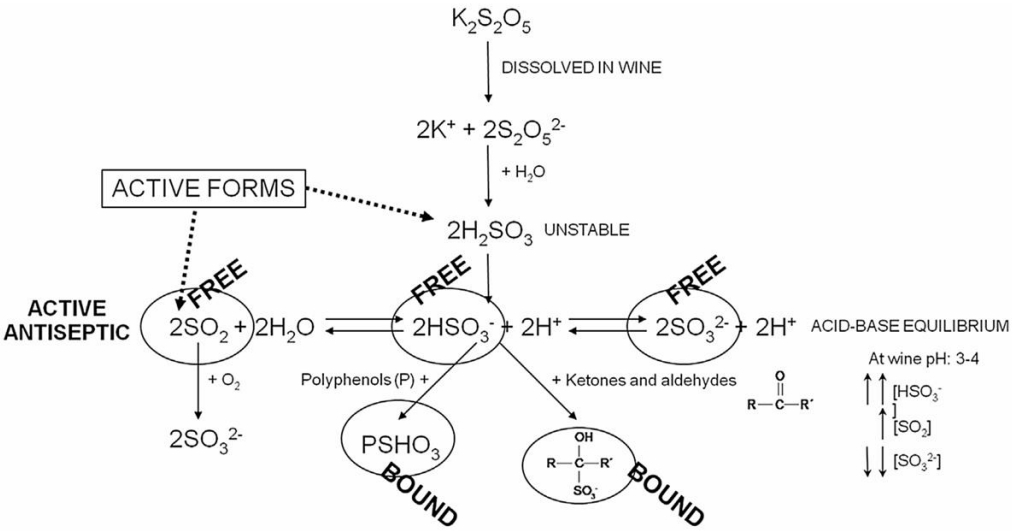

Figure 10. Chemical equilibrium of sulphur dioxide (SO_2) when added as potassium metabisulphite ($K_2S_2O_5$) to wine (Guerrero and Cantos-Villar, 2015).

Figure 10 taken from Guerrero and Cantos-Villar (2015) shows the reactions when $K_2S_2O_5$ is added to the must it rapidly ionizes under acidic conditions. SO_2 is converted into two different forms in the solution. First, the free SO_2, which comprises the molecular or active SO_2, bisulfite ion (HSO_3^-), sulphite ion (SO_3^{-2}). Second, the bound forms of SO_2 (sulphurous acid, H_2SO_3), when bound with other molecules in wine (i.e., aldehydes such as ethanal, aldehydes, ketones, anthocyanins, polyphenols, sugars and acids) (Howe et al., 2018; Ribéreau-Gayon et al., 2006). The most active fraction of free SO_2 is composed of molecular SO_2. Additionally, the basic equilibrium in wine must be considered, as the concentrations of SO_2, HSO_3^- or SO_4^{2-} depend directly on the pH (Mas et al., 2021). The sum of free SO_2 and bound SO_2 gives the value of total SO_2, and this is the parameter used as a reference in the maximum limits set by legislation. The concentration of SO_2 in wine is reported in mg per litre (ppm). The yeasts can form 10-50 mg/L of SO_2 during fermentation, which is usually bound to acetaldehyde during its formation.

The recommended concentrations of free SO_2 during ageing and storage step are 25 mg/L for red wines and 30 mg/L for white wines. OIV (2021b) recommended the following limit during winemaking for SO_2 (Table 4). SO_2 for organic and biodynamics wines are in regulations from EU Council Regulation EC No. 834/2007, EC Reg No. 203/2012 and Demeter protocol.

The correct doses of SO_2 is not easy to calculate due to chemical equilibrium in the must should be considered. The unappropriated doses could be detrimental to human health, and compromise microbiological stability of wine (Esparza et al., 2020). Sulphite exposure has been related to bronchoconstriction, urticaria,

headaches, dermatitis, diarrhoea, or worsening of asthmatic symptoms (Häberle et al., 2017; Witkowski et al., 2022).

Table 4. Limits of sulphur dioxide (SO_2) concentration in different wines according with the residual sugar concentration (OIV, 2021b).

Wine type	Sugar concentration (g/L)	SO_2 Limit
Dry with / Rosé	< 4	200 mg/L
White / Rosé / Red	> 4	300 mg/L
Sweet white / others	> 4	300 mg/L
Red	< 4	150 mg/L
Organic	-	100 mg/L
Biodynamic	-	70 mg/L

On the other hand, some native yeast populations present in the must are sensitive to different concentrations of SO_2, limiting the yeast growth during the fermentation including the DWY, and potentially leading to stuck fermentation (Osborne and Edwards, 2006). Abdo et al. (2020) showed that spontaneous PdC, prepared with the addition of SO_2, resulted in a significant reduction of non-Sce species, particularly within *Hanseniaspora* genus and its relative abundance decreased from 90% at the beginning to 45% after 7.5 days of AF. Additionally, incorrect doses could affect the sensory or stability characteristics of wines (Ribéreau-Gayon et al., 2002). It could neutralise their aromatic profiles, or in higher quantities produce aroma defects, such as sulphur gas or its reduction to malonic acid or mercaptan. As a result, these wines could smell of wet wool that rapidly becomes suffocating and irritating, together with a burning sensation on the aftertaste. Conversely, insufficient doses of SO_2 do not ensure the total

stability of the wine, resulting in wines with excessive oxidation or microbial development (Ribéreau-Gayon et al., 2006; Togores, 2011).

Many efforts are directed at identifying substances to replace or reduce the use of SO_2 during and after AF, maintaining the organoleptic properties of the final wine (Mas et al., 2021; Portillo and Mas, 2022). Several molecules have been studied for oxidation prevention as ascorbic acid, inert gases, oenological tannins, the use of reduced glutathione or inactivated dry yeasts rich in glutathione (Bustamante et al., 2024). Other compounds have been studied for their antiseptic properties such as lysozyme, potassium sorbate, fumaric acid in wine, chitosan, dimethyl dicarbonate, some bacteriocins, hydroxytyrosol, short-/medium-chain fatty acids and polyphenols have been studied (Lisanti et al., 2019; Mas et al., 2021; Portillo and Mas, 2022; Ribéreau-Gayon et al., 2006).

Some yeast species have been studied for their use as biocontrol agents. A biocontrol treatment consists of the use of different species/isolates tested against the target pathogen, and the most active organism, it is studied for potential application. The use of non-Sce yeasts such as *L*. *thermotolerans*, *M*. *pulcherrima*, and *T*. d*elbrueckii*, have been used during fermentation as biocontrol agents. These species showed the ability to limit the growth of spoilage microorganisms such as *Aspergillius*, and prevent both chemical and enzymatic oxidation, thereby contributing to wine quality and stability (Freimoser et al., 2019; Morata et al., 2021).

Other physical methods comprise high pressure, low electric current, microwaves, pulsed electric field, pulsed light, ultraviolet irradiation, and ultrasound, etc (Morata et al., 2017; Portillo and Mas, 2022).

8.2. Ethanol

Ethanol production. Ethanol, a by-product generated by yeasts, is the primary stress factor they encounter during AF. Yeasts degrade glucose via glycolysis by respiration or fermentation. Respiration yields a high amount of ATP (approx. 18 ATP per glucose), whereas fermentation produces less ATP (2 ATP per glucose) but does not require oxygen (De Deken, 1966). These metabolic strategies are associated with the Crabtree effect (De Deken, 1966; Postma et al., 1989; Van Urk et al., 1990). The Crabtree effect occurs when yeasts continue to ferment sugars even in the presence of oxygen and high glucose concentrations (Crabtree, 1929). Crabtree-positive yeasts exhibit the Crabtree effect and use both fermentation and respiration simultaneously. In contrast, Crabtree-negative yeasts rely exclusively on respiration. Yeasts use respiration, fermentation, or a combination of both pathways when are exposed to high sugar and oxygen levels. In both cases, the process starts by the degradation of 1 molecule of glucose into 2 molecules of pyruvate. This process generates energy in form of ATP and NADH.

Pyruvate in the presence of oxygen, is degraded to acetyl-Co A and CO_2. Acetyl-Co A goes into the Krebs cycle, generating other subproducts. In the absence of oxygen, pyruvate is decarboxylated to acetaldehyde and CO_2. Then, acetaldehyde is partly excreted to the medium and reduced as ethanol by the alcohol dehydrogenase (ADH). Or it can be oxidised to acetate by the aldehyde dehydrogenase (ALDH) (Millán and Ortega, 1988).

In fermenting yeasts has been reported the aldehyde $NADP^+$ oxidoreductase EC 1.2.1.4 in the cell cytoplasm. This enzyme offers an alternative pathway if the pyruvate oxidase complex is inhibited, converting the pyruvate to acetyl-Co A and yielding NADPH that is used in the biosynthesis of fatty acids and acetic acid. Acetic acid can be used as a biosynthetic intermediate in the cell metabolism or be excreted into the wine. As the sugar is consumed as the AF progress, the ethanol starts to accumulate in the media (Millán and Ortega, 1988).

Impact of ethanol in yeasts. Ethanol is soluble in aqueous and lipid media and consequently, it can cross the plasmid membrane and change the fluidity and permeability (Torija et al., 2021). Ethanol causes misfolding of proteins and activation of various stress signalling pathways to maintain the pH homeostasis, and finally can lead to cell death (Bauer and Pretorius, 2000).

Strategies to counterpart the ethanol content. Yeast have developed strategies to antagonise fluidity caused by ethanol, by enhancing the stability of proteins and membranes and maintaining intracellular pH homeostasis. These strategies have been based on the Sce model and have been summarized by Torija et al. (2021): (a) inhibition of cell cycle and propagation, (b) accumulation of trehalose and glycogen, (c) increase in heat shock proteins, (d) increase the activity of plasma membrane ATPase, the levels of oleic acid and ergosterol in the membrane, (e) induction of genes encoding vacuolar proteases and their inhibitors and (f) increased activation of genes related with unfolded protein response and their transcription factors. Some yeasts use this accumulated ethanol as a source depending on their genetic makeup and environmental

conditions, through another alcohol dehydrogenase (ADH2) (Thomson et al., 2005).

Ethanol tolerance. Yeast tolerance to ethanol involves multiple genetic, molecular, and physiological processes with complex interactions (reviewed by Saini et al., 2018; Snoek et al., 2016). The ability to grow and survive in the presence of ethanol varies among species and strains. The presence of wine yeast species can change significantly during fermentation, especially as ethanol levels increase during later stages (Bauer and Pretorius, 2000). Most of the non-Sce species cannot survive when the alcohol level reaches 4-8% (v/v) (Ciani et al., 2016b).

Ethanol tolerance is also influenced by temperature; for example, temperatures below 30 °C favour the ethanol resistance to *S*. *bacillaris* and *H*. *uvarum*. Other studies have found some non-Sce yeasts such as *H*. *uvarum*, *T*. *delbrueckii* and *Pichia fermentans*, and *S*. *bacillaris* grow until 10% (v/v) of ethanol and over this value, the Sce has an advantage to growth in high level of ethanol (Torija et al., 2021). For example, wild strains of Sce can tolerate around 8% (v/v) and some commercial strains tolerate from 15% to 20% (v/v) of ethanol level (Sahana et al., 2024; Saini et al., 2018). Benítez et al. (1983) isolated 632 strains of wine yeasts from vineyards in southwest Spain. Among these isolates, they found 35 strains grown in 15% (v/v) ethanol and fermented in 18% (v/v) ethanol.

Thus, yeasts species or strains that can grow, respire, or accumulate ethanol have a significant advantage over other microorganisms in fruit environments. For example, in sparkling wines, the adaptation of yeast is especially important due the yeasts species have to survive in the base wine to conduct the second

fermentation. Base wine presents low pH and contains high ethanol concentration, presence of SO_2 and CO_2, glycerol, and acetic acid. Also, it has limited nutrients, and it is kept at low fermentation temperatures (Borrull et al., 2015).

Ethanol has been used as a supplement in the form of wine to create a fortified PdC to put a selective pressure in yeasts, and then evaluate its impact in AF. Moschetti et al. (2016) compared AFs inoculated with two spontaneous fortified PdCs with addition of 1.5% and 3% (v/v) of ethanol, one spontaneous PdC, and one inoculated with a commercial strain. This study assessed the microbial, chemical, and sensory composition of Nero d'Avola red wine. At the microbial level, AFs inoculated with fortified PdCs showed comparable yeast population growth to that inoculated with a PdC containing a commercial strain, and superior growth than inoculated with a spontaneous PdC. Additionally, the fortified PdCs demonstrated a substantial concentration and high strain diversity of the Sce population during vinification.

8.3.Temperature

Temperature is a critical factor in winemaking. It can influence cell viability and fermentation kinetics of yeasts, affecting the wine quality.

Yeast cells release a significant amount of energy as a form of heat, increasing the fermentation temperature (Ribéreau-Gayon et al., 2006). Temperature fluctuations are perceived for yeasts as stress during AF (Walker and Basso, 2020). For example, a culture of Sce in the exponential growth phase will lead to cell death if it is suddenly exposed to heat from 19 to 35 ºC or has a temporary

growth arrest at 37 °C (Rozèz et al., 1988). Contrary, if yeast culture is subjected to a sudden decrease in temperature (cold shock) it accumulates trehalose, which confers protection and increases cryoresistance (reviewed by Bauer and Pretorious, 2000; Piper, 1997).

The temperature conditions and their variation depend on the type of wine produced (Bauer and Pretorius, 2000). For example, temperatures between 25-28 °C are indicated for red wines and 10-15 °C for white wines (Torija et al., 2021). However, 2-3 °C variations cannot always be avoided in large tanks used on an industrial scale. These temperature variations cause heat stress in yeasts and impact negatively, even in temperature-controlled fermentation (Bauer and Pretorius, 2000; Henschke, 1997b). Therefore, yeasts have adapted to different temperatures, and their growth kinetics vary depending on the species and strains (Fleet and Heard, 1993). Generally, yeasts can ferment sugars at twice the rate at 30 ºC than at 20 ºC, with each 1 ºC increase resulting in a 10% higher rate of sugar transformation within the same timeframe.

Several studies have shown that temperature preferences have shaped dominance patterns among yeast species (Belloch, 2008; Fleet and Heard, 1993; Gonçalves et al., 2011; Kurtzman et al., 2010; Salvadó et al., 2011b). Some *Saccharomyces* species, such as *S*. *cerevisiae* (Sce), *S*. *paradoxus*, and *S*. *mikatae*, can thrive at 37 °C, while others like *S*. *arboricolus*, *S*. *uvarum*, and *S*. *kudriavzevii* are unable to grow at such high temperatures due to their cryophilic nature. Additionally, non-Sce species such as *Kazachstania lodderae*, *K*. *martiniae*, *Tetrapisispora blattae*, *Lachancea waltii*, and *Hanseniaspora vineae*, also show temperature-dependent growth limitations (i.e., *H*. *vineae* cannot grow

at 37 °C) (Kurtzman et al., 2010). Generally, non-Sce species have been reported to grow better at low temperatures or at least at temperatures until 25 ºC (Andorrà et al., 2010; Heard and Fleet, 1988).

Additionally, temperature influences the growth of yeast species that modify the wine product. Low temperatures favour the growth of non-Sce yeasts, which has been associated with enhancing the complexity of white wines due to the production of fruity acetate esters and the decrease of higher alcohols such as solvent-like characters. Low temperatures are associated with wines with an increase of acetate esters, ethyl esters and overall medium-chain fatty acids (Beltran et al., 2008; Molina et al., 2007; Torija et al., 2021). However, low temperatures reduce the diffusion of phenolic compounds (from the skin and pulp to the juice) (Sacchi et al., 2005). Low temperatures affect yeasts causing: a reduction of growth, longer lag phase, decrease in sugar consumption rate, longer fermentations, pathways of modification of secondary metabolite production, change in the permeases of the membrane, and consequently an increase of unsaturated fatty acids to maintain the membrane stability. Also, low temperatures change the enzymatic activity, protein translation and folding rates, heat shock protein regulation and RNA secondary structure stability (reviewed by Torija et al., 2021).

Conversely, high temperatures favour the establishment of Sce, which is preferred for red wines. High temperatures can accelerate the fermentation, shorten the lag phase in yeasts and cause a sharp temperature and the production of other metabolites such as acetic acid, acetaldehyde or glycerol (Fleet and Heard, 1993; Torija et al., 2021). Nonetheless, the yeast growth phase

can be negatively affected by a high initial temperature (26-28°C), because it results in the disruption of the hydrogen bonding and the denaturation of proteins and nucleic acids, which causes damage and late death of yeast cells (Walker and Van Dijck, 2006). It could lead to stuck fermentation and an excessive increase in volatile acidity. Therefore, 20 °C is considered optimal to start the AF as it prevents thermal shock to the yeasts and maximizes the formation of fatty acids, higher alcohols, and their esters (Ribéreau-Gayon et al., 2006).

9. Stuck fermentations

Stuck fermentation is defined as no desired high residual sugar at the end of AF, and it has always been a major problem in winemaking resulting in irreversible effects in wine quality and the hygienic status of the final product leading to economic losses (González and Morales, 2021; Maisonnave et al., 2013; Tedesco et al., 2022). The AF is considered complete, when wines have residual sugar less than 4 g/L, but generally, these sugars are below 2 g/L (Ribéreau-Gayon et al., 2006).

Stuck fermentation is a complex phenomenon that results from many factors acting individually or collectively. These factors are summarized in Figure 11, based on Ribéreau-Gayon et al. (2006), chapter 3, section 3.8., and detailed as follows:

Sugar effect. Total sugar concentration in musts can vary between 150-250 g/L, being higher until 350 g/L in musts used for sweet wine production (Ribéreau-Gayon et al., 2006). The presence of a high concentration of sugars in the must

(> 200 g/L) represents an osmotic pressure for yeasts inducing an increase of glycerol accumulation for osmoprotection. Additionally, it causes dehydration and inhibiting enzyme activity crucial for AF (Torija et al., 2021).

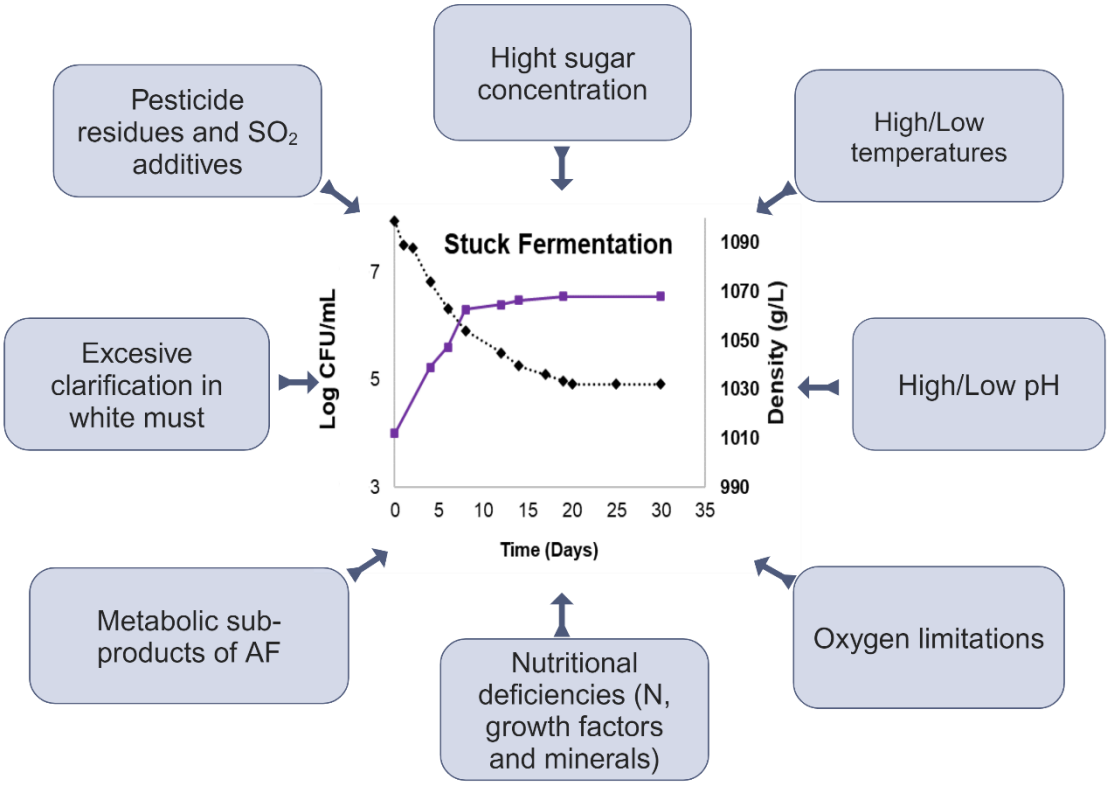

Figure 11. Common causes of stuck alcoholic fermentation (Ribéreau-Gayon and Peynaud, 1960).

Fermentable sugars are mainly glucose and fructose, they are in equal proportion in must. However, during the AF this ratio changes, and it could influence the selection of yeast strains (Guillaume et al., 2007; Torija et al., 2021). For example, Sce is a glucophilic yeast, preferring glucose to fructose but the proportion of fructose therefore increases as AF progresses. Consequently, wine yeasts have to ferment this non-preferred sugar after long periods of starvation and in the presence of large amounts of ethanol. The stress associated with these

conditions amplified by other nutritional imbalances affects the yeast's activity and can lead to sluggish or stuck fermentations (Guillaume et al., 2007).

Temperature. Also plays a key role, as seen in section 8.3.

pH effect. The pH influences the yeast activity. The internal pH of yeasts varies between 5.5-5.7 and they grow better in a slightly acidic environment (Narendranath and Power, 2005). The typical pH range in oenology goes from 2.8 to 4.2 (Heard and Fleet, 1988). Yeast metabolism changes considerably outside this range and could lead to a stuck AF. For example, at a pH of 2.9-3.2, compounds such as acetic, tartaric, succinic, and malic acids present in the growth medium may enter the cells in the undissociated form, because their corresponding pKa are higher than the external pH. This causes the acidification of the cytosol and can stuck fermentation. Also, this cytosol acidified in synergy with ethanol inhibits the AF by decreasing the pH of the internal cell, enolase activity, and the fermentation rate (Torija et al., 2021).

Low pH levels can prolong the yeast lag phase at the beginning of AF. This reduces biomass accumulation, changes the sugar consumption rate, increases the accumulation of organic acids (mainly acetic, succinic, and L-lactic acid) and glycerol, and decreases the final content of ethanol (Liu et al., 2015; Ribéreau-Gayon et al., 2006). At high pH levels such as 3.7-4.2, the antiseptic effect of SO_2 is reduced and could increase spoilage microorganisms such as bacteria and can affect the AF in several stages. For example, LAB affects mainly in ageing or storage (Mas et al., 2021; Torija et al., 2021).

pH modifies the solubility and the chemical stability of nutrients. These changes can make the compounds less accessible to the yeast, causing nutrient depletion.

Oxygen effect. Yeast growth and survival can be affected by lack of oxygen. The anaerobiosis condition inhibits the synthesis of essential cellular compounds like sterols and unsaturated fatty acids, which require molecular oxygen (Torija et al., 2021). Additionally, the anaerobic conditions compromise the maintenance of cellular redox potential and essential mitochondrial functions (reviewed by Merico et al., 2009).

Nutritional deficiency. Deficiencies in nutrients such as sugar, nitrogen, growth factors (vitamins, nucleotides and nucleosides, purines, and pyrimidines), and other molecules considered as survival factors (long chain saturated and unsaturated fatty acids and ergosterol) could lead to stuck fermentation (Bisson, 1999; Torija et al., 2021). A nitrogen deficiency can lead to stuck fermentation due it is necessary to produce the biomass to have a good fermentation rate and complete the AF (Bisson, 1999; Mas et al., 2022). Nitrogen sources in must are present in the form of polypeptides (25-40%), amino acids (25-30%), proteins (5-10%), and ammonium cation (NH_4^+) (3-10%) (Ribéreau-Gayon et al., 2006). Yeast uses mainly NH_4^+, amino acids, and small peptides as nitrogen sources (Torija et al., 2021). The combined availability of these sources is referred as yeast-assimilable nitrogen (YAN) (Bell and Henschke, 2005; Joshi and Ray, 2021). The ammonium NH_4^+ is easily assimilated and can satisfy the yeast nitrogen needs for the synthesis of amino acids.

Nitrogen levels in musts vary between 100 and 1000 mg/L (Torija et al., 2021). However, these values change according to the vineyard culture practices and climatological conditions and grape variety and maturity level (Ribéreau-Gayon et al., 2006). For instance, different nitrogen concentrations have also been found

in individual plots within the same vineyard, i.e., 25-45 mg/L in vines with less vegetative growth and 152-294 mg/L in grapes from more robust vines (Ribéreau-Gayon et al., 2006). Naumov et al. (2000) have reported nitrogen levels of 46-354 mg/L in red musts and 36-270 mg/L in white musts. Nitrogen is often deficient in botrytized musts and has been found that must with less than 100 mg/L of nitrogen led to stuck fermentations (Lleixà et al., 2016). Consequently, nitrogen supplementation is a common practice in winemaking, even without any previous determination of the quantity of nitrogen present in the must.

Excess of nitrogen causes microbiological instability and the formation of toxic substances such as ethyl carbamate and biogenic amines (Torija et al., 2021). The quantity of YAN required for yeasts depends on the quantity of initial sugar in musts, then higher sugar concentrations may result in increased requirements of YAN in Sce (Childs et al., 2015). Nonetheless, a good fermentation performance should have a minimum value of 140 mg/L with a sugar content of 200 g/L in must to complete the AF (Martínez-Moreno et al., 2012). Additional supplementation of nitrogen is preferred at the exponential phase of yeast growth (Beltran et al., 2005; Bely et al., 1990; Lleixà et al., 2016).

Nitrogen requirements also depend on the different wine yeast species (Albergaria and Arneborg, 2016; Kemsawasd et al., 2015). Although, Sce synthesises amino acids and does not require an external supply; however, their addition stimulates growth yeasts (Torija et al., 2021).

Toxic metabolites and oenological practices. Metabolic by-products such as medium chain fatty acids from C6 to C12 are produced by yeasts under hypoxic conditions (Taylor and Kirsop, 1977). High concentrations of them are being

related to stuck fermentations because they are toxic to yeasts (Torija et al., 2021). The toxicity increases when combined with ethanol, making them harmful by themselves (Lafon-Lafourcade et al., 1984).

Excessive clarification. In white must, excessive clarification leads to the removal of the solids and some molecules such as unsaturated fatty acids and sterols that are survival factors for yeasts.

Pesticides and additives. Fungicides such as triazole used in vineyard management can change the composition of sterols and fatty acids altering the integrity of yeast membrane (Doignon and Rozes, 1992). Also, excess of additives such as SO_2 can be toxic for yeasts and lead to stuck fermentation (Ribéreau-Gayon et al., 2006).

10. Consumer preferences

Wine is an important food product that contributes to the global economy, supporting industries like agriculture, hospitality, and international trade. Winemaking practices have evolved according to industry modernisation, changing consumer preferences for newer styles and environmentally sustainable products, international competition within the wine market, demand for innovative fermentation technology, and mitigation of climate change (Bisson et al., 2002; Pretorius and Høj, 2005).

The growing consumer demand for environmentally sustainable products also led winegrowers to adopt *low-input* practices (Baiano, 2021; Carrau et al., 2020). The *low-input* concept surged in the 1980's as a reaction to the detrimental effects of

conventional agriculture. This agriculture is characterised by the excessive use of fertilizers and chemicals in food, affecting human health and the environment (Schaller, 1993). Several studies have suggested that implementing *low-input* strategies can enhance the authenticity of the wine linked to the *terroir* effect, thereby contributing to the wine quality (Carrau et al., 2020). However, consumers and experts still doubt to sensory attributes and intrinsic quality of wines produced using *low-input* strategies (Parga-Dans et al., 2023).

Despite lack of definition and regulation, natural wines are defined by the *Association des Vins Naturels* (AVNas) as wines produced from organic or biodynamic grapes without oenological additives in the cellar and with minimal intervention at all stages of winemaking (Ballester et al., 2024; Sáenz-Navajas et al., 2024).

Ballester et al. (2024) highlighted consumer perceptions of natural wines compared to wines from conventional practices. In their study, eighteen winemakers from Spain (La Rioja) and seventeen from France (Burgundy) evaluated different wines from different Spain origins (Figure 12). Their results are illustrated in the principal component analysis (PCA) plot. On the left side are placed the positive characteristics: very intense aroma, floral and berry, etc. Conventional wines comprise 2/3 of this group placed on the left side, while only 1/3 of natural wines are in this pace. The right side of the plot is dominated by six samples characterised by low-quality ratings and some negative descriptors such as “reduction”, “acetic acid” or “faulty”, etc. Natural wine samples in the proportion of 5/6 are on the right side of the plot.

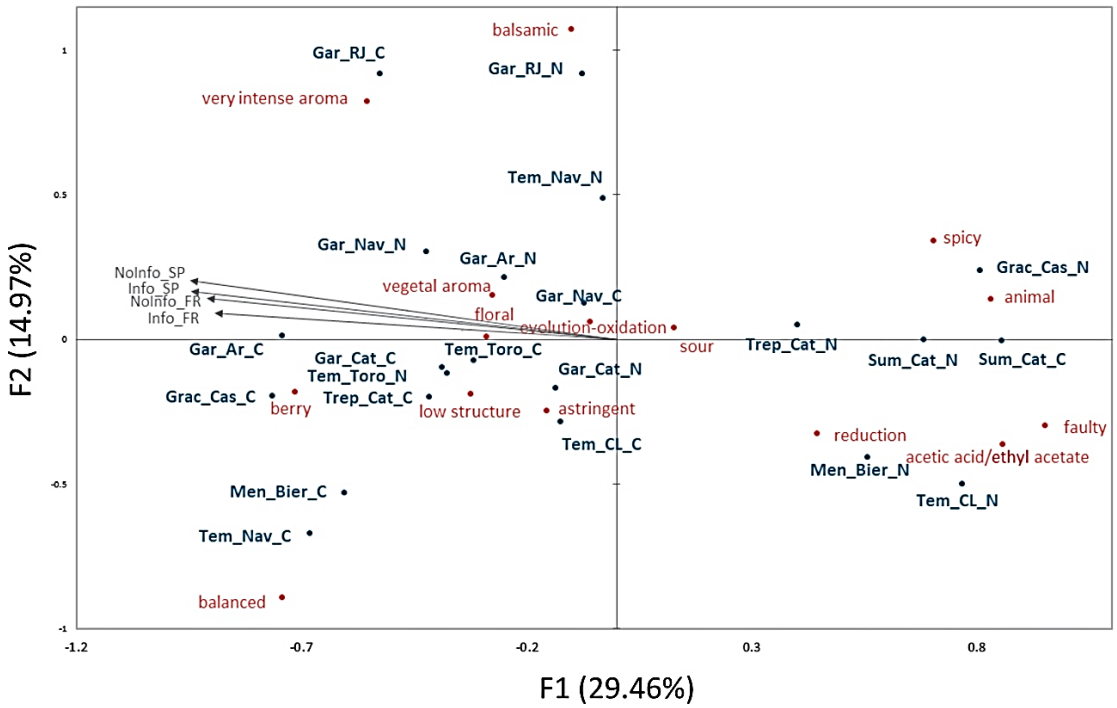

Figure 12. Principal component analysis (PCA) carried out on the citation frequencies of the descriptors generated during the sorting task. The mean quality scores for tasting wines from two types of cultures: conventional (C) and natural (N) on different origin from Spain: Ar, Aragon; Bier, Bierzo; Cas, Castilla-La Mancha; CL, Castilla y Leon; Cat, Cataluña; Nav, Navarra; RJ, Rioja; Toro,Toro;) using different grape varieties: Gar, Garnacha; Grac, Graciano; Men, Mencía; Temp, Tempranillo; Trep, Trepat (Ballester et al., 2024).

Another study carried out by Stanco et al. (2020) showed consumer preferences for wines with several attributes: such as tradition, sustainability, innovation, geographical indications, grape variety, sustainable certification, vintage, and price. These attributes emerged as the most significant factors influencing wine purchases among 419 regular wine consumers in Italy.

Today, unconventional practices are gaining attention as viable methods for producing *low-input* wines while maintaining typicity and promoting sustainability. These wines are perceived by consumers as premium products that align with their environmental consciousness, promising financial returns to wineries. Table 5 represents an analysis of Strengths, Weaknesses, Opportunities, and Threats

(SWOT) of wines produced by conventional or natural practices (Ballester et al., 2024).

Table 5. SWOT (Strengths, Weaknesses, Opportunities, Threats) analysis of wines produced by conventional or natural methods (organic, biodynamic, and *low-input* practices).

	Conventional wines	Natural wines
STRENGHTS	• Reproducibility of wine profiles (Fleet and Heard, 1993; Mas et al., 2016) • Higher quality in tasting sessions (Ballester et al., 2024) • Easily accessible in markets • Homogeneity in chemical composition, sensory characteristics, and typicality (Maioli et al., 2021) • Wide price range (Jorge et al., 2020) • Well-established brands sold worldwide (Jorge et al., 2020)	• Perception of naturalness, artisanal, and value healthiness (Sáenz-Navajas et al., 2024; Vecchio et al., 2021) • Serving as social identity (Urdapilleta et al., 2021) • Reduction of the use of chemical substances (Baiano, 2021) • Environmental consciousness and sustainability (Fabbrizzi et al., 2021) • Ecological and biodiversity conservation (Sáenz-Navajas et al., 2024) • More complex and authentic (Fabbrizzi et al., 2021)
WEAKNESSES	• Perception of less healthy and “artificial” (Mas et al., 2016; Sáenz-Navajas et al., 2024) • Less colour intensity and total polyphenol index (Maioli et al., 2021) • Negative environmental impact (Santos et al., 2020) • Uniformization of sensory profiles (Fleet and Heard, 1993) • Less yeast diversity (Bagheri et al., 2015)	• Uncertain outputs in quality attributes (Ballester et al., 2024) • Higher price (European Commission, 2014) • Less available in markets (Fotopoulos et al., 2003) • Perceived to be of lower quality in tasting sessions (Ballester et al., 2024) • Sensory defaults (Sáenz-Navajas et al., 2024)

OPPORTUNITIES	• Implementation of methods environmentally friendly (Maioli et al., 2021) • Market expansion • Technology integration • Consumers are not able to discriminate between organic and conventional wines (Pagliarini et al., 2013)	• Association with ethical values (Capitello and Sirieix, 2019) • Recovery of the identity and character of the wine (Fabbrizzi et al., 2021) • Necessity of sustainability certifications to gain credibility (Parga-Dans et al., 2023) • Consumers are willing to pay a premium price (Galati et al., 2019) • Awareness between sensory properties and potential faults (Ballester et al., 2024) • Sustainability is trendy (Fabbrizzi et al., 2021)
THREATS	• Growing consumer awareness (Ballester et al., 2024) • Adaptation to negative impacts of climate change on grapes yields (Santos et al., 2020) • Increasing trend for consuming sustainable products such as natural wines (Baiano, 2021) • Regulation of additives uses (Cravero, 2019)	• Lack of legislation and regulation (Ballester et al., 2024; Galati et al., 2019) • High-cost certifications (Scozzafava et al., 2021) • Confusion consumers between organic, biodynamic, and natural wine categories (Alonso González et al., 2022) • Differences in cultural perceptions about the term "naturalness" (Sáenz-Navajas et al., 2024) • Doubt about the quality and taste (Ballester et al., 2024) • Natural winemakers and associations themselves oppose the certification (Alonso González et al., 2022)

Further investigation is required to ensure these *low-input* practices contribute to microbial diversity preservation, microbiological control, and organoleptic quality of the final product. Additionally, these products must be safe and avoid undesirable compounds such as biogenic amines, ethyl carbamate precursors, or ochratoxin A in wine (Comuzzo et al., 2013; Ribéreau-Gayon et al., 2006).

Among these emerging low intervention practices is the PdC method, known for its traditional approach and cost-effectiveness to cover these demands. In this work, the use of PdC could allow to reduce the use of commercial strains and the usual dosis of SO_2 with microbiological purposes, thereby implementing low-cost strategies for wineries.

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KATHERINE BEDOYA ALVIRA

Hypothesis and Objectives

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The hyphothesis of this thesis is that employing *pied de cuve* (PdC) under various conditions can effectively maintain microbial autochthonous diversity while ensuring microbiological control throughout alcoholic fermentation (AF), ultimately resulting in the production of high-quality wines.

General Objective

As a consequence, this study aims to evaluate the microbiological and organoleptic impact in white must vinification when inoculated with different PdC.

Additionally, it proposes modifications to prepare PdC easily in the cellar.

This main goal has three specific objectives divided into three chapters:

- ***Objective 1:*** Evaluation of different stressors applied to PdC and their impact on AF using different matrices (**Chapter I**). To achieve this objective, several yeast communities mimicking natural grape microbiota were tested in the laboratory to test their survival under different oenological stress conditions. The results were tested with synthetic and reconstituted natural concentrated must. Finally, the most promising oenological conditions were tested in a cellar experiment using fresh natural must. In this way, the best conditions for generating a PdC that could maintain microbial diversity were established and the resulting microbial communities were analyzed.

- ***Objective 2:*** To investigate the chemical and sensory effects of the PdC technique on wine production using Muscat of Alexandria and Sauvignon Blanc varieties. Wines inoculated with different PdC with the addition of

different stressors such as sulphur (SO_2), ethanol, and temperatures were evaluated by HPLC and sensory tasting in harvests 2022 and 2023, in two countries Spain and Chile. The best PdC formula was selected as the harvests progressed based on the best chemical with special emphasis on the organoleptic characteristics until obtaining predictable results with desirable fermentation characteristics (**Chapter II**).

- ***Objective 3:*** Evaluation of the level of grape maturity on the microbial diversity and effectivity of a modified PdC (SO_2, ethanol, and temperatures) for AF (**Chapter III**). This includes analyzing fermentation rates, grape microbiome diversity, *Saccharomyces cerevisiae* (Sce) strain diversity during AF, chemical composition of the wines using HPLC, and sensory attributes. Additionally, Sce population diversity using Interdelta-PCR profiles will be assessed and characterize key strains with high abundance during AF for potential future use in winemaking.

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Results

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Chapter I

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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.

Keywords

Pied de cuve; yeast diversity; temperature; ethanol; sulphur dioxide; yeast community structure.

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 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., 2011a). 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 ºC, 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 ºC to preserve aroma compounds in wine or to favour the growth or 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 DWYs has produced concern due to uniformity in the sensorial profile of wines by the inoculation of one single strain (Belda et al., 2017c). 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; Li et al., 2012; Morgan et al., 2019; Moschetti et al., 2016).

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 un-inoculated PdC of Muscat of Alexandria must.

2. Material and Methods

2.1. Laboratory experimentation

Laboratory fermentations were prepared either in the synthetic must (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 (Figure 1. A): i) preparation of a yeast consortium, inoculated at a concentration of 2 x 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., 2015a). 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 ºC. 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 x 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 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 (Figure 1. A).

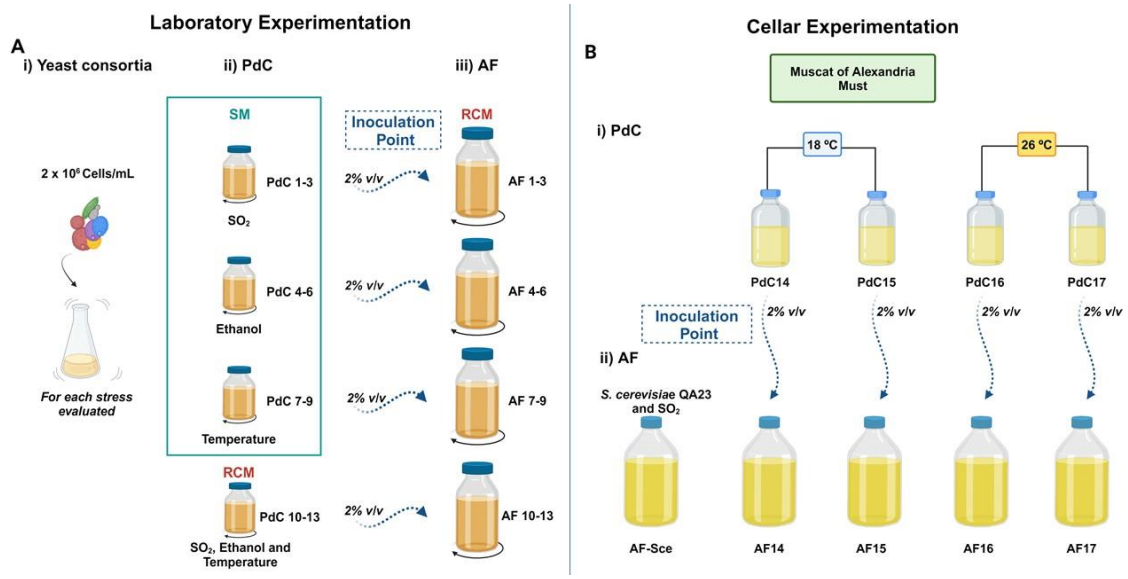

Figure 1. Diagram illustrating the process followed to prepare the different *pied de cuve* (PdC) and alcoholic fermentation (AF) at laboratory experimentation (A) and cellar experimentation (B). Every condition was tested in triplicate. Figure created with BioRender.com.

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 ºC. 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 ºC 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 (Figure 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 ºC (PdC10 and PdC11, respectively), and at 26 ºC (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 ºC 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 ºC and 120 rpm (Figure 1. A). An additional AF inoculated with 2 x 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 ºC. 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 (Figure 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 (Figure 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 ºC (PdC14 and PdC15, respectively), and 26 ºC (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 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 ºC (Figure 1. B).

Additionally, a fresh batch of clarified must supplemented with 40 mg/L of SO_2 was inoculated with 2 x 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 ºC 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 ºC, 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 in 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 ºC 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 (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 (Figure 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 (Figure 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 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 (Figure 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 (Figure 2. A).

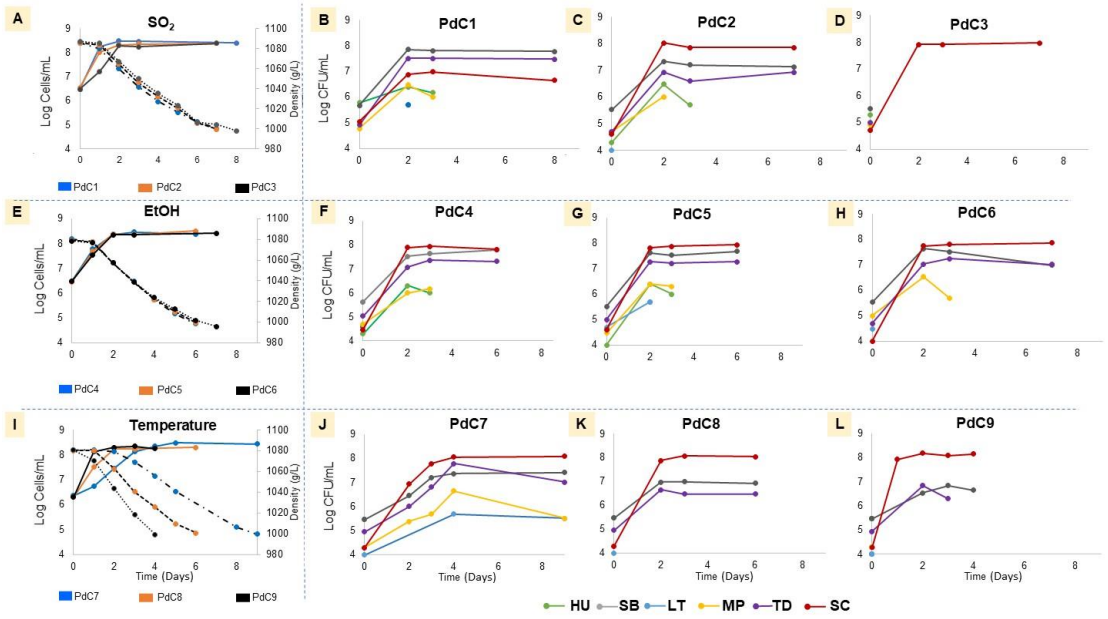

Figure 2. Impact of sulphur dioxide (SO_2, A), ethanol (EtOH, E) and temperature (I) on fermentation kinetics and total yeast population during preparation of PdCs using synthetic must (SM). Solid lines stand for the number of cells quantified at the microscope while dashed lines stand for the density (g/L). PdC1, PdC2, PdC3 were fermented at 22 ºC with SO_2 concentrations of 0, 40, and 80 mg/L, respectively, added as sodium metabisulphite. PdC4, PdC5, and PdC6 were fermented at 22 ºC with 40 mg/L SO_2 and 0, 0.5, and 1% (v/v) of ethanol. PdC7, PdC8, and PdC9 had 40 mg/L SO_2 h and 1% (v/v) of ethanol and were fermented at 16, 22, and 28 ºC, 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*.

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 (Figure 2 B and Figure 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 (Figure 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 (Figure 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 ($p < 0.05$) (Figure 2. E).

As observed by CFU counts on WLN medium of PdC5 and PdC6 (Figure 2. G and Figure 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 (Figure 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 (Figure 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 (Figure S1) and compared the results to the Neubauer and CFU counting. Although the total yeast number assessed by qPCR (Figure S1) was similar to Neubauer counting (Figure 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 Figure 2. I. Thus, the PdC7, PdC8 and PdC9 finished on the 4th, 6th, and 9th days, respectively (Figure 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 x 10^8 cells/mL) and PdC8 and PdC9 achieved that maximum during the second day (Figure 2. H and I). The abundance of non-Sce organisms was found to be impaired at higher temperatures, as shown in Figure 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 (Figure 2. K and Figure 2. L). The Sce strain number, on the other hand, remained unaffected by any of the tested temperatures (Figure 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 ºC and 26 ºC, 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 (Figure 1. A).

Figure 3 shows the PdCs using RCM without SO_2 or ethanol supplementation and fermented either at 18 ºC (PdC10) or 26 ºC (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 ºC (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 (Figure 3. A).

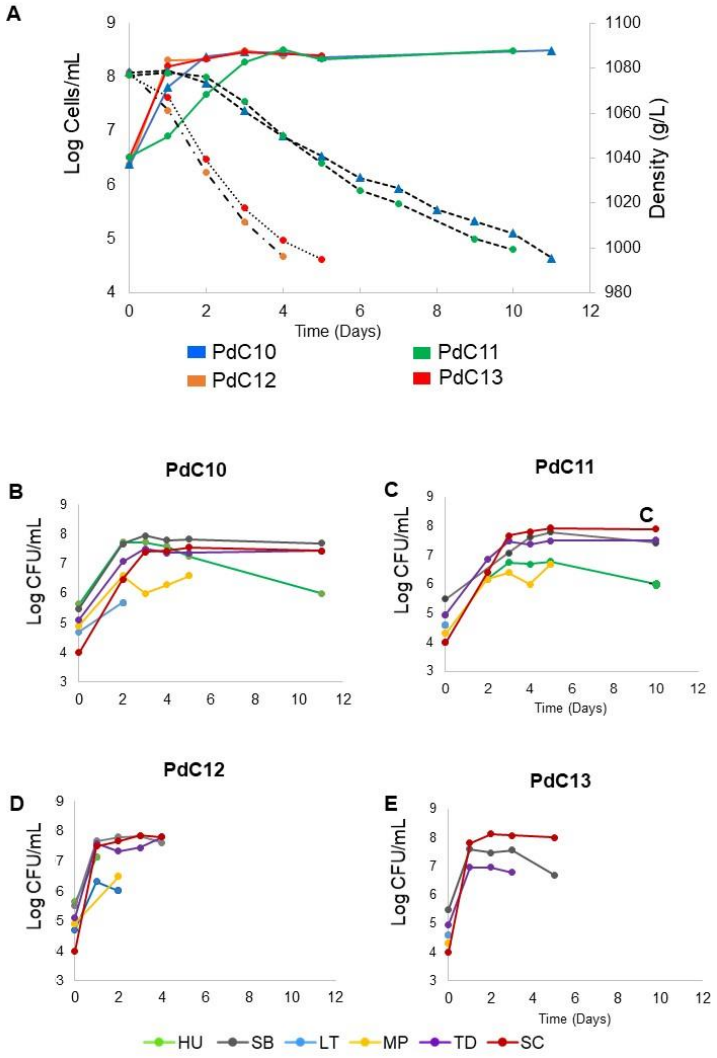

Figure 3. (A) Combined effect of sulphur dioxide (SO_2), ethanol and temperature on PdC10-13 using RCM medium. The PdC10 and PdC12 were carried out spontaneously without SO_2 or ethanol at 18 and 26 ºC, respectively. PdC11 and PdC13 were supplemented with 40 mg/L of SO_2 and 1% (v/v) ethanol. The yeast 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 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 ºC, 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 (Figure 3. B-E). In PdC10, the non-Sce strains were favoured over the Sce strain (Figure 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 ºC. The Sce strain maximum population was favoured by supplementation with SO_2 and ethanol at 18 ºC (Figure 3. C), but its growth rate was slower than at 26 ºC (Figure 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 (Figure 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 (Figure 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 ºC 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 h (Supplementary Figure S2).

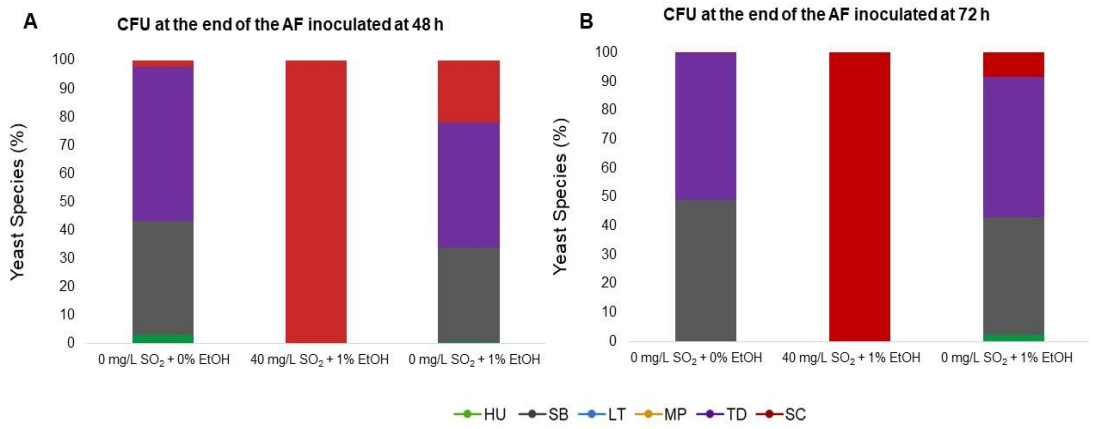

Figure 4. Impact of inoculation time of PdC on the yeast population measured as CFU/mL on WLN medium at the end of alcoholic fermentation (AF) using RCM. The AFs were inoculated at 48 h (A) or 72 h (B) with PdCs prepared with or without the influence of sulphur dioxide (SO_2) and ethanol (EtOH). Every 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.*

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 (Figure 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 ºC (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 (Figure 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 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). These AFs were carried out without additional treatment at 22 ºC. 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 (Figure 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 AF3 (Figure 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 Figure 5. B).

Nevertheless, AF5 and AF6 reached the maximum yeast population with a delay of 24 h with respect AF4 (as shown in Figure 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 (Figure 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 on the kinetics of AF10, AF11, AF12 and AF13 (Figure 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 (Figure 5. D).

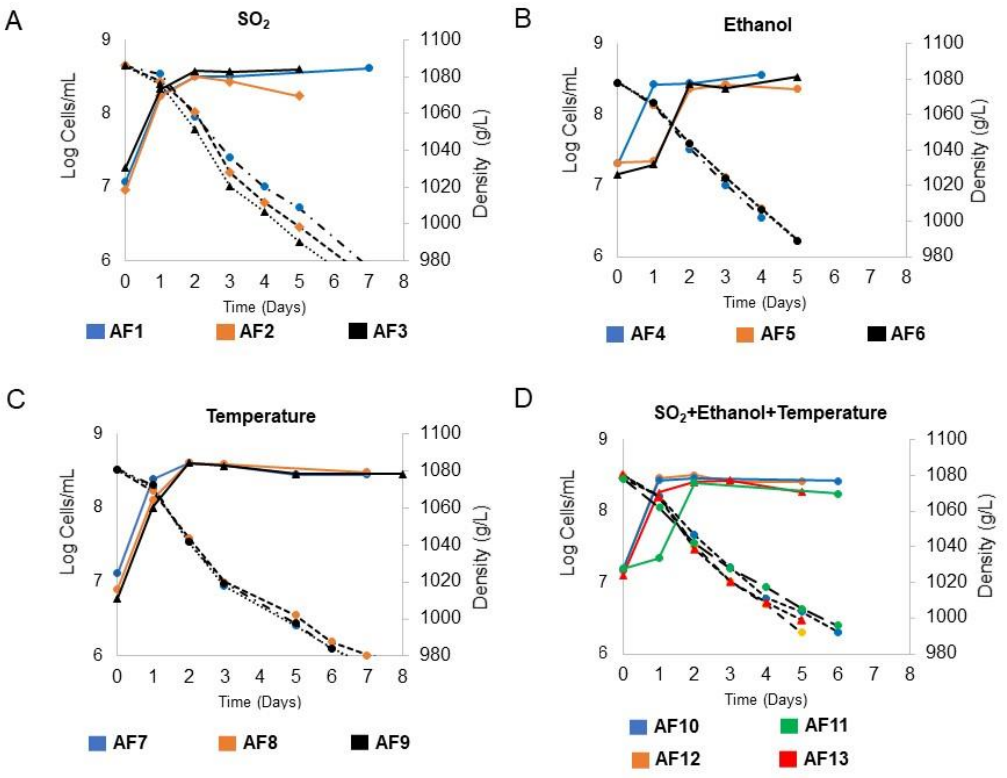

Figure 5. Alcoholic fermentations (AFs) inoculated with different PdCs using reconstituted concentrated must (RCM). Yeast population dynamics as total cells/mL by microscopy count (solid lines) and fermentation kinetics as the density of sugar in g/L (dashed lines). AF1-3 were inoculated with PdC1-3 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.

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 ºC (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 (Figure 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 ºC and the 4th day at 18 ºC (Figure 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 ºC 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 Figure 3. A).

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

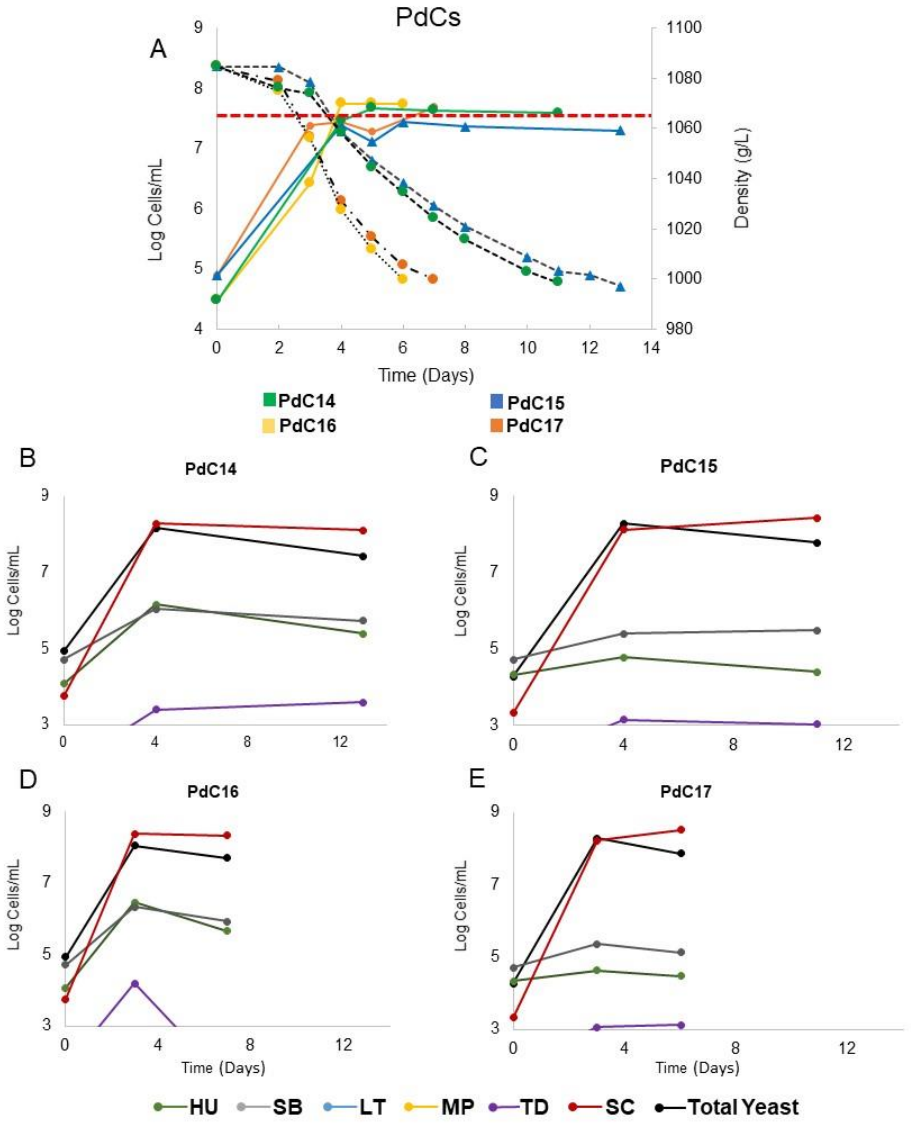

Figure 6. (A) Sulphur dioxide (SO_2), temperature, and ethanol effect on total yeast population and fermentation kinetic during PdC14-17 using natural must of Muscat of Alexandria during cellar experimentation. Solid lines stand for the number of yeasts quantified under the microscope while dashed lines 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 ºC, 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*.

3.5.2. Impact of PdC inoculation on AFs at the cellar

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 assess 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 (Figure 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 (Figure 6. B and D) had a higher number of non-Sce cells than the ones supplemented with SO_2 and ethanol at the IP (Figure 6. C and E).The fermentations inoculated with the different PdC (AF14-17) were conducted in natural must without SO_2 correction at 18 ºC (Figure 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 (Figure 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 (Figure 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.

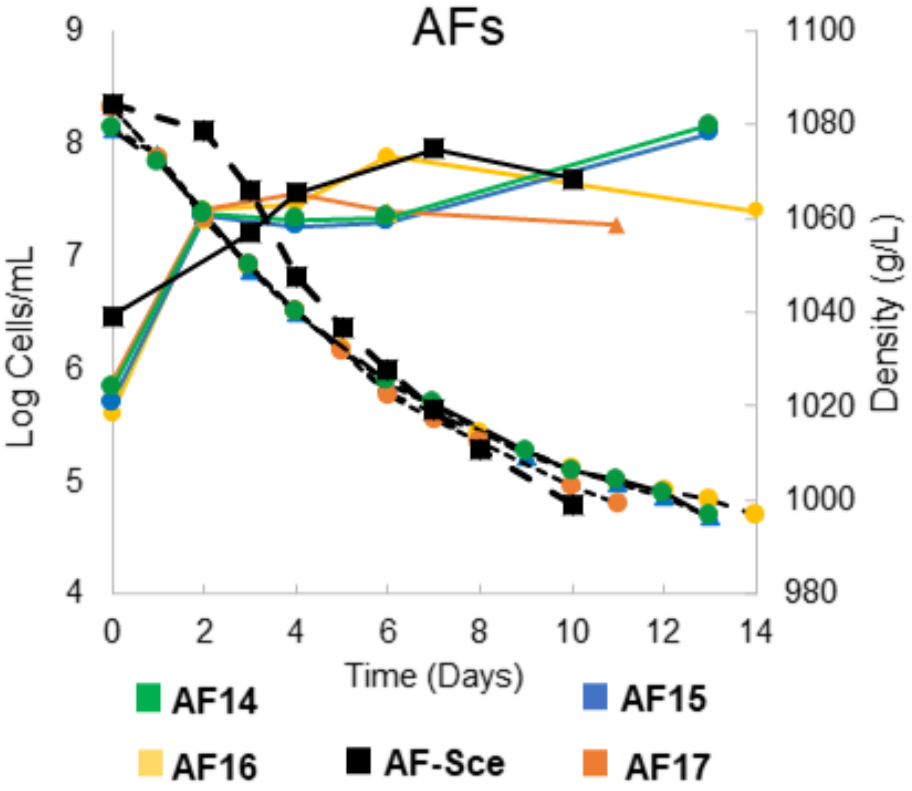

Figure 7. Effect of PdC14-17 inoculation on total yeast population and fermentation kinetic during the corresponding alcoholic fermentation (AF14-17) using natural must Muscat of Alexandria during cellar experimentation. The inoculated control with Sce QA23 (AF-Sce) is also represented. Solid lines stand 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.

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. bacillaris	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. bacillaris	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. bacillaris	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.

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 ºC and 26 ºC) 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 (Figure 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 ºC (PdC16 and PdC17), profile 7 at 18 ºC (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 inoculated in the AF-Sce was the most abundant detected during all stages of this fermentation (Figure 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.

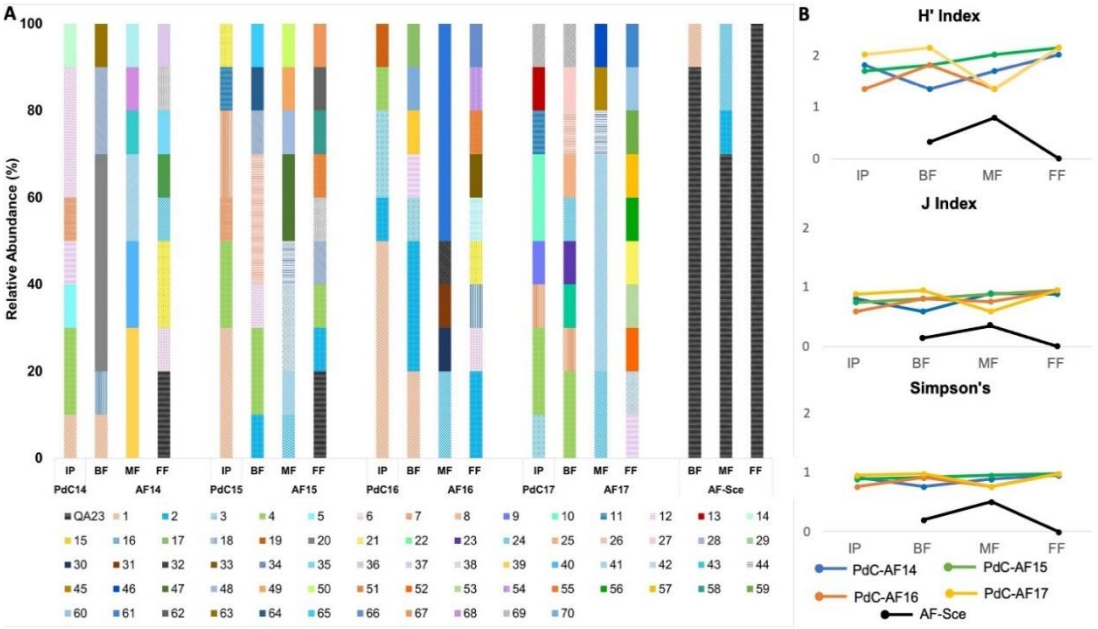

Figure 8. (A) Relative abundance of Interdelta profiles of *Saccharomyces* colonies isolated from YPD plates medium. IP stands for the PdCs sampled at the inoculation point whereas BF, MF, and FF correspond to the beginning, middle, and final fermentation stages of the AFs inoculated with different PdCs.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

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 (Figure 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 (Figure 8. B).

Finally, different and some identical Interdelta profiles of Sce strains were grouped at 90% similarity by a dendrogram into 26 clusters (Supplementary Figure 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., 2003b) 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., 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 uninoculated 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. bacillaris* exhibits favourable 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. bacillaris* 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 ºC. It is known that Sce can grow at a wide range of temperatures, even higher than 32 ºC. 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 ºC and 26-28ºC (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 indices 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 ºC 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) reported that a higher diversity of Sce was observed 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.

Acknowledgments

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

Author contributions Methodology, formal analysis, writing the original draft, review, and editing: K.B. and L.B. Conceptualization, data curation, formal analysis; funding acquisition, project administration, resources, supervision validation, writing, review, and editing different drafts: N.R., A.M., and M.C.P.

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Supplementary data

Table S1. Parameters of the standard curves derived from qPCR (quantitative-polymerase chain reaction) of 10-fold serial DNA dilutions from the yeasts *H. uvarum*, *S. bacillaris*, *L. thermotolerans*, *M. pulcherrima*, *T. delbrueckii* and *S. cerevisiae*. Y-intersections, correlation coefficients (R^2), efficiencies (%), and standard errors (E) are shown.

Yeast	Slope	Intersection	R^2	Efficiency (%)	Error
Total Yeast	-3.291	36.328	0.992	101.311	0.073
H. uvarum	-3.575	37.996	0.996	90.440	0.058
S. bacillaris	-3.447	41.884	0.998	95.040	0.041
L. thermotolerans	-3.126	34.793	0.995	108.872	0.062
M. pulcherrima	-3.145	39.75	0.097	107.972	0.057
T. delbrueckii	-3.291	36.194	0.996	101.301	0.055
S. cerevisiae	-3.389	38.053	0.999	97.295	0.027

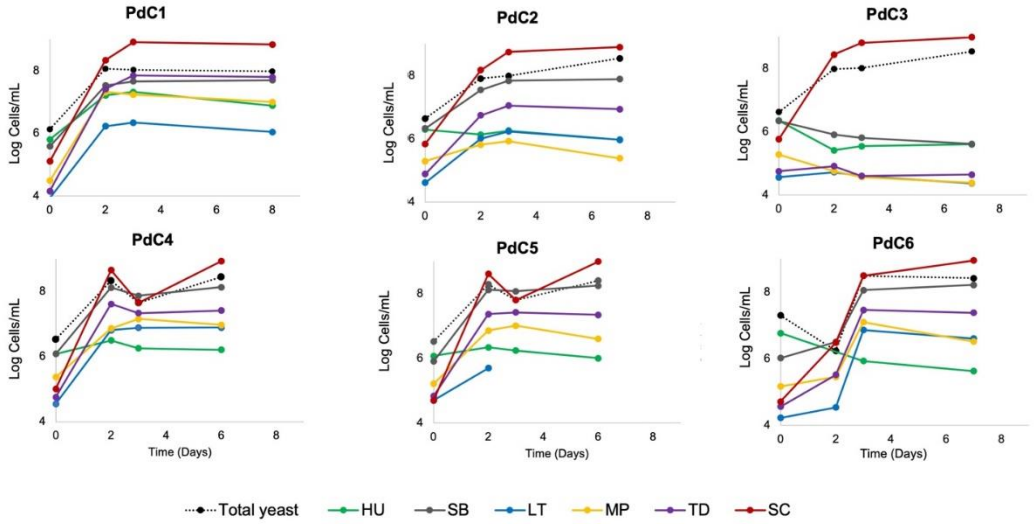

Figure S1: SO_2 and ethanol effect on the yeast population during the *pied de cuve* (PdC) using synthetic must (SM) analysed by qPCR. PdC1, PdC2, and PdC3 were performed with 0, 40 and 80 mg/L SO_2, respectively. PdC4, PdC5, and PdC6 were carried out with 40 mg/L of SO_2 and 0, 0.5, or 1% (v/v) of ethanol. All the PdCs were incubated at 22 ºC.

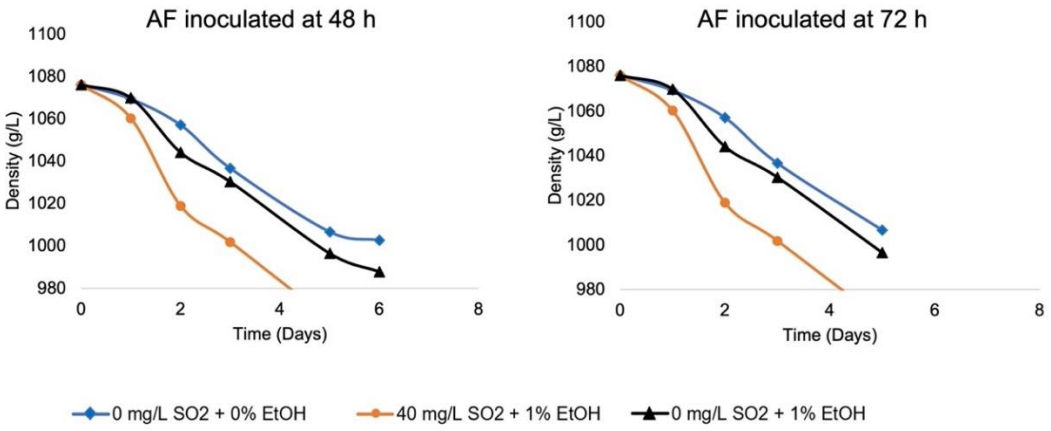

Figure S2: Evaluation of the inoculation time of *pied de cuve* (PdC) into new reconstiotuited concentrated must (RCM) to perform AF. The effect of the inoculation of PdC at 48 and 72 h over the fermentation kinetics is represented by must density (g/L). The PdCs were prepared without SO_2 or ethanol (blue line), with 1% (v/v) of ethanol (black line), and with 1% (v/v) of ethanol and 40 mg/L of SO_2 (orange line). Both PdCs and AFs were carried out in RCM at 22 ºC.

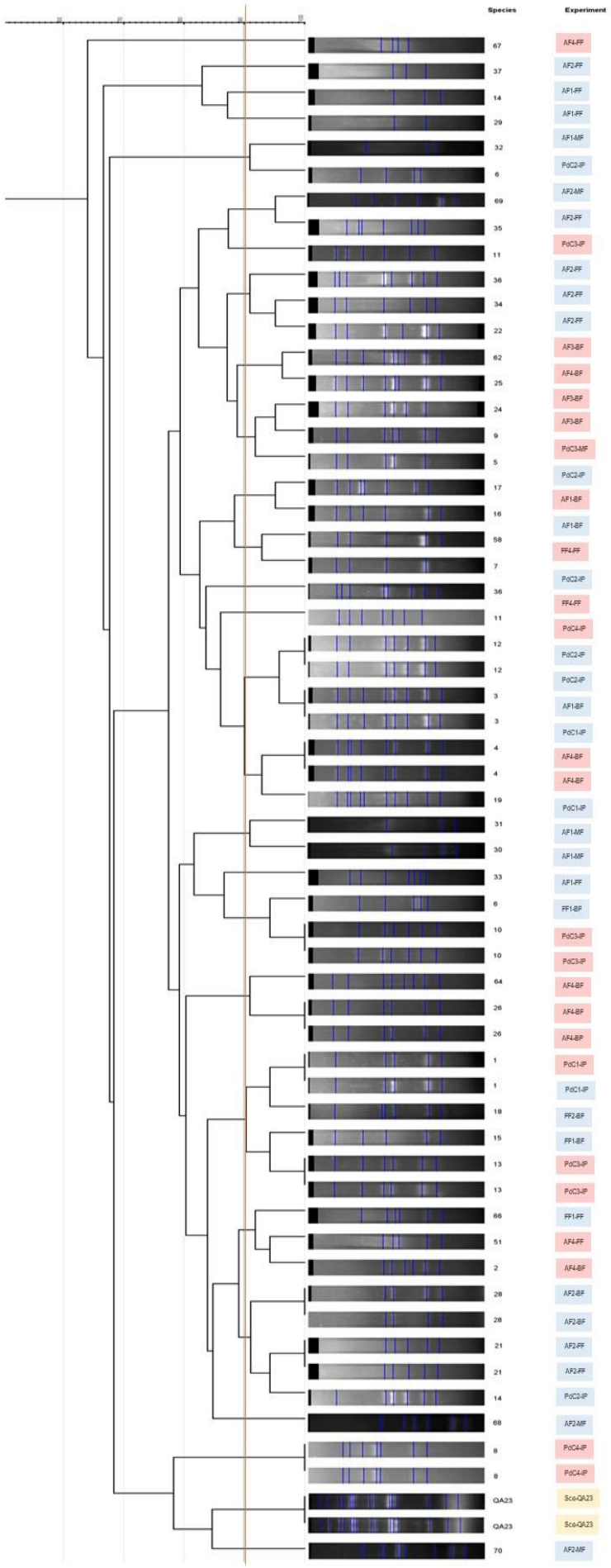

Figura S3. Dendrogram showing the clustering of the different 70 Interdelta profiles of *Saccharomyces cerevisiae* strains isolated during the present study from IP (inoculation point) of the PdCs and the BF, MF, and FF of the corresponding alcoholic fermentation (AFs). The dendrogram is based on the similarity matrix using DICE coefficients and it was constructed with the unweighted pair group method with arithmetic mean (UPGMA). Several identical profiles from different agarose gels and fermentation stages (profiles 1, 3, 4, 10, 12, 13, 21, 26, 28, QA23) were included to test the reproducibility and reliability of the clustering. The red vertical line indicates the clustering when the similarity coefficient was ≥ 90%. The experiments were grouped into blue or red if the PdCs had been

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Chapter II

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The Impact of the Inoculation of Different Pied de Cuve on the Chemical and Organoleptic Profiles of Wines

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Microorganisms

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Microbiological and organoleptic influence of different stresses during the pied de cuve applied to white wine vinification
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Abstract: Controlling the microorganisms involved in alcoholic fermentation during wine production can be achieved by adding a small quantity of spontaneously fermenting must to freshly crushed grapes, a technique known as *pied de cuve* (PdC). This method not only serves as an inoculation starter but also enhances the *microbial footprint* unique to each wine region. Recent studies have confirmed that wines inoculated with PdC exhibit efficient fermentation kinetics comparable to those inoculated with commercial strains of *Saccharomyces cerevisiae*. However, further research is required to draw robust conclusions about the chemical and sensory impacts of PdC-inoculated wines. In this study, we examined the chemical and sensory effects of the PdC technique across three different harvests: Muscat of Alexandria (Spain, harvests 2022 and 2023) and Sauvignon Blanc (Chile, harvest 2023). Each PdC was prepared using various stressors (sulphur dioxide, ethanol, and temperature). Our findings revealed that wines produced with PdC exhibited similar fermentation kinetics and sensory profiles to those inoculated with commercial strains. Notably, PdC fermentations resulted in lower concentrations of acetic acid compared to both the commercial strain and spontaneous fermentations. The sensory analysis indicated that PdC wines significantly differed from those made with commercial strains, with PdC wines displaying more pronounced tropical notes. These results suggest that the PdC technique, particularly when using specific stressors, can maintain desirable fermentation characteristics while enhancing certain sensory attributes, offering a viable alternative to traditional inoculation methods.

Keywords: *Pied de cuve*; alcoholic fermentation; wine quality; sensory analysis; HPLC analysis

1. Introduction

The use of commercial yeast, such as the dry wine yeast (DWY), is a widely adopted inoculation strategy in wineries (Ciani et al., 2016a; Pinu et al., 2023). Its popularity stems from its ability to ensure successful alcoholic fermentation (AF) and achieve desired organoleptic attributes in the final wine, leading to uniformized aromatic profiles (Molinet and Cubillos, 2020). However, DWY usage can be costly, particularly for small and medium-sized cellars, due to the need to purchase additional compounds like nitrogen for adding during the DWY preparation or adding directly into the must to enhance the fermentation performance (Ciani et al., 2016a; Díaz-Hellín et al., 2013; Kontkanen et al., 2004; Rodríguez-Porrata et al., 2008).

Consequently, some winemakers are exploring strategies to integrate native microbiota from the cellar environment into the fermentation process. By this, they are leveraging the adaptability of native yeast strains to specific environmental conditions to produce wines with unique sensory attributes (Belda et al., 2017c; Mas and Portillo, 2022). This approach aligns with the environmentally conscious practices of wineries moving towards sustainability and organic methods, responding to the consumer trends for natural and low-intervention wines that reflect the *terroir* concept (Lappa et al., 2020).

Several strategies exist to maintain microbial diversity in winemaking, including spontaneous fermentation (SF), the selection of autochthonous strains, or the *pied de cuve* (PdC) method (Lappa et al., 2020; Mas and Portillo, 2022). Spontaneous fermentation (SF) is a process carried out by the non-*Saccharomyces* (non-Sce) yeasts and *Saccharomyces* yeasts that are naturally

present in grapevine and winery environments (Varela and Borneman, 2017). Though SF allows you to conserve the natural microbial diversity, it can lead to off-flavour compounds and sluggish or stuck fermentations. Additionally, it usually results in slower fermentation kinetics compared to must inoculated with the DWY of *Saccharomyces cerevisiae* (Sce) strains (Mas and Portillo, 2022). Another strategy involves selecting yeasts from must or vineyard environments to propagate those with desirable oenological features and preserve and use them for future vintages (Pulcini et al., 2022). Traditionally, some cellars have employed the PdC (bottom of the deposit in French) method, which involves using a small volume of already fermenting must to initiate AF when added to fresh must (Börlin et al., 2020; Mas and Portillo, 2022). PdC can be derived mainly from two different strategies: in the first one, a single yeast culture (generally a laboratory-selected strain) is propagated in a suitable medium or must that will be later added as PdC to the main vat of must; the second can be derived from small vats already fermenting spontaneously, either in the cellar or the vineyard. These vats are analysed for organoleptic and physiochemical properties to select the PdC with the best characteristics and absence of off-flavours for inoculation into a new batch of must (Börlin et al., 2020; Mas and Portillo, 2022). While some wineries still use PdC, its impact on yeast populations and wine sensory attributes is not well studied. Some authors highlight the advantages of PdC for microbial control during AF and improved fermentation kinetics compared to SF (Álvarez-Barragán et al., 2023; Börlin et al., 2020). Nonetheless, debates continue regarding its effectiveness and contribution to wine complexity due to the limited number of studies. Existing research has not shown significant differences in global quality characteristics

nor disparities in general regarding chemical parameters like residual sugars, ethanol, and the acidity between the PdC, DWY, or SF (Álvarez-Barragán et al., 2023; Li et al., 2012). Only significant differences were found in the metabolic fingerprint or the volatile composition among the PdC, DWY, and SF (Álvarez-Barragán et al., 2023).

In a previous study conducted by our group (Bedoya et al., 2024a), we evaluated the impact on yeast populations and fermentation kinetics by using combinations of stressors (sulphur dioxide (SO_2), ethanol, and temperature) to create four different PdCs. These PdCs were then added to fresh Muscat of Alexandria must to conduct the AF. The PdCs initiated four separate AFs, which were compared to an AF inoculated with a DWY strain of *S. cerevisiae*. Our results showed that all the PdCs served as robust AF starters, leading to higher total yeast populations than fermentations inoculated with the DWY. Depending on the stressors used for PdC preparation, some AFs inoculated with PdC had fermentation kinetics comparable to those inoculated with DWY. Significant differences were observed in the abundance of non-Sce species, particularly at the initial fermentation stages of the AF. Furthermore, the Interdelta analysis of the isolated *S. cerevisiae* strains during AFs inoculated with the PdC and DWY indicated significantly higher diversity indices in those inoculated with the PdCs compared to those inoculated with the DWY.

In the present study, we hypothesize that PdCs created under stress conditions not only support successful AF and promote the selection of diverse fermentative strains but also potentially result in wines with unique organic compound profiles and favourable organoleptic characteristics. This study will

assess the chemical and sensory impact of wines inoculated with PdC across three harvest campaigns and compare them to those produced through DWY inoculation or SF. The three campaigns were as follows: (i) 2022 harvest using Muscat of Alexandria must in Spain; (ii) 2023 harvest using Sauvignon Blanc must in Chile; and (iii) 2023 harvest using Muscat of Alexandria in Spain. The physicochemical parameters of the wines produced under each treatment will be measured using high-performance liquid chromatography (HPLC), and the organoleptic evaluation will be assessed through a sensory analysis.

2. Materials and Methods

2.1. Experiment Setting and Sampling

Two of the experiments were conducted at the experimental cellar of the University Rovira I Virgili (Tarragona, Spain), using fresh must from the Muscat of Alexandria variety harvested in the 2022 (M2) and 2023 (M3) campaigns. The third experiment took place at the University of Chile's cellar (Santiago, Chile), employing fresh must of the Sauvignon Blanc variety harvested in 2023 (S3). The parameters of the musts for the alcoholic fermentation (AF) are described in Table 1.

All musts were settled according to the method described by Bedoya et al. (2024a) to prepare both the PdC and the must for AF.

Table 1. Main experimental parameters of musts during 2022 and 2023 campaigns.

Harvest	Variety	Density (g/L)	Sugar (g/L)	YAN (mg/L)	pH
M2	Muscat of Alexandria	1082.1	199.8	102.5	3.5
S3	Sauvignon Blanc	1080.0	188.0	98.0	3.3
M3	Muscat of Alexandria	1082.6	217.6	82.0	3.4

The kinetics of PdCs and AFs were measured daily by densitometry using an electronic densitometer (Densito 30PX Portable Density Meter; Mettler Toledo, Barcelona, Spain) until density was under 990 g/L. The total yeast population was monitored using a microscopy counting chamber, and the viable population was tracked by colony-forming unit (CFU) counts on YPD solid medium (1% yeast extract, 2% peptone, 2% dextrose, and 17 g/L Agar, all compounds come from Biogenetics, Milan, Italy); Lysine (LYS, Oxoid Ltd., Basingstoke, UK) and WLN (Difco Laboratories, Detroit, MI, USA).

2.2. Pied de Cuve

After settling for 24 h, 1 L of must was placed in 1.5 L bottles for each treatment. Some of these PdC were treated with SO_2 and ethanol (SE), and others were fermented without additives at two temperatures (Table 2).

We used the code M2-26 for a PdC from the 2022 Muscat of Alexandria harvest, fermented at 26 °C without additives. The code M2-26SE indicates a PdC from the same harvest and grape variety, fermented at 26 °C with SE additions. The rest of the samples were coded similarly (Table 2). Each PdC treatment was

monitored until the density dropped by 15-20 g/L and the microscopy showed approximately 1 × 10^8 cells/mL. At this point, triplicates of PdCs were combined and added to fresh must at 2% (v/v) to start AF.

Table 2. *Pied de cuve* (PdC) treatments and alcoholic fermentation (AF) of musts inoculated with different PdC. The M2 and M3 harvest correspond to Muscat of Alexandria must from vintages 2022 and 2023 at Tarragona. The harvest S3 corresponds to Sauvignon Blanc must collected at the 2023 campaign at Santiago de Chile.

Harvest	**Spontaneous Fermentation for PdC**		**AF at 18 °C and No Stress**	
	Code	***Parameters***	***Code***	***Inoculation modality***
M2	M2-26	26 °C and no stress	P-M2-26	Inoculated with M2-26
	M2-18	18 °C and no stress	P-M2-18	Inoculated with M2-18
	M2-26SE	26 °C with SO_2 and EtOH	P-M2-26SE	Inoculated with M2-26SE
	M2-18SE	18 °C with SO_2 and EtOH	P-M2-18SE	Inoculated with M2-18SE
		Null	C-M2	Control inoculated with Sce QA23
S3	S3-26SE	26 °C with SO_2 and EtOH	P-S3-26SE	Inoculated with S3-26SE
	S3-18SE	18 °C with SO_2 and EtOH	P-S3-18SE	Inoculated with S3-18SE
		Null	C-S3	Inoculated with Sce 1118
M3	M3-18SE	18 °C with SO_2 and EtOH	P-M3-18SE	Inoculated with M3-18SE
		Null	C-M3	Control inoculated with Sce QA23
		Null	SF-M3	Spontaneous fermentation

2.3. Alcoholic Fermentation

Once the grapes reached maturity with a probable alcohol content of 12% (v/v), approximately 60 Kg of grapes was harvested and processed to obtain the must. After 24 h of settling as described previously, 3 L of must was distributed into 5 L containers, and the AF was conducted in triplicate at 18 °C without agitation at the cellars. The different inoculation strategies of AFs are described in Table

2. One set of AFs was inoculated with 2% (v/v) of the PdCs prepared previously and labelled according to the conditions used for the corresponding PdC. The second set of AFs was inoculated with 2 x 10^6 cells/mL of rehydrated DWY *S. cerevisiae* and 40 mg/L of SO_2 to serve as control fermentations (labelled as C-). The DWY of *S. cerevisiae* used in the experiments was obtained from Lallemand Inc., Montreal, Canada, differed for the experimentation in Spain and Chile, reflecting the strains commonly used in each cellar. For the experiments M2 and M3 in Spain, the *S. cerevisiae bayanus* strain QA23 was used. During the Chilean campaign S3, the *S. cerevisiae* (ex-bayanus) strain 1118 was inoculated. Additionally, for the M3 harvest, we included an AF without any inoculation to conduct spontaneous fermentation (SF-M3).

2.3. Chemical Analysis

Samples for the chemical analysis were taken from must, at the beginning and end of each AF. Total sugars and YAN (ammonia and primary amino nitrogen) were quantified using the Y15 Bioanalyzer with the corresponding enzymatic kits (BioSystems S.A, Barcelona, Spain).

The main chemical parameters of the produced wines (glucose, fructose, ethanol, and glycerol) and acids (acetic and succinic acid) were quantified in an Agilent 1100 HPLC (Agilent Technologies, Waldbronn, Germany) following the procedure described by Bedoya et al. (2024a). Briefly, the samples were centrifugated at 13.000 rpm for 5 min, and the supernatant was filtered with 0.22 µm pore filters before injection (Agilent Technologies). The main chemical parameters of the wines (glucose, fructose, ethanol, and glycerol) and acids (acetic and succinic acid) were quantified in an Agilent 1100 HPLC (Agilent

Technologies, Germany). The HPLC had coupled a Hi-Plex H (300 mm × 7.7 mm) column inside a 1260 MCT (Infinity II Multicolumn Thermostat). The column conditions involved maintaining a temperature of 60 °C for 30 min, with the mobile phase at 5 mM H_2SO_4 flowing at a 0.6 mL/min of rate. Additionally, the chromatograph was equipped with two detectors: an MWC detector (G1365B multiwavelength detector) and a RID detector (1260 Infinity II refractive index detector) (Agilent Technologies, Germany). The results of ethanol are expressed as the yield of grams produced per 100 grams of consumed sugar (g/100 g), averaged from biological triplicates.

The results of ethanol are expressed as the % of efficiency in relation to the theoretical maximum ethanol production per gram of sugar (0.51 g), averaged from biological triplicates.

2.4. Sensory Analysis

The wines produced from each experiment were aged in bottles for 2 months at 8 °C before undergoing sensory analysis. Each sensory session involved between 18 and 23 trained and expert tasters in wine that frequently participated in tasting sessions of this type. They came from the Faculty of Oenology (Universitat Rovira i Virgili) for M2 and M3 harvests and from the University of Chile for the S3 harvest. The panelists were informed about the kind of samples they were going to taste and the general conditions of the experiments. The identities of the panellists were protected by assigning random numbers to their answer sheets, corresponding to their place during the analysis. Tasting was carried out with official black glasses to eliminate visual subjectivity related to the colour and intensity of wines. Additionally, the glasses were randomly

numbered with 3-digit codes, and the wines were served anonymously according to a Latin square of Williams design to avoid the range and carry-over effect.

During these sessions, a triangle test was conducted to assess whether the panellists could distinguish the wines inoculated with the PdCs and their respective controls. For the M2 campaign, comparisons included P-M2-26 Vs. C-M2; P-M2-18 Vs. C-M2; P-M2-26SE Vs. C-M2; and P-M2-18SE Vs. C-M2. For the S3 campaign, comparisons included P-S3-26SE Vs. C-S3; and P-S3-18SE Vs. C-S3. For the M3 campaign, comparisons included P-M3-18SE Vs. C-M3; P-M3-18SE Vs. SF-M3; and C-M3 Vs. SF-M3.

Concurrently, a simple descriptive test was conducted to evaluate attributes such as terpene, tropical, vegetal, acidity, bitterness, and quality. Tasters rated the intensity of each attribute using a structured scale ranging from 1 (no detection) to 5 (highest value). Descriptive results from panellists who were able to differentiate the wines in the triangle test were normalized, resulting in 14 panellists for each descriptive test.

2.5. Statistical Analysis

The fermentations were performed in triplicate, and the data were expressed as the mean and standard deviation. The area under the curve (AUC) was used to identify significant differences (p-value < 0.05) in fermentation performance, measured by a decrease in must density (Ruiz-de-Villa et al., 2023). Briefly, AUC values were calculated by the integrating density decrease over two consecutive time points. Statistical analysis was performed using ANOVA and

the Tukey test with XLSTAT version 2023.2.1413 software (Lumivero, New York, NY, USA).

The same software and the statistical test were used to identify significant differences in organic acids and other compounds across all conditions. The data of the triangle test and the mean of scores of the descriptive attributes during the sensory analysis were analyzed statistically using ANOVA, and the spider plots were created with Excel. The level of significance was 0.05.

3. Results and Discussion

3.1. Sugar consumption and population dynamics

The yeast population dynamics and sugar consumption kinetics during the PdC prepared under different stressors (SO_2, ethanol, and temperature) and the AFs inoculated with them were monitored during three harvest campaigns using different grape varieties (M2, S3, and M3). These dynamics are represented in Figures 1–3 with their AUC calculated for the AFs in each harvest (Supplementary Material Table S1). The evaluation of stressors used in the PdC preparation helped us to select the parameters for the following harvests. Our criteria included yeast population composition and quantity, must density decrease rate during fermentation, main organic compound production, and the sensory characteristics of the final wines.

3.1.1. M2 Harvest: antecedents from our laboratory

The fermentation kinetics and population dynamics for M2 harvest were recently published by our group (Bedoya et al., 2024a). In that study, we assessed the effects of stressors individually and the optimal time to inoculate the PdC into

fresh must. We found that 40 mg/L of SO_2 and 1% (v/v) ethanol were effective stressors, prolonging the presence of both moderate fermentative non-Sce and highly fermentative Sce. The best inoculation time using natural must was when the PdC experienced a density drop of 10-20 g/L. A lower temperature during the PdC allowed the non-Sce to be more abundant at the beginning of the AF, significantly increasing Sce diversity compared with the control inoculated with a commercial Sce strain. While yeast growth differences during AFs were minimal, total population growth at the end of the AFs was not significantly different from the inoculated control C-M2 (Figure 1).

AUC analysis for AF kinetics revealed differences for the P-M2-26 and P-M2-26SE (Table S1). In summary, the PdC method using SO_2 and ethanol addition at specific inoculation times effectively managed fermentation kinetics and yeast dynamics (Figure 1). Additionally, it promoted local yeast diversity and maintained fermentation security and efficiency comparable to commercial yeast strains (Bedoya et al., 2024a). However, we did not know if the differences at the population level and diversity of Sce strains had a chemical and sensory impact on the resulting wines or if the organoleptic differences induced by the PdC could be acceptable for the consumers. Herein the present study, we aimed to verify the effectiveness of these selected stressors on PdCs during M2 harvest to conduct the AF in different harvests and grape varieties (M3 and S3). Additionally, we evaluated the chemical and sensory impact of PdC inoculation on wines from the harvests M2, M3, and S3.

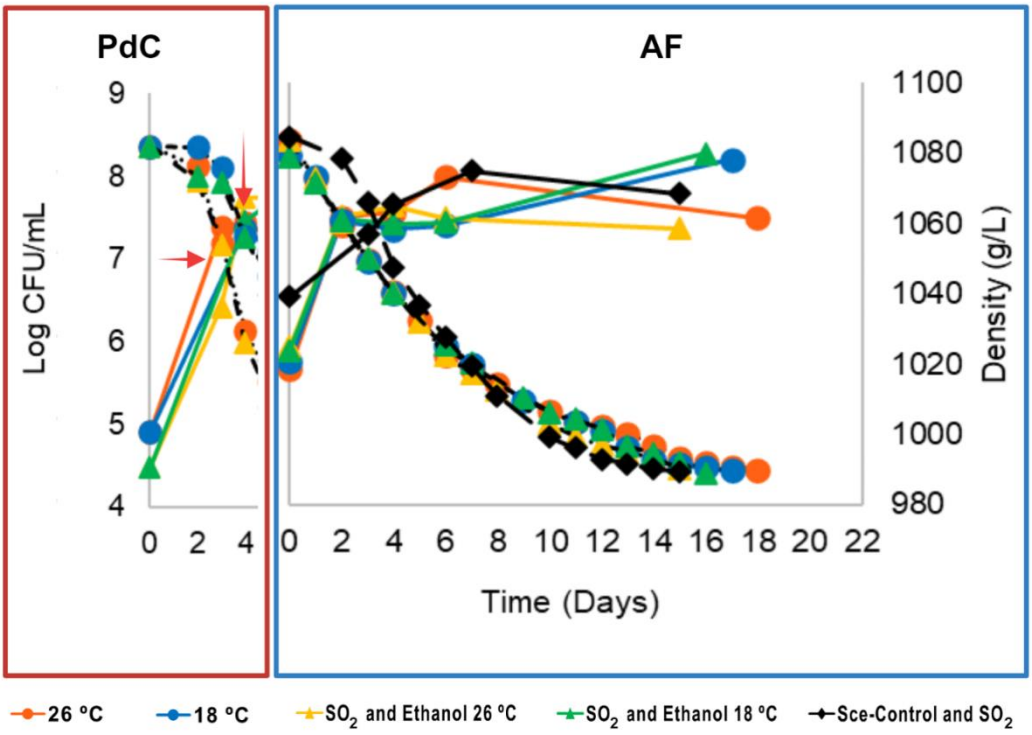

Figure 1. PdC and AF monitoring during M2 harvest, Muscat of Alexandria 2022. Yeast population measured as CFU/mL (solid lines), and fermentation kinetics (dashed lines) represented as must density decrease during *pied de cuve* (PdC) (red rectangle) and the respective alcoholic fermentation (AF) (blue rectangle). Different symbols represent various PdC treatments, and the same colour scheme represents the AFs inoculated with their corresponding PdC. Black rhombuses represent control fermentations inoculated with a commercial strain of *S. cerevisiae*. The red arrow indicates the inoculation point of the PdC into fresh must.

3.1.2. S3 and M3 Harvests

For the S3 harvest, PdCs with SO_2 and ethanol (concentrations selected previously during M2 harvest) were fermented at 26 and 18 °C (Figure 2). Similar to the results obtained during M2 harvest, the temperature was the primary driver of yeast population growth and fermentation kinetics during PdC (Figure 2).

The PdC inoculated into fresh must provided an abundant yeast population, enabling a fast onset of AF comparable to the control (C-S3). However, both P-S3-26SE and C-S3 finished on the 15th day, while the P-S3-18SE finished on the 16th day.

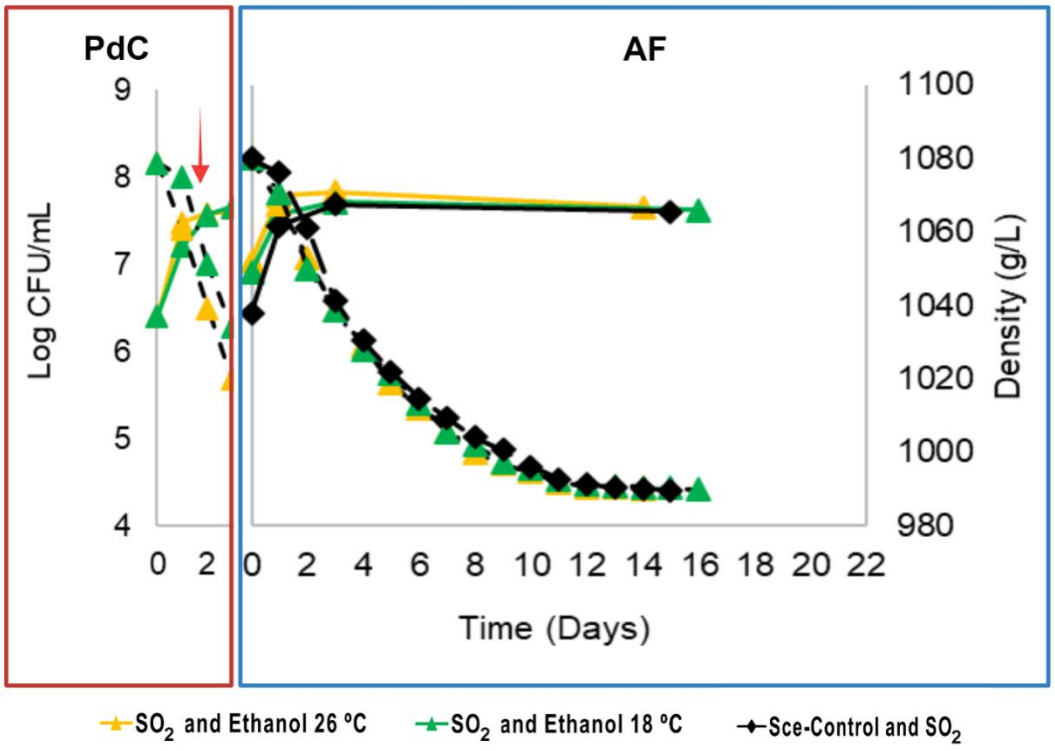

Figure 2. PdC and AF monitoring during S3 harvest, Sauvignon Blanc 2023. Yeast population measured as CFU/mL (solid lines), and fermentation kinetics (dashed lines) represented as must density decrease during *pied de cuve* (PdC) (red rectangle) and the respective alcoholic fermentation (AF) (blue rectangle). Different symbols represent various PdC treatments, and the same colour scheme represents the AFs inoculated with their corresponding PdC. Black rhombuses represent fermentation controls inoculated with a commercial strain of *S. cerevisiae*. The red arrow indicates the inoculation point of the PdC into fresh must.

The AUC analysis of the density loss over time indicated significant differences for P-S3-26SE kinetics compared to P-S3-18SE and C-S3 (Table S1). Thus, the AFs inoculated with PdC containing SO_2 and ethanol and fermented at 26 °C (P-M2-26SE and P-S3-26SE) showed the lowest AUC values (Table S1),

demonstrating consistent fermentation outcomes across M2 and S3 musts inoculated with the same PdC formulation (SO_2 and ethanol at high temperatures).

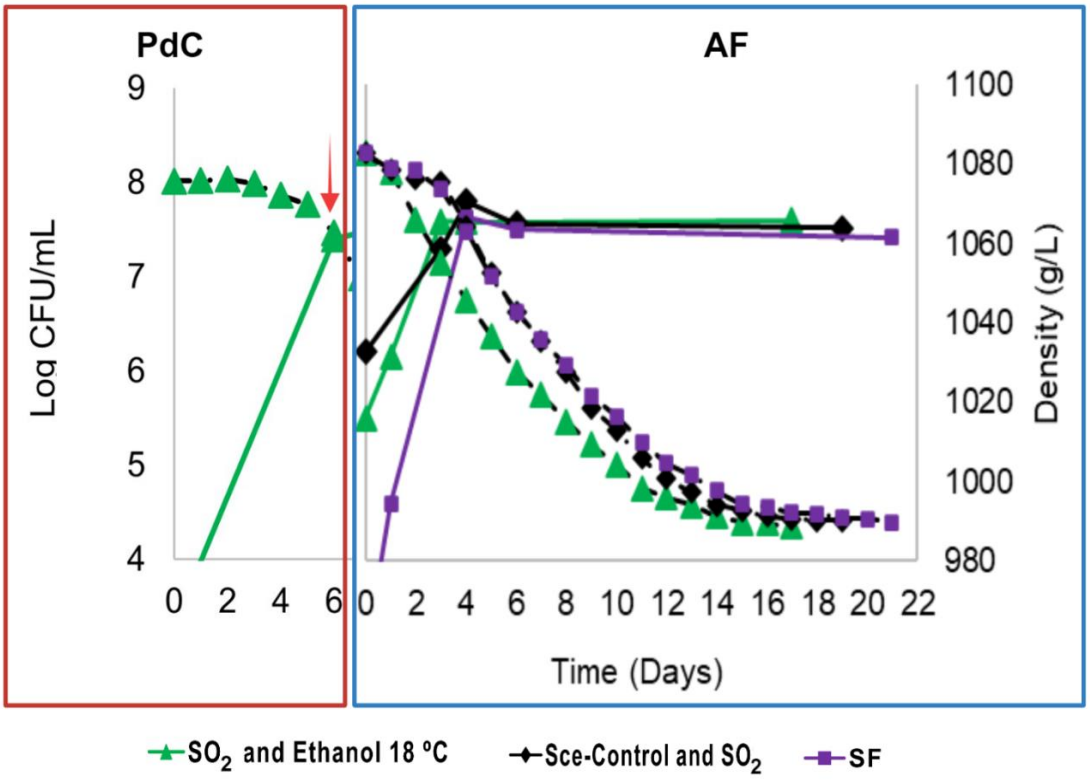

Figure 3. PdC and AF monitoring during M3 harvest, Muscat of Alexandria 2023. Yeast population measured as CFU/mL (solid lines), and fermentation kinetics (dashed lines) represented as must density decrease during *pied de cuve* (PdC) (red rectangle) and the respective alcoholic fermentation (AF) (blue rectangle). Different symbols represent various PdC treatments, and the same colour scheme represents the AFs inoculated with their corresponding PdC. Black rhombuses represent fermentation controls inoculated with a commercial strain of *S. cerevisiae*, while purple squares indicate musts fermented spontaneously. The red arrow indicates the inoculation point of the PdC into fresh must.

Interestingly, during the M3 harvest, the initial yeast population of the must was lower than in M2 and S3 harvests. This fluctuation could be due to the special climatological conditions of the vintage, as the 2023 vintage was particularly dry in Spain. The initial yeast population in AF-C-M3 (1.5 x 10^6 CFU/mL) was higher

than in P-M3-18SE (3 x 10^5 CFU/mL) and SF-S3 (1 x 10^3 CFU/mL). However, P-M3-18SE showed a faster population increase during the beginning of AF, consistent with PdC inoculation during M2 and S3 harvests. SF-M3 had a similar growth rate to P-M3-18SE (Figure 3). P-M3-18SE finished fermentation in 17 days, while C-M3 and SF-M3 finished 2 and 4 days later, respectively. AUCs for density loss kinetics in AFs during M3 harvest (Table S1) indicated significant differences among the three fermentation modalities, with the fastest kinetics belonging to P-M3-18SE. These results corroborate that the PdC technique provided effective microbiological control during AF, even with a low initial yeast population in natural must.

Building on these results and the previous findings (Bedoya et al., 2024a), we investigated if these differences could be reflected in variations in the physicochemical attributes and sensory perception of the resulting wines under various inoculation strategies.

3.2. Wine Chemical Analysis

The main organic acids, ethanol, glycerol, and residual sugars of the resulting wines were determined by HPLC for each AF triplicate. Organic acids are crucial for wine stability and significantly influence its organoleptic features such as flavour, colour, and aroma (Ribéreau-Gayon et al., 2006). We quantified the organic acids focusing on compounds that differed significantly within each harvest (Table 3).

Table 3. Chemical analysis of the final wines from each AF modality. M2, Muscat of Alexandria 2022; M3, Muscat of Alexandria 2023; and S3, Sauvignon Blanc 2023. All data are expressed as the arithmetic average of three biological replicates ± standard deviation (n = 3). The % of theoretical yield for ethanol production is included (maximum of 0.51 g ethanol/g consumed sugar). The added ethanol to PdC corresponds to 0.02% (v/v) over the final quantity.

Harvest	AF Modality	Sugar Consumption (g/L)	Ethanol (g/L)	% Theoretical Ethanol Yield	Glycerol (g/L)	Succinic Acid (g/L)	Acetic Acid (g/L)
M2	P-M2-26	186.79 ± 0.69 ^{a}	89.5 ± 6.2	94.0 ± 6.8	4.41 ± 0.02 ^{a}	1.04 ± 0.10 ^{a}	0.35 ± 0.04 ^{a}
	P-M2-18	176.61 ± 0.13 ^{b}	82.0 ± 7.3	91.1 ± 8.1	3.90 ± 0.19 ^{ab}	1.18 ± 0.03 ^{a}	0.35 ± 0.01 ^{a}
	P-M2-26SE	182.85 ± 2.53 ^{c}	89.6 ± 8.0	96.1 ± 8.9	4.05 ± 0.10 ^{ab}	0.99 ± 0.06 ^{a}	0.35 ± 0.02 ^{a}
	P-M2-18SE	175.39 ± 0.49 ^{b}	88.6 ± 5.2	99.1 ± 6.1	3.10 ± 0.17 ^{c}	1.00 ± 0.03 ^{a}	0.34 ± 0.04 ^{a}
	C-M2	189.35 ± 0.46 ^{a}	83.8 ± 8.9	86.8 ± 9.3	3.54 ± 0.24 ^{bc}	1.06 ± 0.01 ^{a}	0.39 ± 0.01 ^{a}
S3	P-S3-26SE	178.91 ± 0.92 ^{ab}	88.7 ± 1.8	97.0 ± 1.9	5.29 ± 0.05 ^{a}	N.D.	0.70 ± 0.03 ^{a}
	P-S3-18SE	177.19 ± 0.92 ^{b}	87.4 ± 0.6	96.5 ± 1.4	5.29 ± 0.04 ^{a}	N.D.	0.48 ± 0.01 ^{b}
	C-S3	181.50 ± 0.35 ^{a}	88.9 ± 2.8	95.8 ± 3.1	5.17 ± 0.04 ^{b}	N.D.	0.76 ± 0.04 ^{a}
M3	P-M3-18SE	216.13 ± 0.06 ^{a}	95.6 ± 0.3	86.7 ± 0.2	5.10 ± 0.03 ^{a}	1.28 ± 0.03 ^{ab}	0.46 ± 0.03 ^{a}
	C-M3	215.33 ± 0.02 ^{a}	94.8 ± 0.7	86.7 ± 0.6	6.32 ± 0.03 ^{b}	1.39 ± 0.01 ^{b}	0.69 ± 0.01 ^{b}
	SF-M3	215.83 ± 1.03 ^{a}	95.4 ± 0.8	86.7 ± 1.2	6.28 ± 0.08 ^{b}	1.16 ± 0.04 ^{a}	0.86 ± 0.01 ^{c}

N.D. means not determined. Different letters indicate that the values are significantly different within the same harvest (p-value < 0.05).

Higher sugar consumption values were consistently observed for AFs inoculated with commercial strains, while lower values correspond to AFs inoculated with PdC prepared at 18 °C. Interestingly, sugar consumption was not significantly different for AFs inoculated with commercial strains and PdC prepared with stressors at higher temperature (P-M2-26SE and P-S3-26SE). Similar results using the PdC of red grape variety must prepared at 26 °C were reported by Moschetti et al. (2016). The higher temperature of the PdC might have selected highly fermentative yeasts like Sce over the non-Sce yeast (Salvadó et al., 2011a). Our previous results showed that the non-Sce presence was favoured longer at the PdC at the beginning of the AF during M2 harvest when a lower temperature was applied (Bedoya et al., 2024a). In the M3 harvest, sugar consumption was similar across the three modalities (P-M3-18SE, C-M3, and SF-M3).

No significant differences were detected in the efficiency of ethanol production among treatments within each harvest. AFs inoculated with PdCs prepared with SO_2 and ethanol at 18 °C presented the lowest glycerol concentration for the M2 and M3 harvests, while the influence of the PdC over the glycerol production during S3 harvest was not as prominent. The glycerol concentration in harvest from the same grape variety, M2 and M3, ranged from 3.10 to 6.28 g/L, which falls within the normal range of 2 to 10 g/L (Nieuwoudt et al., 2017; Ough et al., 1972; Radler and Schütz, 1982; Ribéreau-Gayon et al., 2006). A significant increase in viscosity could be observed for values around 10 g/L (Gawel et al., 2007; Jones et al., 2008). Our results indicate that the observed variations could hardly influence the sensory experience of our wines. Glycerol production is influenced by factors such as grape variety, yeast strains, fermentation

parameters like temperature, and winemaking processes (Erasmus et al., 2004; Jones et al., 2008; Ough et al., 1972). Ruiz-de-Villa et al. (2023) have reported values of glycerol for Muscat of Alexandria between 4.86 and 5.97 g/L in musts inoculated with *T. delbrueckii* or *S. cerevisiae* at different temperatures (25 and 16 °C). They observed an increase of glycerol concentration in wines inoculated with *T. delbrueckii* at 25 °C. Thus, glycerol production is temperature dependent with higher values at higher temperatures (Ough et al., 1972). In our study, the effect of PdC prepared with SO_2 and ethanol and fermented at low temperatures during M2 and M3 harvest might have limited the growth of glycerol-producing yeast (Jolly et al., 2014). In the wines from S3 harvest, concentrations were between 5.17 and 5.29 g/L, which falls within the previously reported values for wines from the Sauvignon Blanc variety between 5.42 and 6.31 g/L (Nieuwoudt et al., 2017).

Succinic acid concentrations were similar among treatments in M2. Significant differences were found just in the M3 harvest, where the C-M3 had the highest value and the SF-M3 had the lowest. Thus, the effect of PdC was not prominent at the succinic acid levels.

A high concentration of acetic acid in wine is not desirable because of the generation of off-flavours and consequent organoleptic depreciation. SF is frequently associated with high volatile acidity due to the proliferation of spoilage yeasts (Wang and Liu, 2013). Acetic acid yields showed no significant differences between the different treatments of M2 harvest. Interestingly, for the S3 and M3 harvests, the lowest acetic yield corresponded to the AFs inoculated with PdCs prepared at 18 °C with the same stress conditions (P-S3-18SE and P-M3-18SE).

These results suggest that the combination of low temperature and the stressors in the PdC selected native non-Sce and Sce populations with a low production of acetic acid. In general, acetic acid values obtained during our PdC-inoculated fermentations were between 0.34 and 0.48 g/L, which is similar to the range of 0.33 to 0.49 g/L reported previously (Álvarez-Barragán et al., 2023; Börlin et al., 2020). The exception was the AF-S3-26SE (0.70 g/L of acetic acid). This result indicates that the PdC elaborated at 18 °C efficiently kept the acetic acid lower than their respective controls and PdC elaborated at a higher temperature.

Further insights into sensory perception were pursued through the triangle and descriptive tests conducted on the final wines.

3.3. Wine Sensory Analysis

Sensory analysis was conducted on wines inoculated with different PdCs and their corresponding controls within each harvest (M2, S3, and M3). A triangle test was used to assess if panellists were able to distinguish wines inoculated with the PdCs from their respective controls. Additionally, experts were asked to do a descriptive test of the wines. We employed the same attribute and score scale for sensory evaluation in all harvests (M2, S3, and M3). However, our goal was to assess these attributes within each harvest rather than compare them across the three harvests. It is essential to consider the differences arising from various cultural and personal factors when conducting a sensory analysis of wine on an international scale. The preferences and perceptions of wine quality and factors as tropical can vary greatly between nationalities, limiting definitive conclusions. To achieve a comprehensive and balanced understanding, it is beneficial to include individuals from diverse nationalities and backgrounds in tasting panels

and to account for these cultural factors when interpreting the results. The sensory analysis aimed to determine if the PdC-inoculated wines resulted in a consistent variation of the sensory perception and if their overall quality and organoleptic evaluation were positive. Table 4 summarizes the wine comparisons and the statistical results of the triangular test.

Table 4. Results of triangular sensory analysis of wines obtained according to the type of inoculation of natural musts. M2, Muscat of Alexandria 2022; M3, Muscat of Alexandria 2023; and S3, Sauvignon Blanc 2023. Assessors for each sensory session: 14.

Harvest	Comparison	*p*-Value
M2	P-M2-26 Vs. C-M2	**< 0.05**
	P-M2-18 Vs. C-M2	**< 0.05**
	P-M2-26SE Vs. C-M2	0.175
	P-M2-18SE Vs. C-M2	**< 0.05**
S3	P-S3-26SE Vs. C-S3	**< 0.05**
	P-S3-18SE Vs. C-S3	**< 0.05**
M3	P-M3-18SE Vs. SF-M3	0.179
	P-M3-18SE Vs. C-M3	**< 0.05**
	SF-M3 Vs. C-M3	**< 0.05**

Significantly different values (p-value < 0.05) are highlighted in bold.

The results of the descriptive test of wines from each harvest were represented in a spiderweb diagram (Figure 4). In the M2 harvest, the wines from the C-M2 presented higher acidity and vegetal and bitterness attributes, which were the main factors differentiating them from the PdC-inoculated wines, both at the descriptive analysis and triangular test. However, as presented in Section 3.2, the wines of the M2 harvest did not show significant differences in acetic acid or ethanol yields, which could have contributed to these attributes. A combination of higher sugar consumption and lower glycerol observed for C-M2 wines could

make their taste more tart or sour, explaining the differences in sensory description (Jackson, 2008). In contrast, the P-M2-18SE wines showed high scores for tropical and quality attributes. Terpene, a characteristic feature of the Muscat of Alexandria variety, scored higher in PdC-inoculated wines than C-M2 (Figure 4. A).

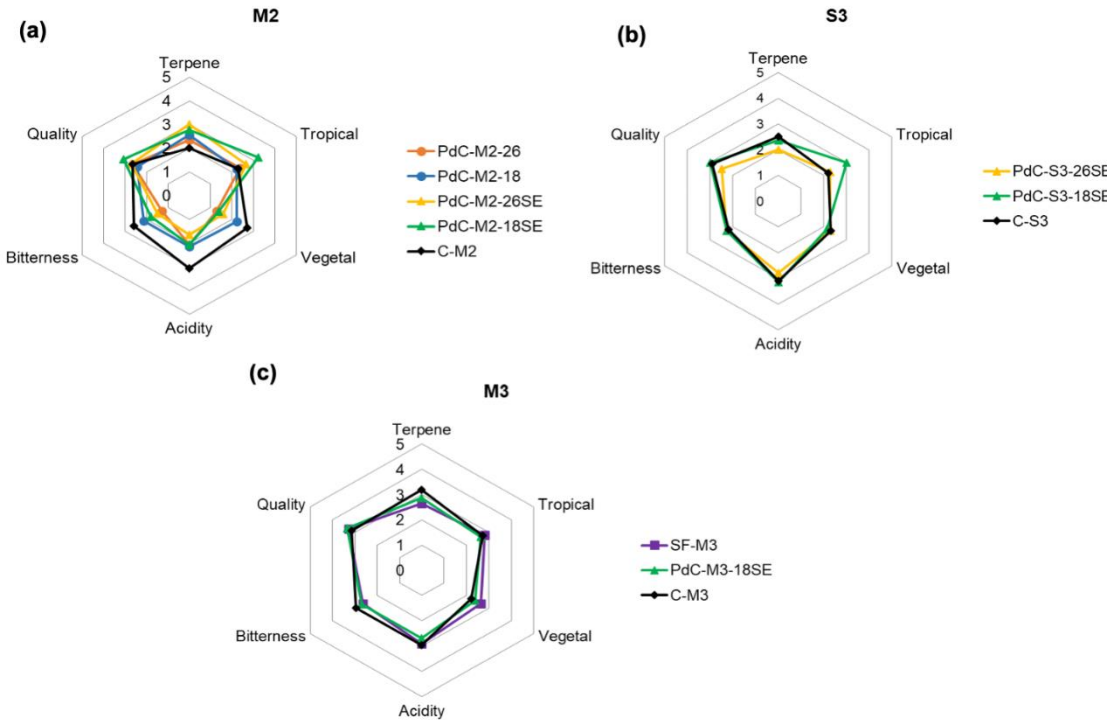

Figure 4. Spiderweb plots for the descriptors terpene, tropical, acidity, vegetal, bitterness, and quality of wines from harvest. (**A**) M2, Muscat of Alexandria 2022; (**B**) S3, Sauvignon Blanc 2023; and (**C**) M3, Muscat of Alexandria 2023. Assessors for each sensory session: 14. Asterisks (*) indicate significant differences between treatments with *p*-value < 0.05.

In the M3 harvest, elaborated with the same grape variety, the wines from P-M3-18SE showed a higher score in terpene than SF-M3 but lower than C-M3 (Figure 4. C). The tropical, acidity, and quality attributes of P-M3-18SE were similar among the treatments during the M3 harvest. The wines resulting from SF-M3 presented the highest vegetal attribute (Figure 4. C), which is generally perceived

as a negative one. The wines from the S3 harvest produced by the inoculation of PdCs were also clearly different from the DWY-inoculated control (C-S3) by its higher tropical features in the case of wines from P-S3-18SE (Figure 4. B). The lowest quality and terpene were found for P-S3-26SE wines, indicating that the higher temperature during the PdC preparation had a negative influence on the organoleptic perception, similar to the M2 harvest wines. The rest of the attributes of PdC-inoculated wines in S3 harvest were comparable to the C-S3, but the mentioned differences were sufficient for the panellists to differentiate them.

Our results generally agree with Álvarez-Barragán et al. (2023), who found differences in some attributes but not in the global variable that included quality when comparing PdC-inoculated wines with a DWY-inoculated control and SF. Nonetheless, it should be noted that the perception of specific concepts, such as tropical and quality, varies among tasters from different countries due to geographical and gastronomic cultural differences, and this can limit definitive conclusions.

Overall, in our study, the sensory analysis confirmed that PdC inoculation, particularly with stressors like SO_2 and ethanol, influenced the sensory attributes of wines, enhancing specific desirable flavours and aromas while maintaining acceptable levels of volatile acidity and glycerol content.

Future studies should explore the dynamics of Sce strains in must inoculated with PdC. It is crucial to extend this research to other grape varieties, including more complex wines such as red and rosé, to gather additional data that could be applied at the cellar level.

4. Conclusions

Our findings have confirmed that the application of selected stressors over PdC can effectively facilitate the AF process. We observed consistent results in yeast population growth and fermentation kinetics across the grape varieties Muscat of Alexandria and Sauvignon Blanc, harvested over three different vintages. These results were comparable to those obtained by the DWY-inoculated controls. Additionally, the stressors applied during the preparation of the PdC, especially temperature, significantly influenced the performance of the PdC and the selection of yeast strains driving the different AFs. This variability in yeast selection and AF performance highlights the critical role of PdC conditions in shaping fermentation outcomes. Wines produced from PdC-inoculated fermentations exhibited significant differences in certain chemical parameters compared to those from spontaneous and DWY-inoculated fermentations. These differences were also perceptible at the sensory level. Notably, the quality scores of the PdC-inoculated wines were comparable or higher than those of the respective controls at each harvest. Thus, the use of PdC with selected stressors ensured a consistent AF process across different vintages, demonstrating the robustness of this methodology in maintaining desirable fermentation characteristics and wine quality. Future research should be directed to test the effectiveness of the PdC methodology with the selected stressors in other grape varieties and more complex wines. Also, the evaluation of the effect of parameters such as the grape ripeness or viticultural practices on the initial microbiology of the must and the selection of yeasts during PdC is key knowledge to test the robustness of our proposed methodology to control the AF while preserving the autochthonous microbiota.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Table S1: Area Under the Curve (AUC) of different inoculation modalities to conduct the alcoholic fermentation (AF).

Author Contributions: Conceptualization, A.M., N.R. and M.C.P.; methodology, K.B and M.C.P.; validation, N.R., A.M., K.B. and M.C.P.; formal analysis, N.R., A.M., K.B., C.J. and M.C.P.; investigation, A.M. and C.J.; resources, A.M., N.R. and C.J.; data curation, K.B. and M.C.P.; writing—original draft preparation, K.B. and M.C.P.; writing—review and editing, N.R., A.M., K.B., C.J. and M.C.P.; visualization, N.R., A.M., K.B., C.J. and M.C.P.; supervision, N.R., A.M. and M.C.P.; project administration, A.M.; funding acquisition, A.M. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Conflicts of Interest: The authors declare no conflicts of interest.

Supplementary data:

Table S1. Area Under Curve (AUC) of different inoculation modalities to conduct the alcoholic fermentation (AF). Different superscript letters indicate that the values are significantly different within the same harvest (*p*-value < 0.05).

Harvest	AF modality	AUC
M2	P-M2-26	9206.41 ± 3.91 [a]
	P-M2-18	9193.40 ± 4.46 [ab]
	P-M2-26SE	9184.90 ± 7.39 [b]
	P-M2-18SE	9198.70 ± 3.52 [ab]
	C-M2	9203.19 ± 13.98 [ab]
S3	P-S3-26SE	15212.62 ± 5.69 [a]
	P-S3-18SE	15223.62 ± 19.73 [b]
	C-S3	15254.75 ± 4.16 [b]
M3	P-M3-18SE	18401.62 ± 9.30 [a]
	C-M3	18570.98 ± 9.67 [b]
	SF-M3	18725.48 ± 5.16 [c]

UNIVERSITAT ROVIRA I VIRGILI
Microbiological and organoleptic influence of different stresses during the pied de cuve applied to white wine vinification
KATHERINE BEDOYA ALVIRA

Chapter III

UNIVERSITAT ROVIRA I VIRGILI
Microbiological and organoleptic influence of different stresses during the pied de cuve applied to white wine vinification
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Multi-Approach Analysis of Pied de Cuve Across Different Levels of Grape Ripeness

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Manuscript in preparation

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Microbiological and organoleptic influence of different stresses during the pied de cuve applied to white wine vinification
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Abstract: Grape ripening impact is evaluated in the application of spontaneous *pied de cuve* (PdC) fermented with sulphur dioxide (SO_2), ethanol, and temperature fermentation of 18 ºC. PdC involves adding a small proportion of fermenting must to the bottom of the vat to initiate alcoholic fermentation (AF). The effectiveness of PdC inoculation was assessed by monitoring fermentation kinetics and yeast population dynamics, using potential alcohol content (PAC) as a ripening indicator at 10%, 12%, and 13% (v/v) PAC. Microbial communities, including yeast and bacteria, from grapes and select PdC points were identified through next-generation sequencing (NGS). The diversity of *Saccharomyces cerevisiae* strains at different stages of PdC and AF was analyzed using Interdelta-PCR.

Fermentation density, yeast population kinetics, *S. cerevisiae* strain diversity, as well as chemical and sensory profiles were compared across PdC-inoculated, commercial strain QA23-inoculated, and spontaneous fermentation (SF). Optimal fermentation kinetics and yeast population growth were observed at 12 % (v/v) PAC level across all treatments. However, the higher strain diversity of *S. cerevisiae* was found in 13 % (v/v) PAC. At this PAC level, the PdC-inoculated wines resulted in the lowest acetic acid production and the highest glycerol concentration. Overall, the PdC-inoculation strategy consistently mantained higher yeast strain diversity at all PAC levels, with better sensory attributes than SF for lower PAC levels and than commercial-inoculated fermentation at 13% (v/v).

In conclusion, PdC inoculation is an effective strategy for preserving yeast strain diversity comparable to or higher than SF, while ensuring robust fermentation kinetics across all PAC levels evaluated.

Keywords: Muscat of Alexandria, *pied de cuve*, population yeast dynamics, grape ripening, Interdelta-PCR

1.Introduction

The *pied de cuve* (PdC) technique, derived from the French term meaning *bottom of the deposit*, was a traditional practice in winemaking before the commercial availability of dry yeast wine (DWY) strains (Mas and Portillo, 2022). This technique involves adding a small proportion, generally 2-10 % (v/v) of must that is already fermenting to the bottom of the vat, which is then filled with the main batch of must to initiate alcoholic fermentation (AF).

One approach to prepare PdC is by harvesting and pressing a small proportion of grapes a few days before the main harvest. Under aseptic conditions, this method minimizes cross-contamination from the winery's resident yeasts and activates fermentation in the PdC primarily through the natural microbiota present in the must coming from the soil and the vine phyllosphere (Sturm et al., 2006). When PdC is initiated by the natural microbiota present in the must, it is referred to as spontaneous or uninoculated PdC (Ubeda Iranzo et al., 2000).

Recently, PdC technique has gained renewed interest as a good strategy for preserving the *microbial footprint* while controlling AF and preventing microbial spoilage in wines (Belda et al., 2017d; Mas and Portillo, 2022; Portillo and Mas, 2022). The concept of vine *microbial terroir* is associated with stable differences

in microbial populations in grape musts, which are influenced by grape variety, geographical location, climatic factors, vine and grape health, viticultural practices, and winery environment (Belda et al., 2021; Bokulich et al., 2016b; Portillo and Mas, 2022; Renouf et al., 2005).

Winemakers are increasingly interested in leveraging autochthonous microbiota to produce wines that preserve the unique *microbial terroir*, offering distinctive aromatic profiles to consumers (Belda et al., 2021; Bokulich et al., 2014). It is well established that the production of aromatic compounds such as acetate or fatty acids ethyl esters, is closely related to the microbial species and strains involved in AF (Mas et al., 2022). Furthermore, microbial diversity serves as a genetic reservoir for novel fermentation products and plays a crucial role in mitigating the undesirable effects of imbalances in technological, phenolic, and aromatic maturity caused by rising temperatures due to climate change (Mira de Orduña, 2010; Molinet and Cubillos, 2020; Tronchoni et al., 2022).

The PdC method could contribute to ongoing efforts to preserve yeast diversity, an important factor in producing wines with distinctive aromatic profiles (Belda et al., 2017c). Although some studies have evaluated the impact of PdC inoculation on AF, further research is needed to assess its effect on microbial populations, fermentation rates, chemical composition, and aromatic profile across different grapes varieties and conditions. To date, fewer than ten studies have examined the impact of PdC inoculation in AF (Álvarez-Barragán et al., 2023; Bedoya et al., 2024a, 2024b; Börlin et al., 2020; Li et al., 2012; Morgan et al., 2017; Moschetti et al., 2016).

Our research group has investigated the influence of various stressors in PdC such as sulphur dioxide (SO_2), and ethanol at different temperatures to exert selective pressure on the yeast microbial population, focusing on the optimal timing for PdC inoculation in AF. Optimal AF inoculated with PdC is characterized by the conservation of yeast diversity, aromatic complexity, efficient fermentation rates, and, most importantly, positive sensory evaluation by tasters (Bedoya et al., 2024a). Also, we explored the impact of PdC inoculation during different harvests of the same grape varietal in Spain and compared the results with a harvest in Chile using a different grape variety. The results indicated that wines inoculated with PdC exhibited controlled AF, lower acetic acid levels across all harvests, and positive attributes that distinguished them from wines inoculated with commercial *Saccharomyces cerevisiae* (Sce) strains (Bedoya et al., 2024b).

The ripeness level of grapes at harvest is a critical factor that significantly influences the AF process, ultimately affecting the chemical composition, sensory attributes, and overall quality of the wine. As grapes ripen, there are notable changes in sugar concentration, acid content, phenolic compounds, and aromatic precursors, all of which play essential roles in fermentation kinetics and the resulting wine profile (Bisson, 2001; Vilanova and Martínez, 2007). Technological maturity in winemaking is characterized by sugar content, which determines the potential alcohol content (PAC) of the future wine, as well as the titratable acidity and pH of the grape must (Rajha et al., 2017).

In this study, we aim to evaluate the impact of grape ripeness on PdC creation and its subsequent inoculation into a fresh must for AF. Our objective is to assess the influence of three different maturity levels on PdC creation and performance

of PdC-inoculated AF, comparing it with AF inoculated with a commercial strain QA23 and with spontaneous fermentation (SF). This multi-approach assessment includes fermentation rates, diversity of grape microbiome for each harvest level and some point of the PdC, diversity of Sce strains during AF, chemical composition analysis using high-performance liquid chromatography (HPLC) for key organic compounds (i.e., ethanol, glycerol, and citric, acetic, tartaric and succinic acids), some acids with enzymatic kits, and sensory evaluation of the resulting wines.

Additionally, we have focused on the population diversity of Sce using diversity indices calculated from the Interdelta-PCR profiles. Finally, we conduct a brief characterisation of strains with high relative abundance during AF or those present from the beginning to the end of AF.

2. Material and Methods

2.1. Experimentation settling in the cellar

Grapes from the Muscat of Alexandria variety were harvested in 2023 randomly from three parcels of the experimental vineyard Mas dels Frares, affiliated with the Faculty of Oenology at the University Rovira i Virgili in Tarragona, Spain. For each experiment, berries were collected at three different ripening levels based on the probable alcohol content (PAC): 10%, 12%, and 13% (v/v). The pressing and settling process were standardized for *pied de cuve* (PdC) preparation and the alcoholic fermentation (AF). After pressing, the must was drained and collected in a clean vat. The must was treated with 40 mg/L of SO_2 and 20 mg/L

of pectolytic enzymes (Lallzyme, Lallemand Inc., Montreal, Canada) and allowed to settle down for a minimum of 24 h at 8 ºC before use.

Following settling, the must was carefully racked and distributed into specific containers to conduct the fermentations in triplicate. Fermentation density was monitored daily using an electronic densitometer (Densito 30PX Portable Density Meter; Mettler Toledo, Barcelona, Spain). Yeast population kinetics were tracked using a Neubauer chamber for counting and plating on solid media. PdC and AF were conducted at 18 ºC, a typical temperature for fermenting white must. Fermentations were considered finished when the must density reached 990 g/L and residual sugars were ≤ 2g/L.

2.1.1. Pied de cuve (PdC) preparation

PdC was prepared by harvesting randomly within each parcel 10 kg of grape berries 2-3 days before the main harvest at each PAC level (10%, 12%, and 13 % (v/v)). The berries were crushed, pressed, and drained using clean equipment, and the must was settled as described in section 2.1. After 24 h, SO_2 levels (total and free) were measured, and the must was adjusted with metabisulfite to achieve a final concentration of 40 mg/L of free SO_2. Additionally, 1% (v/v) ethanol was added using wine containing 10% (v/v) ethanol. This preparation was labelled as PdC- and was distributed in triplicate into sterile 1.5 L bottles containing 1 L of the batch PdC.

Fermentation of PdC was monitored until the density dropped by 15-20 g/L, and the yeast population determined by microscopy reached approximately 10^7-10^8 cells/mL, both criteria were considered optimal conditions for using PdC as a starter (Bedoya et al., 2024a). Once these criteria were met, each triplicate of the

PdC was mixed in equal proportions to obtain the volume required for inoculation. However, each triplicate continued to be monitored until the end of the fermentation.

Each PdC was labelled according to the harvest PAC level: 10%, 12%, and 13% (v/v). For instance, PdC10 represents the PdC prepared from must with a PAC of 10% (v/v), while PdC12 and PdC13 represent those prepared from musts with PACs of 12% and 13% (v/v), respectively (Table 1).

Table 1. Codes for the *pied de cuve* (PdC) and alcoholic fermentation (AF) modalities of must obtained from Muscat of Alexandria grapes variety at different maturity levels measured as probable alcohol content (PAC): 10%, 12%, and 13% (v/v). Different strategies of inoculation were used in alcoholic fermentation (AF): with PdC (AF-PdC), with commercial strain QA23 (AF-C), and spontaneous fermentation (AF-S).

		PAC level		
		10% (v/v)	12% (v/v)	13% (v/v)
	Type of fermentation	**Code**	**Code**	**Code**
PdC 18 °C + SO_2 + EtOH	Spontaneous	PdC10	PdC12	PdC13
AF 18 °C	Inoculated with PdC	AF-PdC10	AF-PdC12	AF-PdC13
	Inoculated with Sce QA23 + SO_2	AF-C10	AF-C12	AF-C13
	Spontaneous	AF-S10	AF-S12	AF-S13

2.1.2. Alcoholic fermentation (AF)

The must for AF was prepared by harvesting 60 kg of grape berries at each ripening stage. The berries were processed as described in section 2.1. After 24 h, 3 L of the must was distributed into 5 L containers for fermentation. Three trials in triplicate were conducted for each PAC level using different fermentation

modalities: one set was inoculated with the PdC prepared in section 2.1.1 (AF-PdC-), another set was inoculated with a commercial strain (AF-C-), and the last set was left to ferment spontaneously (AF-S-) as detailed in Table 1.

The different AF-PdC- were inoculated with 2% (v/v) of a combination of the triplicate PdC prepared at each PAC level. For example, AF-PdC10, AF-PdC12 and AF-PdC13 represent musts inoculated with PdC10, PdC12, and PdC13, respectively. The AF-C- were inoculated with 2 x 10^6 cells/mL of a rehydrated strain of *S. cerevisiae bayanus* QA23 (Lallemand Inc., Montreal, Canada) with the addition of 40 mg/L SO_2 as a control. The AF-S- code represents spontaneous fermentation (SF) at each PAC level. After AF completion, triplicates were combined, treated with 30 mg/L of SO_2, and bottled for aging at 8 ºC. Sensory testing was conducted after 2 months of aging.

2.2. Sampling and yeast isolation

Yeast populations from AF-PdC were plated on different solid media at four fermentation stages based on must density, taking into account that the original density of the must was between 1080 and 1070 g/L: (i) inoculation point (IP), when the PdC was ready to inoculate into the must to conduct the AF (density decrease of 10-20 g/L); (ii) beginning of fermentation (BF), original must density of AF; (iii) half fermentation, (HF), density decrease of 35-40 g/L and (iv) final fermentation (FF), density decrease of ≥ 90 g/L with residual sugars ≤ 2 g/L).

In the case of AF-C and AF-S, three sampling points were considered: BF, HF, and FF.

Plating involved serially diluting 100 µL of each triplicate sample and spreading it on three different solid culture media for yeasts, each supplemented with 100 mg/L chloramphenicol and 200 mg/L biphenyl (both from Sigma-Aldrich, Steinheim, Germany) to inhibit bacterial and mould growth, respectively.

The yeast population was quantified using YPD medium (1% yeast extract, 2% peptone, 2% dextrose, 17 g/L agar; all from Biogenetics, Milan, Italy). Lysine-agar medium (LYS, Oxoid Ltd, Basingstoke, UK) was used for quantifying the non-*Saccharomyces* (non-Sce) yeast (De Angelo and Siebert, 1987), since *Saccharomyces* yeasts grow slower using L-lysine as the sole nitrogen source (Fleet and Heard, 1993). Although it does not provide an exact value, the difference between the cells quantified in YPD and LYS media is a good approximation of *S. cerevisiae* (Sce) cells (See supplementary Figure S1). For this media, addititional samples were taken for PdC: (BS), when PdC was before settling; (BF), beginning fermentation, and (IP) as it is described previously.

Finally, the Wallerstein Laboratory Nutrient Agar (WLN) medium (Difco Laboratories, Detroit, MI, USA), was used to differentiate and selecte Sce species based on the colour and morphology of colonies (Feghali et al., 2019; González et al., 2023).

Mesophilic lactic acid bacteria (LAB) were cultured in Man–Rogosa–Sharpe medium (50% MRS Broth, 10% fructose, 4% D-L Malic, 25% agar, pH 4.8) and acetic acid bacteria (AAB) on GYC media (10% glucose, 10% yeast extract, 10% calcium carbonate, 20% agar). Both media were supplemented with 100 mg/L cycloheximide. Media and the supplements used were obtained from Sigma-Aldrich, Steinheim, Germany.

All plates were incubated at 28 ºC and counts of colony-forming unit by mL (CFU/mL) were performed after 2-4 days of culture (Feghali et al., 2019).

2.3. S. cerevisiae typification

A total of 540 Sce colonies were molecularly typified and, 20 colonies showing the colour and morphology of Sce were randomly picked from WLN plates resulting from each of the sampling points described in section 2.3. The identification of the isolated strains was confirmed by molecular analysis using the ITS-PCR technique (Esteve-Zarzoso et al., 1999).

DNA was extracted following the method described by Querol et al. (1992) and Interdelta-PCR was conducted using primers delta 12 and 21 (Legras and Karst, 2003). PCR products were analysed with GelJ v2.0 (Department of Mathematics and Computer Science at the University of La Rioja, Logroño, Spain), and dendrograms were constructed using the UPGMA method (Guzzon et al., 2018). Strains with a similarity coefficient of ≥ 90% were clustered following the methodology described in Bedoya et al. (2024a). A 100-bp DNA ladder marker (Thermo Fischer Scientific Inc., Madrid, Spain) was used and the electrophoresis was conducted at 100 V.

2.4. Genetic diversity

Interdelta-PCR patterns were used to calculate the genetic diversity of Sce strains within each sampling point. The comparisons of diversity at each PAC level and fermentation modalities were analyzed through by the Shannon (H') and the inverse Simpson indexes (D), calculated as described in Börlin et al. (2016).

2.5. DNA extraction and sequencing from natural samples

A barcode sequencing was performed with selected samples in order to compare the influence of the grape ripeness level on the grape microbiota and the PdC before and after its inoculation into a fresh must to perform AF.

Berries and other sampled points: 250 g of healthy berries from the batch destinated to prepare different PdC were randomly collected using sterile material (gloves, plastic bags, and scissors). The intact berries in sterile plastic bags were treated with 500 mL of an isotonic solution containing 9 g/L of NaCl, 0.1% (v/v) peptone, and 0.01% (v/v) Tween 80. Subsequently, they were subjected to orbital shaking at 100 rpm for 30 min. Afterward, 50 mL of the liquid supernatant was collected. 50 mL of samples from berries supernatant, and from PdC before (PdCB) and after (PdCA) its inoculation were frozen at -20 ºC until DNA extraction and sequencing.

2.6. Chemical parameters analysis

Besides PAC, total acidity and pH were measured for fresh musts used for PdC preparation and subsequent AF. Sugars, ethanol, glycerol, and organic acids were quantified at the end of the AFs using the equipment of Agilent 1100 HPLC (Agilent Technologies, Waldbronn, Germany) following the protocol of Quiros et al. (2014). The HPLC had coupled a Hi-Plex H (300 mm × 7.7 mm) column inside a 1260 MCT (Infinity II Multicolumn Thermostat). The column conditions involved maintaining a temperature of 60 ºC for 30 min, with the mobile phase at 5 mM H_2SO_4 flowing at a rate of 0.6 mL/min. Additionally, the chromatograph was equipped with two detectors: an MWC detector (G1365B multi-wavelength detector) and an RID detector (1260 Infinity II refractive index detector) (both from

Agilent Technologies, Waldbronn, Germany). The results are shown as an averaged from biological triplicates.

Additionally, residual sugars, citric, acetic, and malic acids were quantified at the beginning and at the end of AF using the Y15 Bioanalyzer with the corresponding enzymatic kits 267 (BioSystems S.A, Barcelona, Spain). Ammonia, and primary amino nitrogen (PAN), were analysed only at the beginning of the AF also using the Y15 Bioanalyzer.

2.7. Sensory analysis

Two months post-bottling, sensory analysis was conducted by 16 professional oenologists who came from Universitat Rovira i Virgili. A triangle test to assess the ability of the panellists to distinguish between wines produced under each fermentation modality. Additionally, a simple descriptive test evaluated attributes such as terpene, tropical, vegetal, oxidation, acidity, bitterness, and overall quality using a structured scale from 1 (no detection) to 5 (the highest).

2.8. Micro-fermentations of selected strains

Following genetic characterisation, 12 Sce strains were selected for micro-fermentations conducted using sterile concentrated must (Concentrats Palleja S.L., Riudoms, Spain) reconstituted with sterile water at 1:4 (v/v). The must had 154 mg/L of yeast-assimilable nitrogen (YAN), 103 g/L of glucose, and 119 g/L of fructose. The volume of the fermentations was 100 mL in 250 mL sterile glass bottles with a loose lid incubated in static at 18 ºC. Each triplicate was inoculated with 2 x 10^6 cells/mL of the individual selected strains. Fermentations were monitored daily by densitometry and were considered finished when the residual

sugar was below 2 g/L. Cell concentration was measured by optical density at 600 nm (OD600). Chemical parameters such as sugars (glucose and fructose), ethanol, glycerol, and organic acids were measured by HPLC as described in section 2.5.

2.9. Statistical analysis

Fermentations were conducted in triplicate and the data are presented as the mean ± standard deviation. Chemical parameters were analysed using a one-way ANOVA, followed by Tukey's test analyses with XLSTAT software (version 2023.2.1413, Lumivero, New York, USA). Significance was set at p-value < 0.05. Sensory data were further analysed using spider plots and principal component analysis (PCA) in Excel (Microsoft Excel, Microsoft 365 MSO version 2406).

3. Results

3.1. Microbioma results

Results under preparation.

3.2 Must chemical parameters

Three different levels of PAC were considered for experimentation: 10%, 12%, and 13% (v/v). For each PAC level, standard chemical parameters such as initial density, total acidity and pH were measured for must used for both the PdC and the AF processes (Table 2).

Table 2. Initial parameters of musts used to prepare *pied de cuve* (PdC) and alcoholic fermentation (AF) at each harvest based on the probable alcohol content (PAC) 10%, 12% and 13% (v/v).

	PAC 10% (v/v)	PAC 12% (v/v)	PAC 13% (v/v)
PdC preparation			
Density (g/L)	1069.7 ± 1.44	1076.2 ± 0.10	1079.5 ± 0.08
Total acidity	6.07	4.57	3.97
pH	3.18	3.29	3.26
AF			
Density (g/L)	1072.3 ± 1.15	1082.6 ± 0.55	1086.3 ± 0.48
Total acidity	9.00	5.02	3.75
pH	3.19	3.32	3.31
Glucose (g/L)	95.13 ± 7.51	112.53 ± 3.00	101.63 ± 1.14
Fructose (g/L)	85.36 ± 5.71	105.06 ± 3.28	121.66 ± 2.23
PAN (g/L)	34.00 ± 0.00	49.33 ± 0.47	48.78 ± 1.30
Ammonia (g/L)	52.00 ± 0.82	32.67 ± 1.70	26.56 ± 3.81
YAN (g/L)	86.00 ± 0.82	82.00 ± 1.63	75.33 ± 4.50

Total acidity, PAN, and ammonia levels decreased as maturity advanced in both musts. The increase in sugar level was mainly reflected in fructose, which rose from 105 g/L to 121 g/L as PAC increased from 12% to 13% (v/v). Changes in pH were in concordance with those of the total acidity for each must (Table 2).

3.3. PdC dynamics

The fermentation dynamics of PdC were monitored daily to determine the optimal timing for inoculating the must for the AF at each PAC level (10%, 12% and 13% (v/v)). The criteria for readiness was a density decrease between 10-20 g/L and yeast population growth to 10^7-10^8 cells/mL. Each PdC triplicate of PdC was monitored until fermentation completion to obtain full kinetics curves.

Before inoculation into the must for AF, each PdC- met the optimal criteria at different times: 7 days for PdC13, 6 days for PdC12, and 5 days for PdC10

(Figure 1. A). Yeast population trends showed that the PdC12 population initially grew faster but PdC13 equaled it by day 2. PdC12 ultimately reached the highest population, exceeding 10^8 cells/mL. Notably, PdC10 showed slower initial growth, maintaining populations slightly over 10^5 cells/mL for the first 5 days before accelerating to reach similar levels as PdC13. Complete fermentation for PdC12 concluded by day 20, while PdC10 and PdC13 concluded 2 and 9 days later, respectively.

3.4. AF dynamics

The performance of three inoculation strategies (PdC, commercial yeast QA23, and spontaneous fermentation) was evaluated during AF at each PAC level (Figure 1. B-D) and supplementary Figure S2. The quickest fermentation for PAC 10% (v/v) was observed in AF-PdC10, completed in 1 day earlier than AF-C10 and 6 days earlier than AF-S10 (Figure 1. B). Yeast populations were similar across all modalities, though AF-S10 exhibited a longer adaptation period before reaching 10^7 cells/mL. At 12% (v/v) PAC, the AF-PdC12 fermentation was fastest, concluding 2 days before AF-C12 and 4 days before AF-S12 (Figure 1. C). The longest fermentation time occurred at 13% (v/v) PAC, with AF-PdC13, AF-C13, and AF-S13 concluding at 30, 35, and 43 days, respectively (Figure 1. D).

Across all trials, musts inoculated with PdC (AF-PdC-) exhibited faster density decrease and yeast population growth, particularly at 13% (v/v) PAC, and outperformed SF in terms of fermentation speed and yeast growth.

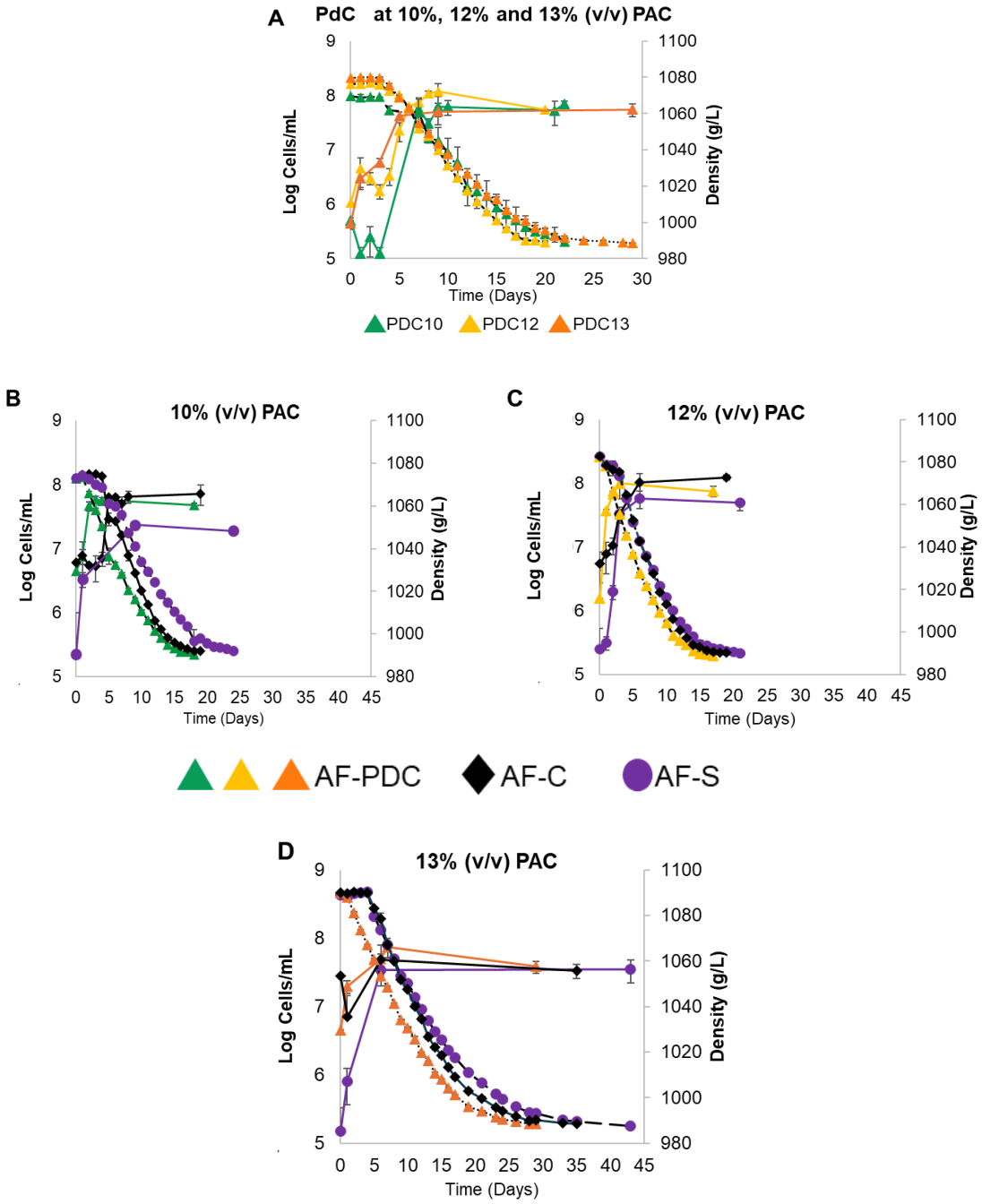

Figure 1. Fermentation kinetics at three maturity levels, measured as potential alcohol content (PAC). Green triangles represent 10% (v/v) PAC, yellow for 12% (v/v) PAC, orange for 13% (v/v) PAC. (A) *pied de cuve* (PdC). (B,C,D) Alcoholic fermentations (AFs) performed using different inoculation strategies: PdC (triangles: green for 10% (v/v) PAC; yellow for 12% (v/v) PAC; orange for 13% (v/v) PAC), a commercial yeast strain (diamonds: black) and spontaneous fermentation (circles: purple). Solid lines depict yeast population dynamics as measured by microscopy cell counts via microscopy, while dashed lines represent changes in sugar density over time. Each data point represents the mean of biological triplicates with its respective error bars.

Neither AAB nor LAB bacteria were detected in culture media sampled in the PdC treatments or across AFs at all PAC levels. These results indicate the healthy status of the grapes (data not shown).

3.5. S. cerevisiae strains diversity through Interdelta-PCR analysis and diversity indices

Phenotypic diversity at key fermentation stages was assessed using Interdelta-PCR analysis (Supplementary material Figure S3). A total of 540 colonies of Sce were grouped into 52 distinct profiles, analysed for diversity and of relative abundance in PdC and AFs across (data not shown). Diversity indices were calculated based on the different strains founded for each inoculation point.

Table 3. Genetic diversity indices of *Saccharomyces cerevisiae* strains across different maturity levels and inoculation strategies. Maturity levels are defined by the potential alcohol content (PAC) in % (v/v). The inoculation strategies in alcoholic fermentation (AF) include: *pied de cuve* inoculation (AF-PdC-), inoculation with a commercial *S. cerevisiae* strain QA23 (AF-C-) and spontaneous fermentation (AF-S-). The highest values obtained within each PAC level and fermentation stage are highlighted in bold.

		Shannon's indices			**Simpson's indices**		
Harvest	Treatment	BF	HF	FF	BF	HF	FF
	AF-PdC10	**1.92**	**1.58**	**1.01**	**0.87**	**0.79**	**0.57**
10% (v/v)	AF-C10	0.00	0.97	0.82	0.00	0.54	0.49
	AF-S10	1.07	0.90	0.20	0.68	0.57	0.10
	AF-PdC12	**1.34**	1.30	**1.48**	**0.72**	0.72	**0.77**
12% (v/v)	AF-C12	0.00	0.69	0.61	0.00	0.52	0.46
	AF-S12	0.77	**1.44**	1.20	0.49	**0.77**	0.63
	AF-PdC13	**2.29**	**2.03**	1.37	**0.94**	**0.89**	0.74
13% (v/v)	AF-C13	0.00	0.69	0.33	0.00	0.52	0.19
	AF-S13	1.93	1.46	**1.55**	0.86	0.73	**0.81**

Table 3 shows that AFs inoculated with PdCs had the highest diversity index in most and at all the maturity levels, except for musts fermented spontaneously in AF-S12 at HF stage and AF-S13 at FF stage. Treatments from the 12% (v/v) PAC did not exhibit higher diversity values than those from the 10% (v/v) PAC, contrary to expectations. All treatments inoculated with the commercial strain QA23 showed the lowest diversity index values, yet this strain did not dominate the FF stages, as previously commented.

3.6. Main oenological parameters

The organic acids and other key oenological parameters of the wines from all treatments and harvests were analyzed using HPLC and enzymatic kits. The HPLC analysis focused on sugars, ethanol, glycerol, and acids. Specifically, enzymatic kits measured citric, acetic, and malic acids. Significant differences were evaluated for treatments within each PAC level (Table 5).

Significant differences were observed across treatments within harvest at PAC of 10% (v/v). Ethanol production was highest in AF-PdC10 and lowest in AF-S10. Conversely, glycerol levels were highest in AF-S10 and lowest in AF-C10. AF-C10 had the highest values of citric, acetic, and succinic acids.

Harvest at 12 % (v/v) PAC did not result in significant differences in ethanol and tartaric production among treatments. Glycerol levels were similar in AF-C12 and AF-S12. The highest values of citric and succinic acid were observed in AF-C12. AF-PdC12 treatment had the lowest acetic acid value, while AF-S12 showed the highest value.

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Table 5. Main chemical parameters in Muscat Alexandria wines in different maturity levels that corresponds to % (v/v) of potential alcohol content (PAC): 10%, 12% and 13% (v/v). Different inoculation strategies were: AF-PdC, alcoholic fermentation (AF) inoculated with PdC (*pied de cuve*); AF-C, AF inoculated with *Saccharomyces cerevisiae* strain QA23; AF-S, AF inoculated with spontaneous fermentation.

	PAC 10% (v/v)			PAC 12% (v/v)			PAC 13% (v/v)		
HPLC									
	AF-PdC10	**AF-C10**	**AF-S10**	**AF-PdC12**	**AF-C12**	**AF-S12**	**AF-PdC13**	**AF-C13**	**AF-S13**
Glucose (g/L)	0.49 ± 0.9 ^{a}	0.54 ± 0.38 ^{a}	0.47 ± 0.38 ^{a}	0.81 ± 0.23 ^{a}	0.76 ± 0.99 ^{a}	0.76 ± 1.22 ^{a}	0.20 ± 0.45 ^{a}	1.65 ± 2.01 ^{a}	1.13 ± 2.15 ^{a}
Fructose (g/L)	1.16 ± 0.03 ^{a}	1.53 ± 0.40 ^{a}	1.24 ± 0.03 ^{a}	0.87 ± 0.06 ^{a}	1.64 ± 0.04 ^{a}	0.89 ± 1.11 ^{a}	2.54 ± 0.12 ^{a}	1.97 ± 0.05 ^{b}	1.94 ± 0.08 ^{b}
Ethanol (% (v/v))	11.75 ± 0.02 ^{a}	11.62 ± 0.05 ^{b}	11.29 ± 0.02 ^{c}	12.12 ± 0.04 ^{a}	12.01 ± 0.09 ^{a}	12.09 ± 0.11 ^{a}	12.85 ± 0.07 ^{a}	13.35 ± 0.04 ^{b}	13.35 ± 0.08 ^{b}
Glycerol (g/L)	4.98 ± 0.18 ^{a}	4.86 ± 0.02 ^{a}	7.74 ± 0.21 ^{b}	5.10 ± 0.06 ^{a}	6.32 ± 0.06 ^{b}	6.28 ± 0.13 ^{b}	5.86 ± 0.03 ^{a}	5.63 ± 0.03 ^{b}	5.61 ± 0.03 ^{b}
Citric acid (g/L)	0.55 ± 0.50 ^{a}	0.73 ± 0.04 ^{b}	0.36 ± 0.03 ^{c}	0.98 ± 0.03 ^{a}	1.08 ± 0.01 ^{b}	0.79 ± 0.02 ^{c}	0.37 ± 0.02 ^{a}	0.41 ± 0.07 ^{a}	0.69 ± 0.09 ^{b}
Acetic acid (g/L)	0.47 ± 0.03 ^{a}	0.62 ± 0.01 ^{b}	0.38 ± 0.04 ^{c}	0.46 ± 0.05 ^{a}	0.69 ± 0.02 ^{b}	0.86 ± 0.03 ^{c}	0.84 ± 0.05 ^{a}	0.80 ± 0.03 ^{a}	1.03 ± 0.04 ^{b}
Tartaric acid (g/L)	3.25 ± 0.11^{a}	3.28 ± 0.02 ^{a}	3.49 ± 0.04 ^{b}	2.04 ± 0.11 ^{a}	1.97 ± 0.16 ^{a}	2.63 ± 0.71 ^{a}	2.78 ± 0.04 ^{a}	2.77 ± 0.07 ^{a}	2.95 ± 0.06 ^{b}
Succinic acid (g/L)	0.87 ± 0.01 ^{a}	1.52 ± 0.09 ^{b}	1.12 ± 0.14 ^{c}	1.28 ± 0.04 ^{ab}	1.39 ± 0.08 ^{b}	1.16 ± 0.05 ^{a}	1.82 ± 0.06 ^{a}	1.73 ± 0.05 ^{a}	2.43 ± 0.05 ^{b}
Enzymatic kit									
Citric acid (mg/L)	216.33 ± 31.01^{a}	221.33 ± 6.66 ^{a}	222.00 ± 5.29 ^{a}	251.67 ± 2.31 ^{a}	258.00 ± 8.89 ^{a}	261.00 ± 9.64 ^{a}	226.33 ± 5.69 ^{a}	221.33 ± 2.08 ^{a}	222.33 ± 2.52 ^{a}
Acetic acid (g/L)	0.24 ± 0.06 ^{a}	0.31 ± 0.02 ^{a}	0.42 ± 0.01 ^{b}	0.29 ± 0.03 ^{a}	0.51 ± 0.01 ^{b}	0.50 ± 0.04 ^{b}	0.63± 0.01 ^{a}	0.62 ± 0.02 ^{a}	0.71 ± 0.04 ^{b}
L-Malic (g/L)	1.21 ± 0.18 ^{a}	1.50 ± 0.02 ^{b}	1.17 ± 0.02 ^{a}	1.35 ± 0.03 ^{a}	1.32 ± 0.03 ^{ab}	1.27 ± 0.03 ^{b}	0.71 ± 0.03 ^{a}	0.74 ± 0.01 ^{a}	0.62 ± 0.05 ^{b}

Different letters indicate that the values are significantly different within the same harvest (p-value < 0.05).

Harvesting at 13% (v/v) PAC, resulted in significantly lower ethanol production in AF-PdC13, but the highest glycerol concentration. Both AF-PdC13 and AF-C13 had the lowest levels of acetic, tartaric, and succinic acid, with no significant differences between them. AF-S13 had the highest concentration of citric, acetic, and tartaric acid.

Overall, the AF-PdC treatments exhibited the highest ethanol levels at 10% (v/v) PAC, but these levels declined to the lowest with higher maturity (13% (v/v) PAC). At 13% (v/v) PAC, AF-PdC13 and AF-C13 showed similar values for most parameters, except for ethanol and glycerol. AF-C treatments had higher citric acid levels at 10% and 12% (v/v) PAC, but AF-S treatments surpassed these levels at 13% (v/v) PAC. A similar trend was observed for acetic acid, where AF-C10 showed the highest levels initially, but spontaneous fermentations (AF-S12 and AF-S13) ultimately achieved the highest values (Table 5).

The highest value of malic acid at 10% (v/v) of PAC was observed in AF-C10. At 12% and 13% (v/v) of PAC, treatments inoculated with the commercial strain (AF-C12 and AF-C13) and those inoculated with PdCs (AF-PdC12 and AF-PdC13) exhibited the highest malic acid levels, with no significant differences among these two treatments.

3.7. Sensory analysis of wines

The sensory evaluation aimed to assess whether wines inoculated with PdC were distinct, acceptable, and had notable features. Tasters were tasked with identifying and differentiating the odour and flavour attributes of wines subjected to various inoculation strategies at each maturity level. The triangle test results indicated that tasters could successfully differentiate between treatments at the

10% (v/v) PAC with statistical significance ($p < 0.001$) (Table 4). However, as the maturity level increased, distinguishing between treatments became more challenging. At 12% (v/v) PAC, tasters could not differentiate between AF-S12 and AF-PdC12. By 13% (v/v) PAC, only AF-S13 and AF-C13 were distinguishable.

Table 4. Triangle test results for sensory analysis of wines at different maturity levels based on probable alcohol content (PAC): (A) 10% (v/v); (B) 12% (v/v) and (C) 13% (v/v). Each treatment corresponds to alcoholic fermentations (AF) using different inoculation strategies: AF-PdC (AF inoculated with *pied de cuve* (PdC)); AF-C (AF inoculated with a commercial strain *Saccharomyces cerevisiae* QA23); AF-S (spontaneous fermentation). S = Significant, NS = Not Significant. Assessors = 14 for each sensory session.

PAC level	Triangle 1 AF-PdC Vs. AF-S	Triangle 2 AF-C Vs. AF-S	Triangle 3 AF-PdC Vs. AF-C
10% (v/v)	< 0.001 S	< 0.001 S	< 0.001 S
12% (v/v)	0.327 NS	0.016 S	0.003 S
13% (v/v)	0.541 NS	0.016 S	0.327 NS

Attributes of the wines are illustrated in the spider plots shown in Figure 3 (A, B, C), while the distribution of all treatments across the three maturity levels is depicted in the PCA component plot (Figure 3. D). At the 10% (v/v) PAC level, AF-PdC10 wine scored the highest for terpenes and displayed similar scores for tropical, bitterness, and quality attributes compared to AF-C10. AF-S10 was characterized by its bitterness, oxidation, and vegetal notes.

At the 12% (v/v) PAC level, wines inoculated with PdC and the commercial strain QA23 exhibited similar results for terpene, tropical, and quality attributes. All

treatments showed comparable bitterness, acidity, and oxidation characteristics, although AF-S12 had the highest score for vegetal attributes.

By the 13% (v/v) PAC level, AF-PdC13 and AF-S13 treatments were similar in terpene and quality attributes, with AF-PdC13 showing the highest score for the tropical attribute. All treatments presented similar acidity, vegetal, and oxidation levels. However, wines inoculated with the commercial strain QA23 had the lowest scores for both terpene and quality attributes.

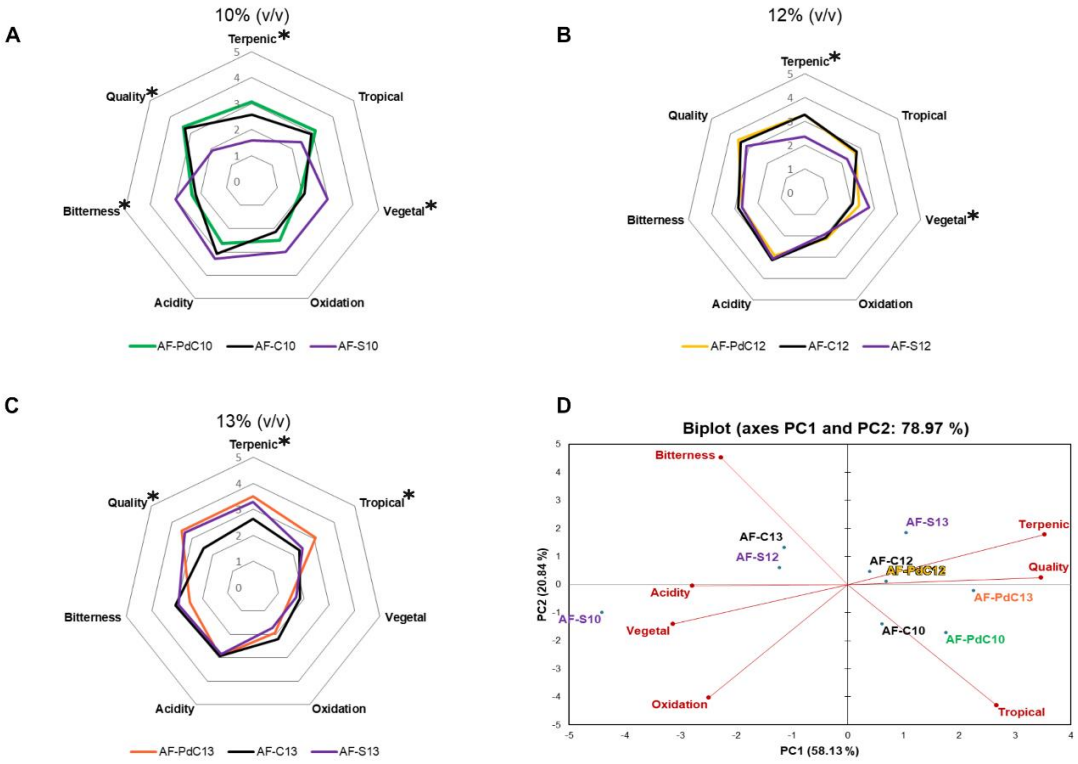

Figure 3. Spider plots and principal component analysis (PCA) of wines at different maturity levels based on probable alcohol content (PAC): (A) 10% (v/v); (B) 12% (v/v), and (C) 13% (v/v). Each treatment corresponds to alcoholic fermentations (AF) using different inoculation strategies: AF-PdC (AF inoculated with *pied de cuve* (PdC)); AF-C (AF inoculated with a commercial strain *Saccharomyces cerevisiae* QA23); AF-S (spontaneous fermentation).(D) PCA analysis of all samples tested in the experiments. The asterisks denote significant differences ($p < 0.05$).

The PCA analysis (Figure 3. C) shows that PC1 and PC2 explain 58.13% and 20.84% of the variability. The right side of the plot highlights attributes considered positive, such as quality, terpene, and tropical notes. Treatments inoculated with PdC tend to cluster near these positive atributes, indicating the benefits of this technique, even at less optimal maturity levels like 10% (v/v) of PAC. Treatments inoculated with the commercial strain, AF-C10, and AF-C12, as well as AF-S13, also fall on the positive side of the PCA plot.

3.8 Micro-fermentations of selected S. cerevisiae strains and chemical analysis

Eleven of the 52 different Sce strains, together with the QA23 strain as control, were selected based on their relative abundance and prevalence during the AFs with natural must to perform micro-fermentations using reconstituted concentrated must (Figure 3). The objective was to obtain a brief characterization of their fermentation performance and their individual influence over the chemical composition at the end of AF. Additionally, the screening would be useful to evaluate the oenological potential of these strains. The fermentation kinetics of the individual strains were similar with slight variations in cell concentrations during the process. Ten out of twelve AFs were completed after 10 days, with strains 6 and 29 taking one day longer. 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 Sce strains or other microorganisms.

Because the Sce strain 7 was present in all three PAC levels and imposed itself in all the AFs inoculated with the commercial strain QA23, an additional micro-fermentation was conducted co-inoculating the strains QA23 and 7 (Figure 3).

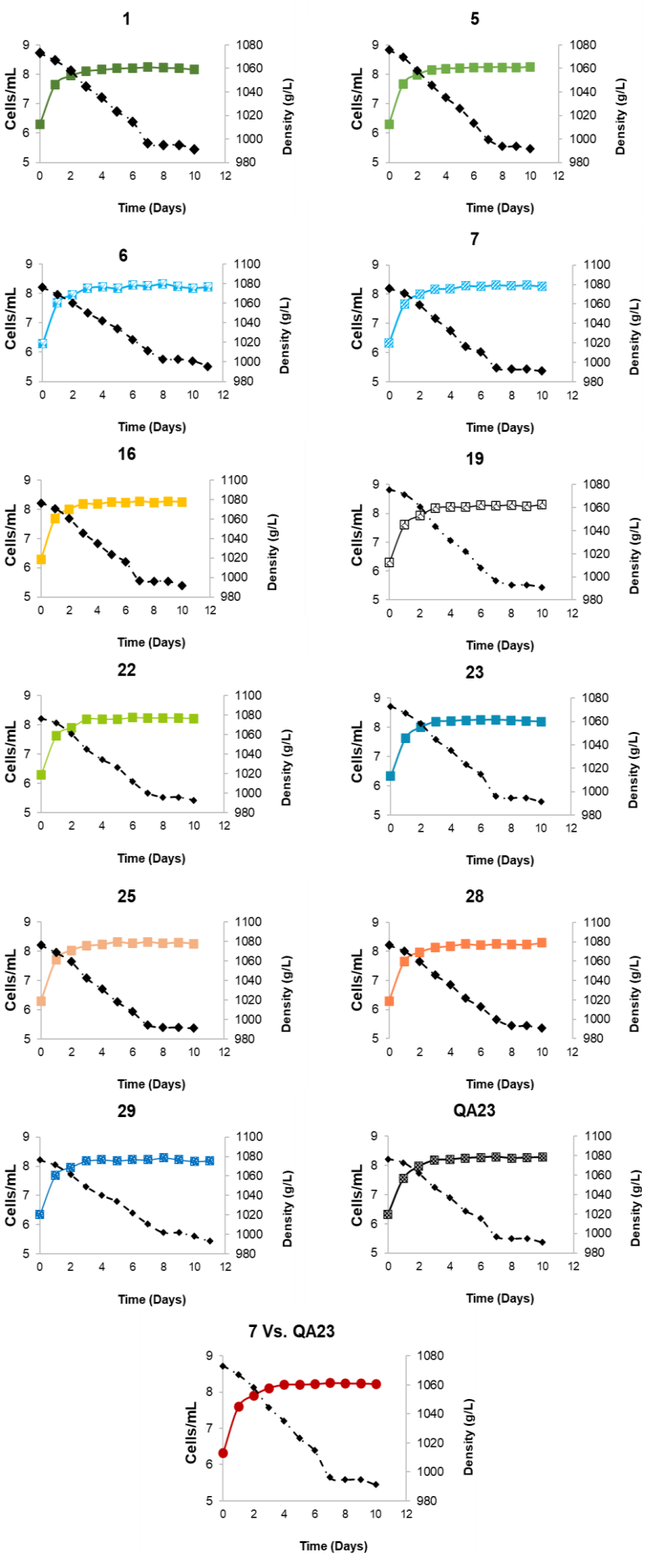

Figure 3. Alcoholic fermentation (AF) of stains of *Saccharomyces cerevisiae* (Sce) inoculated in individual (1,6,7,16,19,22,23,25,28,29, QA23) or co-inoculation (7 Vs. QA23) from the cellar experiments, and cultured in reconstituited concentrated must at 18 ºC. The yeast population is represented by the number of cells quantified indirectly by optical density at OD600 (solid lines) and fermentation kinetic is represented by the density (g/L) of the fermenting must (dashed lines).

The kinetic was highly similar to the individual fermentations of each of these strains. However, during the co-inoculation, the number of total cells was lower at the end of the fermentation, probably due to competition. Interdelta-PCR analysis of 15 random colonies picked from YPD medium inoculated at the end of fermentation, resulted in 13 profiles belonging to strain 7 and 2 to QA23 thus confirming the imposition of strain 7 over QA23, as previously observed in the natural must.

Results of the chemical metabolites production at the end of each micro-fermentation are shown in Table 4. Significant differences were found in the compared parameters except for ethanol and glycerol values. Strain 6 produced the highest concentrations of citric, acetic, and succinic acid levels which is in concordance with the lowest pH at the end of AF. The lactic acid production had the lowest value for strain 7 with 0.21 g/L and the highest value for the QA23 strain with 0.28 g/L.

Table 4. Main chemical parameters in of micro fermentations realized with strains selected from the three harvest points (10, 12 and 13% (v/v).

Strain	Ethanol (% v/v)	Glycerol (g/L)	Citric acid (g/L)	Acetic acid (g/L)	Tartaric acid (g/L)	Succinic acid (g/L)	Lactic acid (g/L)	pH
Sce1	10.60 ± 0.05 ^{ab}	5.96 ± 1.16 ^{a}	0.66 ± 0.10 ^{a}	0.82 ± 0.04 ^{ab}	1.15 ± 0.02 ^{a}	2.23 ± 0.03 ^{ac}	0.23 ± 0.03 ^{abd}	3.31
Sce5	10.66 ± 0.04 ^{ab}	5.12 ± 0.77 ^{ab}	0.61 ± 0.06 ^{a}	0.62 ± 0.02 ^{bc}	1.17 ± 0.04 ^{a}	2.19 ± 0.09 ^{ac}	0.26 ± 0.03 ^{abcd}	3.31
Sce6	10.42 ± 0.01 ^{b}	5.85 ± 1.00 ^{ab}	1.56 ± 0.37 ^{b}	0.87 ± 0.10 ^{a}	1.19 ± 0.03 ^{a}	2.84 ± 0.15 ^{b}	0.27 ± 0.02 ^{acd}	3.12
Sce7	10.55 ± 0.05 ^{ab}	4.64 ± 0.17 ^{ab}	0.72 ± 0.04 ^{ac}	0.71 ± 0.07 ^{abc}	1.15 ± 0.01 ^{a}	2.55 ± 0.11 ^{ab}	0.21 ± 0.01 ^{b}	3.32
Sce16	10.52 ± 0.13 ^{ab}	4.69 ± 0.08 ^{ab}	0.74 ± 0.01 ^{ac}	0.64 ± 0.02 ^{bc}	1.15 ± 0.01 ^{a}	2.37 ± 0.08 ^{ac}	0.23 ± 0.01 ^{abcd}	3.30
Sce19	10.59 ± 0.12 ^{ab}	4.48 ± 0.19 ^{b}	0.65 ± 0.09 ^{a}	0.60 ± 0.03 ^{c}	1.16 ± 0.01 ^{a}	2.51 ± 0.12 ^{ab}	0.23 ± 0.01 ^{abcd}	3.31
Sce22	10.65 ± 0.05 ^{ab}	4.79 ± 0.18 ^{ab}	1.35 ± 0.11 ^{bd}	0.57 ± 0.04 ^{c}	1.17 ± 0.01 ^{a}	2.34 ± 0.11 ^{ac}	0.26 ± 0.03 ^{abcd}	3.26
Sce23	10.67 ± 0.09 ^{ab}	4.68 ± 0.07 ^{ab}	1.07 ± 0.08 ^{cd}	0.71 ± 0.07 ^{abc}	1.15 ± 0.01 ^{a}	2.20 ± 0.07 ^{ac}	0.27 ± 0.01 ^{ac}	3.31
Sce25	10.43 ± 0.26 ^{b}	4.95 ± 0.03 ^{ab}	0.67 ± 0.02 ^{a}	0.68 ± 0.15 ^{abc}	1.13 ± 0.03 ^{a}	2.03 ± 0.25 ^{c}	0.22 ± 0.02 ^{bd}	3.34
Sce28	10.51 ± 0.06 ^{ab}	4.81 ± 0.07 ^{ab}	0.74 ± 0.03 ^{ac}	0.65 ± 0.10 ^{bc}	1.15 ± 0.01 ^{a}	2.35 ± 0.17 ^{ab}	0.25 ± 0.00 ^{abcd}	3.30
Sce29	10.54 ± 0.01 ^{ab}	4.58 ± 0.22 ^{ab}	0.59 ± 0.06 ^{a}	0.60 ± 0.02 ^{c}	1.13 ± 0.06 ^{a}	2.03 ± 0.28 ^{c}	0.25 ± 0.00 ^{abcd}	3.24
QA23	10.77 ± 0.07 ^{a}	4.55 ± 0.01 ^{ab}	0.66 ± 0.06 ^{a}	0.61 ± 0.11 ^{bc}	1.14 ± 0.01 ^{a}	1.95 ± 0.03 ^{c}	0.28 ± 0.01 ^{c}	3.38
QA23 Vs. Sce7	10.65 ± 0.13 ^{ab}	4.94 ± 0.16 ^{ab}	0.70 ± 0.05 ^{a}	0.57 ± 0.02 ^{c}	1.15 ± 0.00 ^{a}	2.32 ± 0.10 ^{ac}	0.23 ± 0.01 ^{abcd}	3.35

Different letters indicate that the values are significantly different within the same harvest (p-value < 0.05).

4. Discussion

Traditionally winemaking practices often involved inoculating a PdC from a must fermenting spontaneously to initiate AF. However, this method has been decreased over time in favour of using DWY due to its reliability in achieving predictable fermentation kinetics and consistent aromatic profiles (González and Morales, 2021). Nonetheless, the widespread use of DWY has contributed to a uniformization of wine styles across the market (González and Morales, 2021; Mas et al., 2016).

Consumer's preferences increasingly favour wines that exhibit regional distinctiveness and complexity, while also being produced with minimal inputs and using environmental-friendly practices (Belda et al., 2021). The development of unique wine profiles depends on the complex interaction of abiotic factors (such as soil, water, solar light, and climate) and biotic factors (including the microbiome in the soil and phyllosphere, grape variety, vineyard biodiversity, and winemaking practices) (Mezzasalma et al., 2018). Therefore, researchers are exploring strategies to enhance the microbial and organoleptic complexity of wines while minimizing the risks of microbial spoilage.

One promising approach involves the use of modified PdC with selective pressures such as SO_2 and ethanol, and temperatures as a *cellar-friendly* strategy to regulate yeast population in PdC, thereby affecting fermentation outcomes (Bedoya et al., 2024a). Our previous research focused on optimizing PdC modifications comparing their efficiency over different harvests, geographical locations, and using two grape varieties (Bedoya et al., 2024b). We found that a PdC with 40 mg/L of SO_2 and 1% (v/v) ethanol at 18 ºC exhibited the

most favourable organoleptic characteristics and the lowest acetic acid levels in most of the harvest evaluated.

Maturation and ripening processes shape the fungal diversity and richness in grape surface (Barata et al., 2012; Liu and Howell, 2021; Martins et al., 2014) with sugar accumulation affecting microbial diversity from fruit set to harvest, where the exudation of these sugars proceeds (Liu and Howell, 2021; Renouf et al., 2005). The microbiota on the grape surface is transferred to the grape must and, consequently, influences the wine's flavour, aroma, and quality of wine (Morrison-Whittle and Goddard, 2018). Previous studies have demonstrated the importance of this microbiota in shaping fermentation characteristics (Bokulich et al. (2016). This study is pioneering for its comparison of inoculation strategies at different maturation levels (10%, 12% , and 13 % (v/v) PAC), including a modified PdC, DWY, and SF. Our findings indicate that PdC inoculations musts, especially those with 12% (v/v) PAC, led to faster yeast population growth and accelerated fermentation kinetics compared to DWY or SF inoculations. The PdCs modified with SO_2 and ethanol exhibited better adaptation and higher yeast populations in new musts, resulting in improved fermentation kinetics. This aligns with findings by Moschetti et al. (2016) and our previous studies Bedoya et al.(2024a, 2024b), which reported that similar PdC formulations enhanced yeast populations and fermentation performance.

The time required for fermentation completion varied with PAC levels, with 13% (v/v) PAC taking longer due to the higher sugar concentrations. The slower fermentation at this PAC level was attributed to changes in sugar ratios and YAN levels, affecting fermentation kinetics. Specifically, higher fructose concentrations

at 13% (v/v) PAC could have favoured non-Sce species, which are less efficient fermenters compared to Sce, which prefers glucose (Kayikci and Nielsen, 2015; Varela and Borneman, 2017). The diminishing YAN availability at higher PAC levels also negatively impacted fermentation kinetics (Ribéreau-Gayon et al., 2006). Despite this, PdC inoculations consistently outperformed DWY and SF in fermentation kinetics, especially at higher PAC levels.

Sce diversity was assessed using Interdelta regions, revealing distinct patterns of yeast strain composition across different inoculation strategies and PAC levels, with just some profiles shared in certain modalities. In general, PdC inoculated fermentations exhibited higher yeast strain diversity (except for some stages of the AF-S fermentations), which could have contributed to the observed differences in sensory attributes. This result agrees with Moschetti et al. (2016) who found a high diversity of Sce strains in AFs inoculated with PdC with the addition of SO_2 and ethanol and with Börlin et al. (2020) that reported the highest diversity indices for barrels conducting the AF spontaneously.

Although the commercial strain inoculated in AF-C fermentations was detected in AF-PdC10 fermentations, this contaminant strain was outperformed by other strains. In fact, the AF-C fermentations were not dominated at any of the PAC levels by the commercial strain inoculated at the beginning. This result indicates better adaptation and competition of the autochthonous strains (Clavijo et al., 2010; Fleet, 2008). The only Sce strain detected in all the modalities of fermentations and all PAC level, was strain 7. This strain outcompetes the inoculated QA23 during the experiments with natural musts as well as the micro-

fermentations with reconstituted concentrated must, proving to be a good candidate for its oenological use.

SF, while simpler and often resulting in high-quality wines, was less consistent in its microbial diversity and fermentation outcomes compared to PdC inoculations. Our results showed that SF sometimes had lower relative abundance of Sce strains, particularly at higher PAC levels, which could affect wine quality.

Chemical differences were observed for several parameters across modalities within each PAC level. AF-PdC modality showed the highest sugar consumption at the end of AF, probably associated with the higher wild Sce strains selected under pressure stress factors previous to inoculation had good fermentative capacities. These indigenous strains reproduce quickly in fermentation and are very efficient in converting glucose into biomass (Spor et al., 2008). Significant differences in ethanol production were observed just for AF-PdC10, thus, we could not conclude that the PdC technique served as a strategy for decreasing ethanol in AF. In micro-fermentations, the levels of acetic acid can be considered within the normal values for laboratory fermentations. However, the values obtained for strains 1, 2, and 3 would be considered significantly different and with a detrimental concentration.

Sensory characteristics of the wines depend on the variability of the microbiota in the vineyard and cellar each harvest (González and Morales, 2021). In general, as grape maturity levels increased, the perceived differences by the assessors among treatments decreased. At 10% (v/v) PAC, the AF-PdC10 showed a higher score for positive attributes than AF-S10, which exhibited the highest scores for vegetal, oxidation, acidity and bitterness attributes in the sensory test.

Interestingly, the Sce strain 1, which showed the second highest acetic acid production during micro-fermentations, dominated the FF of AF-S10 and could have been responsible for the higher acidity reported by assessors. Regardless of the PAC level, AF-PdC resulted in wines with a consistent attributes profile showing better or equal organoleptic characteristics than AF inoculated with DWY and better positive attributes than SF at PAC 10% and 13% (v/v).

5.. Conclusion

In summary, PdC guarantees rapid kinetics fermentation over SF, and comparable to AFs inoculated with DWY. Also, PdC inoculations offer a higher diversity of Sce strains than AFs inoculated with DWY, and in some cases equal to or higher than SF. The presence of several Sce strains has been reported for improving the complexity of the sensory profile of wine (Fleet, 2003). This has been observed in the different PAC levels, where PdC showed higher scores for attributes such as terpene, tropical and quality. The PdC modified with SO_2, ethanol and fermented at low temperature, guaranteed a rapid kinetic, safe fermentation and complexity in diverse microbiome and in sensory tests.

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CRediT authorship contribution statement

Katherine Bedoya: Writing – review & editing, Writing – original draft, Validation, Methodology. Luis Buetas: 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.

Data Availability Statement: the original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Conflicts of Interest: The authors declare no conflicts of interest.

Acknowledgments: None

Supplementary data:

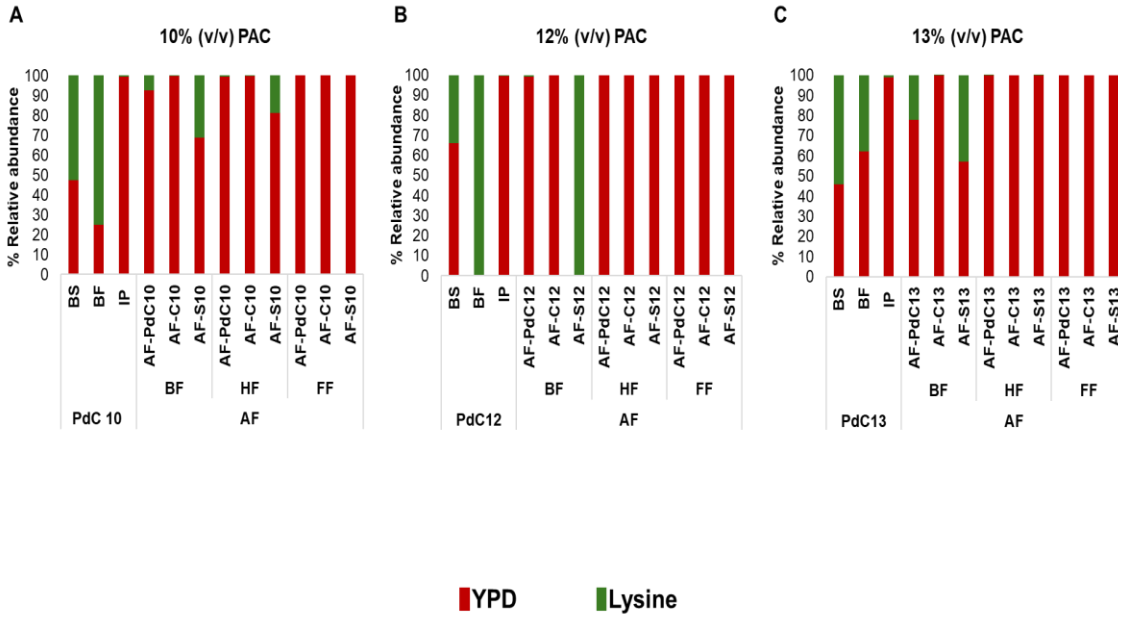

Figure S1. % Relative abundance of total yeast recovered in YPD medium (red barrels) and in Lysine medium for non-*Saccharomyces* in all PAC levels (A) 10% (v/v) PAC. (B) 12% (v/v) PAC. (C) 13% (v/v) PAC. In each PAC were sampled were took from the **(i) *pied de cuve*** (PdC) at different points: (BS), beffore settling; (BF), beginning fermentation; (IP), inoculation point when is ready to be used as starter and **(ii) alcoholic fermentation** (AF) at different points: (BF), beginning fermentation; (HF), half fermentation; (FF), final fermentation. Incoulation strategies used were: AFs inoculated with the PdC (AF-PdC-), with a commercial strain QA23 (AF-C-), spontaneous fermentation (AF-S-).

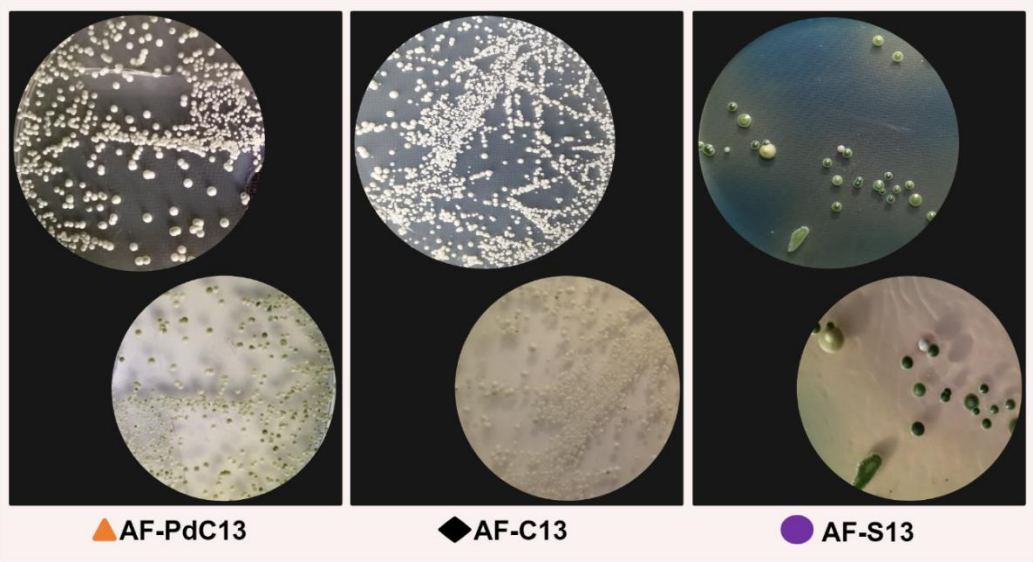

Figure S2. Colony-forming unit (CFU/mL) in Wallerstein Laboratory Nutrient (WLN) medium recovered at the beginning of alcoholic fermentation (AF) at maturity level of 13% (v/v) PAC (probable alcohol content) using different inoculation strategies: *pied de cuve* (PdC) (triangle, orange), with a commercial strain QA23 (diamond, black), and spontaneous fermentation (circle, purple). For each treatment same medium is showed with different background to appreaciate better the colour contrast of the colonies.

General Discussion

Winemaking has been facing an increasing demand for wines that preserve the microbial yeast biodiversity and the aroma complexity, associated both terms with the *terroir* concept (Belda et al., 2017c; Carrau et al., 2020; Fleet, 2003). At the same time, consumers are interested in products from environmentally friendly practices with low intervention during winemaking. Several strategies have been developed to maintain this microbiota as SF, the use of local non-Sce and Sce consortia, and PdC (Mas and Portillo, 2022).

The PdC as a traditional technique, has emerged as a promising solution to meet wine's consumer demands. Research is crucial to understand the impact of the PdC on AF, including microbial dynamics, strain diversity preservation, fermentation rates, microbial safety, and both chemical and sensory quality.

The objective of this thesis is to biotechnologically assess the impact of various *cellar-friendly* stressors on the PdC preparation and its subsequent inoculation into a fresh must. Specifically, it seeks to ensure reproducible, high-quality results in terms of microbiological, chemical, and sensory attributes, comparing these outcomes with those of wines inoculated with DWY and those produced by SF.

- **Impact of stressors on PdC preparation**

Different practices in winemaking and environmental factors impact microbial yeast community and sensory characteristics in wines (Gilbert et al., 2014; Sacchi et al., 2005). Processes such as settling, additive use (SO_2), temperature, and the fermentation itself modify the microbial composition of the must.

Additives like ethanol and SO_2 can intentionally be used to modify the yeast population in the PdC, and consequently, influence the performance of AF

inoculated with it. For example, Moschetti et al. (2016) found that PdC preparation with added ethanol exhibited a higher number of Sce strains compared to PdC without ethanol and those inoculated with DWY. While non-Sce diversity decreased, Sce strains were more abundant.

Traditionally, the PdC has been considered a low-intervention practice carried out by spontaneous microbiota. However, the application of stressors related to wineries environment, can modulate yeast population in PdC, yielding more predictable results. In **Chapter I** of this thesis, different stressors (SO_2, ethanol, and temperatures) were evaluated to prepare different PdC in both laboratory and cellar settings. At laboratory scale, different concentrations of SO_2, ethanol and temperatures altered populations of an inoculated consortia of non-Sce and Sce in PdC, also impacting the kinetics of the AFs. Temperature was found as the major yeast stressor. Additionally, the inoculation time was evaluated to preserve desirable yeast diversity of the consortia used in laboratory experiments to mimic a natural must microbiota. Our results showed that, within 48 h the PdC, some non-Sce could remain, and at this point, the total yeast population reached 10^7-10^8 cells/mL and with the initial must density dropped in 10-20 g/L. These results provided a criterion to use the PdC method in the cellar, where natural musts variability is an additional factor.

Significant differences in yeast populations were observed by qPCR at the initial stage of the AF using natural must. The diversity of Sce strains varied among different PdC modalities (**Chapter I**). Additionally, significant differences in both chemical and sensory analysis from different PdC with and without stressors, fermented at different temperatures, impacted fermentation kinetics, chemical

composition, and sensory appreciation (**Chapter II**). These findings showed that the selected stressors can modulate yeast populations, provide different patterns of Sce strains, and consequently, can alter the aromatic profile in wines. Nonetheless, more research is needed to evaluate other stressors in PdC preparation, using different grape varieties, and searching strategies to evaluate accurately and widely other yeasts that could participate in this process.

- **PdC and kinetics of AF**

Good fermentation kinetics in winemaking include a fast fermentation onset and rate, measured by quick sugar consumption kinetics. These traits are directly related to tolerance to osmotic and ethanol stress and efficient nitrogen utilization (González and Morales, 2021). Traditionally, PdC has served as a good inoculation strategy to inoculate AF. Robust evidence has consistently concluded that PdC guarantees a quick fermentation onset and a good rate compared to SF, and comparable to AFs inoculated with DWY (Börlin et al., 2020; Clavijo et al., 2011; Li et al., 2012; Moschetti et al., 2016).

In **Chapter I**, the inoculation of AFs with certain PdC modalities resulted in a delay of only 1-2 days compared to those inoculated with DWY. In **Chapter II**, the effectiveness in rate kinetics of PdCs (with SO_2, and ethanol at two different temperatures) was consistent in different grape varieties, vintages, and geographic locations. PdC-inoculated AFs resulted in better kinetics than those inoculated with commercial strain QA23 and significantly better than SF. In **Chapter III**, PdC inoculation confirmed that PdC generally exhibited superior fermentation kinetics compared to SF and was slightly better than DWY across

three different maturity levels These results confirm that the PdC method guarantees a quickest start in AFs, showing good fermentation rates.

- **Microbiological control using PdC**

Controlling wild microorganisms in grape must is an advisable oenological practice to ensure the imposition of yeast starter cultures during AF and to prevent the growth of undesirable yeasts, LAB, and AAB that lead to spoilage of wine (Tedesco et al., 2022).

The PdC method has been described as a good starter culture that guarantees microbiological control because it promotes viable and adapted yeast cells to start the AF (Moschetti et al., 2016). For instance, Moschetti et al. (2016) did not detect spoilage microorganisms such as *Dekkera* or *Brettanomyces* spp., or LAB and AAB in any PdC modalities used during the PdC fermentation or AFs inoculated with them. More research is needed to study the presence of spoilage microorganisms during the application of the PdC method.

Some studies evaluated the microbial safety status of PdC indirectly by assessing the presence or absence of off-flavours or off-odours. In this sense, some studies have related the increase of volatile acidity related with the presence of spoilage microorganisms, however, none of these studies has confirmed their presence by means of identification or quantification techniques (Álvarez-Barragán et al., 2023; Börlin et al., 2020). Morgan et al. (2019) identified only the genera *Tatumella* in high proportion in one of the SF and the AF inoculated with spontaneous PdC. However, this *Tatumella* was associated with the total absence of use of SO_2 during AFs. These bacteria possibly caused the conversion of L-Malic to Lactic, an undesired process during some vinifications.

All treatments of this thesis did not show off-favours or off-odours associated with spoilage microorganisms (**Chapter II** and **III**). No LAB and ABB population were recovered during PdC preparation or in any of the AFs during the three PAC levels evaluated (**Chapter III**). The small volume of grapes collected in all experiments of this thesis to elaborate the PdC and conduct the AFs, implied a careful handled of grapes to preserve their health status. This prevents the entrance of undesirable microorganisms through wounds such as powdery mildew, *B. cinerea*, *Cladosporium* spp., *Aspergillus*, and *Penicillium* spp (Barata et al., 2012). Still, this carefulness is not possible in an industrial or traditional cellar where a larger volume of grapes is processed.

As the microbiome present in vineyard changes each season, the use of stressors in the preparation of the PdC such as SO_2 and ethanol, could limit or prevent the growth of spoilage microorganisms. These stressors could select the most suitable yeasts in the PdC, which quickly dominate the AF once inoculated into the fresh must. Our results confirm that PdC is a good inoculation strategy to exert microbiological control in AF.

- **PdC as a source of microbial diversity**

PdC inoculation has been recognized as an important strategy to preserve the microbiological diversity present in vineyards (Börlin et al., 2020). Studies using Interdelta regions or NGS have highlighted the role of PdC in maintaining diverse yeast strains. Moschetti et al. (2016) found the highest concentration strains isolated in the spontaneous PdC with the addition of ethanol, reflected also in trials of the AF inoculated with them. Similarly, Álvarez-Barragán et al. (2023) reported different selections of indigenous Sce in AFs inoculated with

each PdC modality, and the patterns of strains were different from those observed for SF.

Our research, detailed in **Chapters I** and **III**, found that PdC maintained a higher diversity of Sce strains compared to DWY. PdC with added stressors showed increased Sce strain diversity and reduced non-Sce competition, potentially allowing more adapted Sce strains to dominate AF (Moschetti et al., 2016). Indigenous Sce strains have been described to display cooperative behaviour; therefore, it is not expected that a single strain dominates the entire fermentation (Spor et al., 2009, 2008). The results of **Chapters II** and **III** show that no single Sce strain dominates any of the PdC inoculated fermentations.

- **PdC impacted on chemical profile of wines.**

The main chemical parameters of interest in wines are ethanol, glycerol, and organic acids, as they confer stability and contribute to the sensory properties of wines. In this thesis, the use of different modalities of PdC using various stressors affected the microbial composition of the PdC and consequently impacted the chemical characteristics of the resulting wines. Significant differences in chemical parameters among AFs inoculated with PdC, DWY, and SF were found in **Chapters II** and **III**.

In **Chapter II**, the sugar consumption was higher in AFs inoculated with DWY compared to those inoculated with PdC prepared at 18 ºC AFs in harvest M2 and M3. In **Chapter III**, only at the harvest level 13% (v/v) PAC, AFs inoculated with PdC showed a higher and significantly different value of fructose. This increase may be attributed to the low-temperature fermentation of PdC, which

allowed the growth of non-Sce yeasts which could not be able to consume the remaining sugars in the fermentation. For instance, Börlin et al. (2020) found that *H. uvarum* conducted the fermentation in all PdC modalities, while Morgan et al. (2019) found higher values of fructose associated with lower ethanol concentration and slower fermentation kinetics. These results suggested that a higher proportion of vineyard-derived non-Sce yeasts may result in sluggish or stuck fermentation. However, both studies did not use any stressor or additive to control the growth of non-Sce or other microorganisms in any of the PdC used.

One of the critical parameters in winemaking is acetic acid. High concentration of acetic acid levels has been related to the SF and values than exceeds 1 g/L could lead to stuck fermentation. Acetic acid acts independently or in synergy with ethanol, inhibiting the growth and fermentation activity of Sce, particularly in the last stages of fermentation when the concentration of these compounds are the highest (Eglinton and Henschke, 1999). A high concentration of acetic is responsible for the generation of the off-flavours or off-odours, conferring a bitter, vinegar-like aroma acid in wine (Campaniello and Sinigaglia, 2017).

The literature reveals varied results for acetic acid levels among AFs inoculated with different PdC. Börlin et al. (2020) found an increase of 0.6 g/L of acetic acid for one modality of AFs inoculated with a spontaneous PdCs. Similarly, Álvarez-Barragán et al. (2023) reported 0.45 g/L of acetic acid for AF inoculated with a spontaneous PdC, which was high compared to AF inoculated with DWY (0.35 g/L) or a SF (0.33 g/L). However, other studies did not show significant differences for this parameter across PdC inoculation strategy.

In contrast, **Chapter II** reports similar values of acetic acid for all treatments in the 2022 harvest. However, in both S3 and M3 harvests, the AFs inoculated with a PdC containing SO_2 and ethanol and fermented at 18 ºC showed the lowest values for acetic acid in all the essays. Similarly, Moschetti et al. (2016) observed lower acetic acid concentration in wines inoculated with PdC containing 1 and 3% (v/v) of ethanol compared to both AFs inoculated with a PdC without ethanol or inoculated with a PdC containing a DWY. They added systematically 30 mg/L of SO_2 to all PdC modalities. Their PdC conditions were similar to our PdC modalities tested in this chapter (40 mg/L SO_2 and 1% (v/v) ethanol). Moschetti et al. (2016) and our experiments differed uniquely in the fermentation temperatures of the preparation of the PdC and the AFs.

In agreement with **Chapter II**, **Chapter III** also found that AFs inoculated with PdC containing SO_2 and ethanol and fermented at 18 ºC, exhibited the lowest values of acetic acid, except in early harvest level of 10% (v/v) of PAC. As expected, in both PAC levels of 12% and 13% (v/v), SF showed the highest values in acetic acid. This may be due to SF harbouring non-Sce that vary in the acetic acid and ethyl acetate production or the presence of bacteria that could produce high acetic acid (Ciani and Maccarelli, 1998; Song et al., 2017). The results suggest that PdC with SO_2 and ethanol, fermented at lower temperatures, exert pressure that selects for yeasts that produce low yields of acetic acid (Moschetti et al., 2016). Nonetheless, more evidence is needed applying stressors in PdC to start AFs using other grape varieties.

Glycerol values were generally similar among all AFs within each harvest, with the exception of P-M2-18SE and P-M3-18SE. Low fermentation temperatures

may favour the growth of high glycerol-producer yeasts, as *S*. *uvarum* or *S*. *kudriavzevii* or their hybrids, as well as non-Sce strains like *T*. *delbrueckii* (**Chapter II)** (Alonso-del-Real et al., 2017b; Belda et al., 2015). However, literature related to PdC and its impact on glycerol is sparse. Moschetti et al. (2016) quantified this metabolite but they did not find significant differences among treatments.

In **Chapter II**, succinic acid concentration was higher in AFs inoculated with a DWY during harvest M3. In **Chapter III**, AF-C at 10% (v/v) PAC obtained the highest values of citric and succinic acid, with SF showing the second highest value at 13% (v/v) PAC. Succinic acid can increase for various strains (cryotolerant species *S*. *uvarum*, *S*. *eubayanus*, and *S*. *kudriavzevii*) when nitrogen is limited (Sainz et al., 2022; Su et al., 2019). At higher PAC levels, lower nitrogen is available in the must, plus the lower temperature of fermentation may favour the growth of these *Saccharomyces* non-*cerevisiae* species that are capable of producing more succinic acid.

The increased glucose levels is associated with an increase of citric acid production, which may result from non-Sce such as *Hanseniaspora*, *Lachancea*, and *Metschnikowia* spp. or the presence of LAB during the fermentation process. However, no LAB colonies were recovered in any trial.

- **PdC results in sensory profile complexity**

The complexity of wine's sensory profile is directly related to the interactions of microorganisms and winemaking practices (Fazio et al., 2023; Fleet, 2003). Several research studies have demonstrated that different yeast species and strains can influence the sensory characteristics of wines (Albertin et al., 2017; Suárez-Lepe and Morata, 2012).

Differences in sensory profile are expected with the application of PdC, as it provides a source for indigenous strains, such as SF. Both PdC and SF contribute to the profile complexity in wines due to their inherently variable nature. However, sensory results can differ significantly, even when both techniques did not use a commercial yeast starter. For example, Morgan et al. (2019) found similitudes and differences between SF and AFs inoculated with a spontaneous PdC without additives. Both wines were similar by having the highest scores for body and sweetness attributes. The perception of sweetness could be due to the remaining level of fructose in AF inoculated with PdC and the SF. Both treatments also exhibited low acidity and higher scores for pome fruit and tropical fruit aromas/flavours, but they were separated by many other attributes. Wine inoculated with PdC without stressors had a distinct sensory profile compared to SF wines.

Conversely, Álvarez-Barragán et al. (2023) reported undesirable characteristics in wines inoculated with spontaneous PdC without additives, such as glue/solvent-like attributes while other authors have not significant differences in sensory evaluations (Börlin et al., 2020; Ubeda Iranzo et al., 2000). This inconsistency underscores the unpredictability of PdC as a winemaking technique.

To address this variability and assess the impact of PdC on sensory attributes with more reliability, this thesis evaluated sensory attributes in three different harvests, (**Chapter II** and **III**). In **Chapter II**, significant differences were revealed between AFs inoculated with various PdC and those inoculated with DWY, except for P-M2-26SE in harvest M2. Differences were found in bitterness, acidity, and vegetal attributes showing the highest scores in the wines inoculated with DWY. In harvest S3 and M3, panellists could differentiate between PdC-inoculated wines and those inoculated with DWY or SF through triangle tests. Although no significant differences were found among attributes, the inoculation of PdC with SO_2 and ethanol, and fermented at 18 ºC resulted in wines with higher scores in tropical attributes during harvests M2 and S3. Additionally, wines resulting from the PdC technique had similar global quality scores to those inoculated with DWY in the M3 harvest.

In **Chapter III**, the panellists were able to differentiate between some treatments at different PAC levels, however, the difficulty of differentiation increased as PAC levels rose. Wines produced by PdC and DWY inoculation at PAC levels of 10% and 12 % (v/v) had similar global scores. In contrast, SF at these PAC levels received higher negative scores for attributes such as vegetal, oxidation, acidity, and bitterness. For wines produced at PAC 13% (v/v), PdC and SF modalities were quite similar, but the tropical attribute was again more pronounced in the PdC-inoculated wines.

As a conclusion, the modification in parameters for the preparation of the PdC and its inoculation to initiate the AFs significantly impacted the wines. The PdC modified with SO_2, and ethanol and fermented at 18 ºC ensured fermentation with

good kinetics, preserving microbial diversity of Sce strains and reducing acetic acid concentration in AF. Wines inoculated with this strategy showed consistent sensory characteristics, such as tropical attributes, without sensory defects for the majority of treatments, across different harvests, geographical locations and grape varieties.

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Conclusions

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1. The *pied de cuve* (PdC) has proven to be an effective method to facilitate alcoholic fermentation (AF), demonstrating a rapid onset and high fermentation rate that is comparable to the inoculation with selected commercial yeast strains.
2. Proper handling of the PdC not only promotes optimal fermentation but also microbial diversity that allows for unique flavor profiles and distinctive characteristics.
3. At the final point of fermentation with PdC, several different *Saccharomyces cerevisiae* strains can be isolated/detected, highlighting the ability of the methodology to promote a range of yeast strains throughout the fermentation process.
4. Using an appropriate combination of SO_2, ethanol, and temperature, it is possible to modulate the microbial population to enrich the PdC in non-*Saccharomyces* or *S. cerevisiae* yeasts. In this way, winemakers can obtain wines with appropriate complexity or improve fermentation efficiency.
5. Regardless of the maturity level of the grapes, the using of PdC methodology resulted in wines with a consistent attribute profile, showing better or equal organoleptic characteristics than wines produced by the inoculation with commercial strains and better positive attributes than spontaneous fermentations when the maturity level was not optimal.
6. The present thesis found the practicalities for achieving different microbial populations using controlled conditions, such as varying different SO_2 concentrations, ethanol addition, or temperatures, all tested under strict oenological conditions

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KATHERINE BEDOYA ALVIRA

Appendix

Publications derived from this PhD thesis:

- Influence of different stress factors during the elaboration of grape must's *pied de cuve* on the dynamics of yeast populations during alcoholic fermentation Bedoya, K., Buetas, L., Rozès, N., Mas, A., and Portillo, M. C. ***Food Microbiology*** (2024).
- The impact of the inoculation of different *pied de cuve* on the chemical and organoleptic profiles of wines**.** Bedoya, K. Mas, A., Rozès, N., and Portillo, M. C. ***Microorganisms*** (2024).
- Multi-approach analysis of *pied de cuve* across different levels of grape ripeness**.** Bedoya, K., Buetas, L., Rozès, N., Mas, A. and Portillo, M. C. Manuscrip in preparation.

Contribution to national and international meeting

- Optimización del *pie de cuba* para favorecer el desarrollo de la fermentación alcohólica mediante levaduras autóctonas. Bedoya, K., Portillo, M. C. and Mas, A. VIII **Congreso Nacional de Microbiología Industrial y Biotecnología Microbiana**. Valencia, Spain (06/2022)

Poster communications

- Pied-de-cuve optimization for grapemust alcoholic fermentation. Mas, A., Bedoya, K. and Portillo, M. C. **FoodMicro 2022 Conference**. Athens, Greece (08/2022)

- Evaluation of the chemical and organoleptic profiles of wines inoculated with different pied de cuve Bedoya, K., Rozès, N., Jara, C., Mas, A. and Portillo, M. C. **XXIII Congreso Nacional de Microbiología de los Alimentos de la SEM**. Cartagena, Spain (09/ 2024).