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RESEARCH ARTICLE

Pied-de-cuve: A sustainable approach to microbiological control in organic and natural wines

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Abstract – The use of selected yeast is one of the main achievements of microbiological control in the wine industry. However, the reliance on single-strain *Saccharomyces cerevisiae* starters and limited strain diversity has led to objections to its use. Recent trends in winemaking emphasize the importance of microbiological "terroir" and challenged the use of commercially selected starters sourced from other regions. Among the approaches to fight this concept are the wines produced with spontaneous alcoholic fermentation which preserves the native microbiota. However, spontaneous fermentation poses significant risks, including sluggish or stuck fermentation and increased spoilage potential due to the lack of microbial control. To address these challenges, we are proposing the application of the "*Pied-de-cuve*" (PdC) method as an alternative. PdC has been traditionally used in sparkling wine production to acclimate yeasts to the alcohol content of the base wines for the second fermentation. Our proposal is the preparation of the PdC using spontaneous fermentation in diverse winemaking conditions, such as addition of SO_2, fortification with ethanol and temperature adjustments. We performed laboratory trials to optimize PdC preparations, ensuring effective fermentation while preserving the diversity of the yeast consortia used to mimic the natural populations in grape must. The optimal selection was validated at cellar scale with natural must and its microbiota. Under these conditions, the PdC methodology exhibited fermentation kinetics comparable to, or better than, those achieved commercial inoculum. Furthermore, wines fermented using PdC demonstrated greater microbial diversity during and after fermentation and resulted organoleptically different compared to the inoculated controls. Here, we highlight the PdC as a viable method for achieving microbial control of alcoholic fermentations if the preliminary fermentation is appropriately handled. The proposed PdC protocol can be easily adapted to organic, biodynamic, or natural winemaking practices, offering a balanced approach to microbial management and "terroir" expression.

Keywords – *Saccharomyces*, Non-*Saccharomyces* yeasts, microbial footprint, microbial fingerprint

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1. INTRODUCTION

The main process of the transformation of grape juice (must) into wine is alcoholic fermentation. This transformation involves the conversion of sugars into ethanol and carbon dioxide (CO_2). In this process a single molecule of glucose yields two molecules of each product. Although this process is a well understood phenomenon, it has taken humanity more than 15,000 years since it was observed initially (production of beer and primitive forms of wines, not necessarily from grapes) until the comprehension of the process. Fermentation was long regarded as a mysterious or divine phenomenon. How to explain that sweet liquids started spontaneous "boiling" without the application of heat and got transformed into a liquid that helps communication and happiness? In the ancient Egypt, winemakers were considered priests, because they had the capacity to handle the miracle and communicated with higher powers. Similarly, Greeks and Romans had their corresponding wine Gods, Dionysus and Bacchus that symbolized the mystique of fermentation. Throughout the Middle Ages, the production of wines was closely related to monasteries and handled by monks (Mas, 2019).

Since the 19th Century, the responsibility of the process was unveiled: there was no divine intervention but the work of a humble group microorganisms, the yeasts, primary

Saccharomyces cerevisiae, responsible of alcoholic fermentation. This understanding revolutionized not only winemaking but also the broader food industry through different fermentative processes, as the role of some bacteria were identified for other fermentative processes (mostly lactic acid fermentations), including one that could appear in the production of wines (malolactic fermentation). Since then, nearly two centuries of scientific exploration have aimed to control and optimize the alcoholic fermentation representing a new era of winemaking (Mas et al, 2021). This review highlights the advances made by our research group in studying the microbial populations involved in fermentation, the limitations of traditional methods, and innovative alternatives for improving fermentation outcomes while preserving the unique microbial "terroir" of wines. From pioneering molecular biology techniques for microbial identification to proposing novel approaches such as the *Pied-de-Cuve* (PdC) method, we aim to bridge the gap between tradition and scientific precision in modern winemaking.

2. METHODS FOR ANALYSIS DE MICROBIAL POPULATION

Our research group (Wine Biotechnology) has spent the last three decades contributing to the understanding and control of microbial dynamics in winemaking through molecular biology-based identification methods (Constantí et al, 1997, 1998; Torija et al, 2001; Beltran et al, 2002). This work has both confirmed prior findings and led to new insights.

Notably, in grape must from our local regions, *S. cerevisiae* (the main microorganism responsible for completing the fermentation), was hardly detected in grape must or on grapes (Constantí et al, 1997; Torija et al, 2001). However, it could occasionally be detected in very healthy grapes with low fungal loads (approximately 10^4 cells/mL) (Beltran et al, 2002). Early stages of fermentation were instead dominated by a non-taxonomic group of yeast named generally non-*Saccharomyces*. Among them, *Hanseniaspora uvarum* and *Candida stellata* (later reclassified as *Candida zemplinina* and subsequently as *Starmerella bacillaris*) were regarded as the most prevalent genera (Constantí et al, 1997; Torija et al, 2001: Beltran et al, 2002).

Initially, these findings were based on the recovery of microorganisms using culture media, primarily Yeast Extract Peptone Dextrose (YPD), which allows broad recovery of yeasts (Mas et al, 2021). However, it soon became evident that culture-based methods had limitations: many microorganisms could not grow in these media, did not grow fast enough or were in a "viable but not culturable" (VBNC) state, a condition related to wine processes (Millet & Lonvaud Funel, 2000; Andorrà et al, 2010a; Wang et al, 2015a; Navarro et al, 2020). In response to this situation, we started to adapt and apply different culture-independent methods, including quantitative PCR (both RT-PCR and direct QPCR) (Hierro et al, 2004, 2006a; Andorrà et al, 2008, 2010b; Sunyer-Figueres et al, 2018), DGGE-PCR, PCR coupled with fluorescent dyes (Andorrà et al, 2010a; Wang et al, 2015b) and flow cytometry paired with species-specific dyes (Andorrà et al, 2011; Wang et al, 2014). In recent years, we have employed various omics approaches to monitor and study alcoholic fermentation during winemaking (Wang et al, 2015b; Portillo & Mas, 2016; Kirioglou et al, 2018). These culture-independent methods have allowed the ratification of the previous observations but increasing the detection of many microorganisms that previously were almost undetected (Lleixà et al, 2018; Kirioglou et al, 2019a, 2020). Some of them include minority species that were previously overlooked due to their low abundance and the limited number of colonies analyzed by traditional methods. Such advancements have proven especially valuable for studies requiring extensive sampling across multiple locations or fermentation stages. Furthermore, omics technologies have allowed the establishment of correlations between metabolomics and metataxonomic, although it must be considered that statistical correlations do not necessarily imply causation (Kirioglou et al, 2020b).

3. THE MICROBIAL POPULATION DURING ALCOHOLIC FERMENTATION

All the previous studies allowed us to draw an accurate picture of the microbial dynamic during alcoholic fermentation. The process begins with a dominance of different non-*Saccharomyces* yeasts (Constantí et al, 1997; Torija et al, 2001; Beltran et al, 2002; Mas et al, 2021) and the yeast-like mold *Aureobasidium pullulans* (Padilla et al, 2016; Wang et al, 2015b) also identified as prominent by culture-independent methods. However, the presence of different molds quickly disappears due to the challenging conditions of the grape must include low pH, high osmotic pressure, low water activity and nutrient imbalance. Under these conditions, *S. cerevisiae* stablished dominance, driven by its rapid nutrient uptake capabilities (Beltran et al, 2005; Andorrà et al, 2008; Lleixà et al, 2016a; Roca-Mesa et al, 2020, 2022; Torija et al, 2021). However, this is not the only element that helps *S. cerevisiae* to take over the fermentation but also competitive interactions with other microorganisms including cell-to-cell contact, secretion of killing and inhibitory molecules to the media and other antagonistic reactions (Andorrà et al, 2011; 2012; Wang et al, 2015c, 2016; Navarro et al, 2020). Another critical factor is the appearance and increasing production of the main byproduct of fermentation: ethanol. The release of ethanol during alcoholic fermentation allows a quick disappearance of many yeast species, although this effect has been broadly overestimated, as many yeast species can survive increasing concentrations of ethanol (Wang et al, 2015c) and even reach to the end of the alcoholic fermentation without many troubles (Llauradó et al, 2002; Hierro et al, 2006; Padilla et al, 2017).

4. THE ROLE OF HUMAN PRACTICES

This dominance of the fermenting yeast *S. cerevisiae* is greatly impacted by some human related practices. One of the oldest and most influential practices is the use of Sulfur

Dioxide (SO_2), a compound with several positive actions on wine as antioxidant, helps settling the must and it is well-known as antimicrobial for many microorganisms that alter the wine, such as some yeasts, bacteria, and other fungi. While SO_2 has been widely used, it has faced criticism from modern winemakers for its potential to limit microbial diversity. We have analyzed the effects of SO_2 on the evolution of the microbial population during winemaking and observed how it facilitates the reduction of biodiversity in the grape must and the quick imposition of *S. cerevisiae* (Constantí et al, 1998; Andorrà et al, 2008; Bedoya et, 2024a). Trials without SO_2 demonstrated that biological interactions of other yeasts could push *S. cerevisiae* into a status of VBNC, while the usual effect had been that *S. cerevisiae* induces this state in other non-*Saccharomyces* yeast (Navarro et al, 2020; Zhu et al, 2021). Temperature control is another influential human element in winemaking with a very strong effect on the ecology of the microbiota. In our studies we have shown that fermentation temperature affects the survival and competitiveness of various yeast species (Llauradó et al, 2002; Torija et al, 2003a, b; Beltran, 2006, 2007; Hierro et al, 2006; Andorrà et al, 2010c; Redón et al, 2011; Hierro et al, 2007; Bedoya et al, 2024a). This effect has been also observed at the level of *S. cerevisiae* strains which displayed varying temperature tolerances (Torija et al, 2003a).

One of the most transformative human practices in winemaking that determines the prevalence of *S. cerevisiae* during alcoholic fermentation is the inoculation of selected strains of this species (Constantí et al, 1997; Andorrà et al, 2008; Llauradó et al, 2005). A big step forward in the microbiological control of alcoholic fermentation during winemaking was the development of Active Dry Wine Yeast (ADWY) products. The ADWY is a cellar-friendly presentation of selected strains of *S. cerevisiae* that allows a rapid fermentation and a fast take over by the selected strain (Constantí et al, 1998; Andorrà et al, 2010b; 2011; González-Royo et al, 2015; Lleixà et al, 2016b).

5. THE CHALLENGE OF UNIFORMITY

Although the use of ADWY its widespread in industrial winemaking, it has been challenged because of uniformity by many wine makers, particularly in the concepts of organic, biodynamic, and natural wines. Spontaneous fermentation relies on the microbiota present in the grape juice, which can be considered the microbial fingerprint of a given place and the so called microbial "terroir" (Wang, 2015b; Padilla et al, 2016; Jara et al, 2016). It has been described that the spontaneous fermentation can result in wines with unique microbial and sensory profiles, contributing to the concept of "microbial terroir". This autochthonous microbial footprint should be considered as well as a part of the typicality of the wine produced in each area, which forms a part of the identity of the wines (Padilla et al, 2017). Obviously, the use of ADWY often suppresses this diversity, leaving only thee selected strain's footprint in the final wine as its activity is very fast and takes over almost immediately the alcoholic fermentation (Constantí et al, 1997; Andorrà et al, 2008), Andorrà et al, 2010b. Thus, this risk of uniformity of the microbial characteristic is what has led the search for other alternatives.

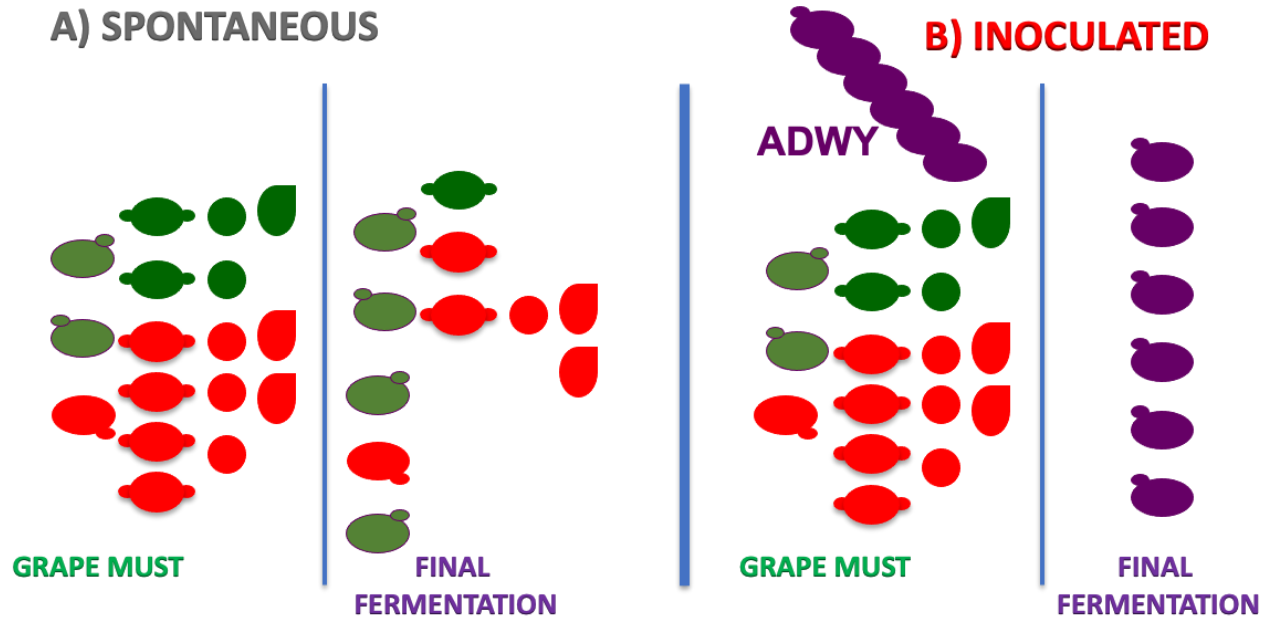

Figure 1: Changes in the composition of the microorganisms after spontaneous and inoculated fermentations. Green color: yeast strains with positive attributes in the final wine. Red color: yeast strains with deleterious impact in the final wine. *Saccharomyces cerevisiae* ( ). The ot shapes are different non-*Saccharomyces* species.

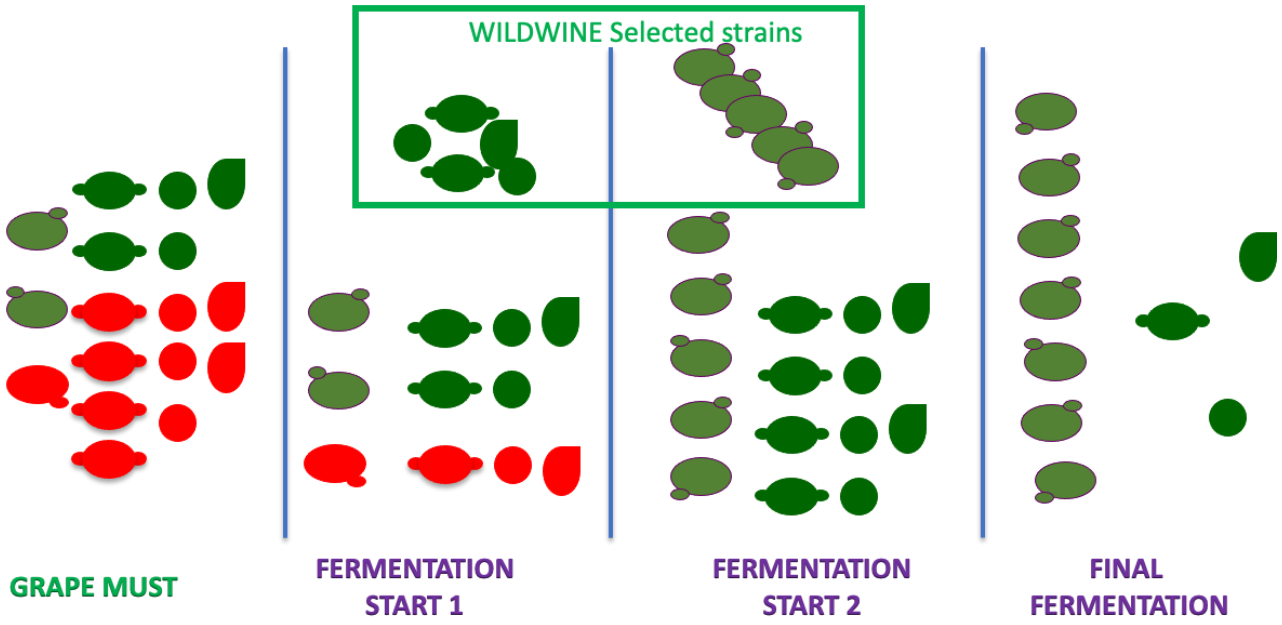

Figure 2: The WILDWINE effect on the populations of alcoholic fermentations. Colors and shapes as in Figure 1.

6. ALTERNATIVES TO ADWY

The first alternative is the return to fully spontaneous fermentations. This practice preserves microbial diversity and the typicality of the area but comes with risks of stuck or sluggish fermentations and undesirable microbial activity with possible unwanted effects. This approach would be only recommendable for experienced winemakers managing small-scale productions with good knowledge of corrective practices when some of the indicated problems might occur (Mas et al, 2020).

A second alternative would be inoculating grape must with locally selected yeasts strains. Nowadays, some yeast producers can offer a portfolio of locally selected yeasts to maintain the typicality. Some companies offer a service of local selection and production of "terroir" *S. cerevisiae* strains. This approach maintains regional microbial characteristics but may still face uniformity issues, although restricted to a more local level. Furthermore, the possible footprint of other non-*Saccharomyces* yeast is also an unsolved problem. There are also some commercial presentations of non-Saccharomyces species available, but there are not region-specific selected and there are only a few of them in the market. A solution to this problem was proposed in the WILDWINE project, in which we were involved together with the Universities of Torino, Bordeaux and Athens, who was acting as coordinators of the project. Several wine producing areas (Priorat, Bordeaux, Piedmont, Peloponnesus) were involved (Mas et al, 2016). The project aimed to replicate the microbial fingerprint already present in the grapes and cellars, select the ones that provided better characteristics to the wine and develop a mixture or "cocktail" of yeasts (different strains and species, all of them selected from those areas) to reproduce the best combination of yeast. Thus, the work involved an initial description and isolation of different strains and species (WILDWINE), selection of the ones that could leave a positive footprint in the wines (selection of strains) and then validate the different combinations and mixtures both at laboratory level and at cellar level (Padilla et al, 2016; Wang et al, 2015b). The resulting wines were distinctive from the commercial ones and completely respectful with the origin of the strains (typicality) (Padilla et al, 2017). However, this method has its main limitation: the production strains cocktails for commercial use remains complex and costly.

7. THE PIED-DE CUVE PROPOSAL

The final alternative we propose is the introduction of the Pied-de-Cuve (PdC) (Portillo & Mas, 2022; Mas & Portillo, 2022). The PdC is traditional approach, often is mistaken with back-sloping although it has considerable modifications. Unlike back-sloping, which uses a sample from a nearly finished product as inoculum, the PdC involves the inoculation of a highly active population of fermenting microorganisms. The term "Pied-de-Cuve" means literally "bottom of the deposit" because the active fermenting yeast are deposited at the bottom of the vat and fresh must is added, initiating fermentation almost immediately with the imposition of the microorganisms present in the PdC.

The PdC has practical applications in winemaking, such as the transfer of isolated laboratory yeast strains to natural must or acclimating yeast to the base wine for the second fermentation during production of sparkling wines (Martí-Raga et al, 2015). The base wines have normally ethanol levels around 10-11% ethanol (v:v) and this is a concentration normally lethal for any yeast unless it is adapted to these levels of ethanol (Martí-Raga et al, 2016, González-Royo et al, 2015). Acclimated *S. cerevisiae*

strains can effectively perform the second fermentation needed for sparkling wine production (Martí-Raga et al, 2015). Figure 3 highlights comparisons between spontaneous fermentation, fermentations inoculated with PdC, and those using ADWY. (Figure 3).

In our proposal, the PdC is initiated using grapes of the same vineyard that are harvested few days before optimal ripening time. This must is spontaneously fermented to achieve high yeast population density (typically $>10^8$ cells/ml). A portion of this fermenting must (1-5% v/v) is then used as PdC for subsequent fermentation. PdC preparation is performed under several stressors common in winemaking such as SO_2, temperature and ethanol (Bedoya et al, 2024a, b).

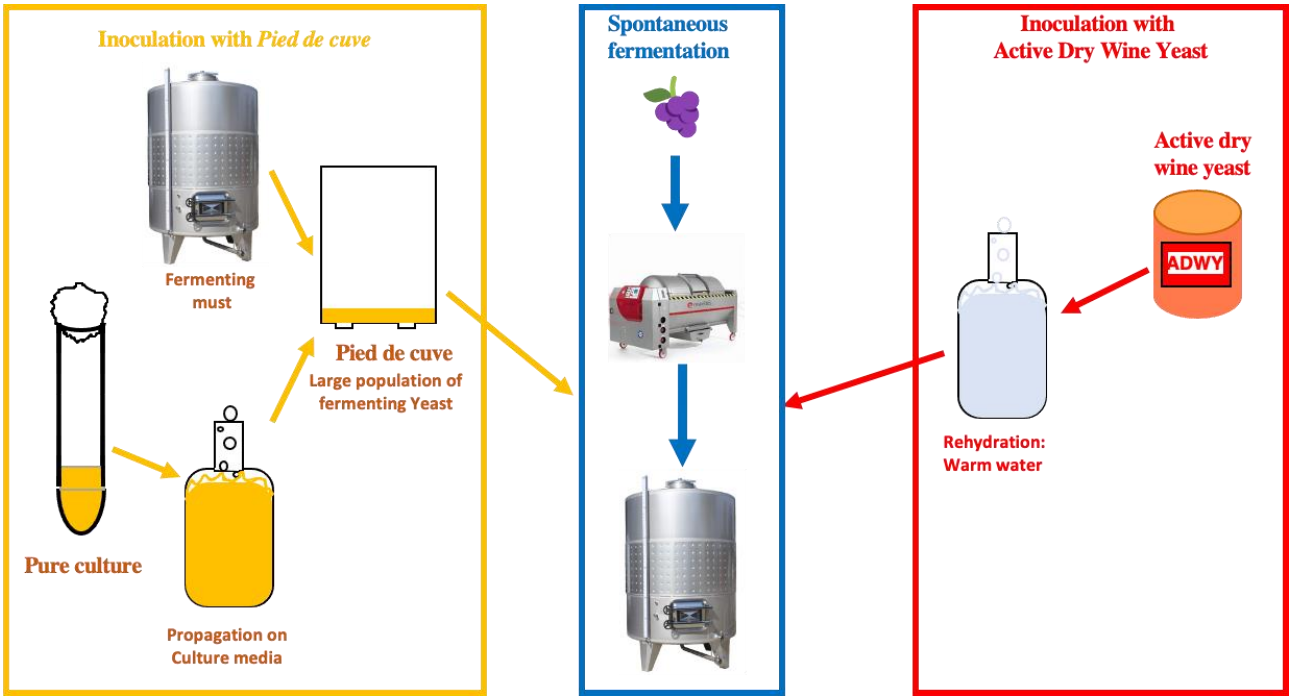

Figure 3: Comparison between spontaneous fermentation, ADWY inoculated fermentation and fermentation with *Pied de Cuve*

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Laboratory trials tested various combinations of stressors to observe their impact on microbial communities and yeast survival (Bedoya et al., 2024a). We first optimized laboratory conditions to cultivate a microbiota in the PdC that that closely resembled the initial microbial community found in the grape must. The laboratory fermentations were designed to replicate microbial profiles observed in previous studies conducted by our group (see above) and the fermentations were performed in the different conditions. After monitoring the microbial population under various conditions and combinations, we identified an optimal protocol involving the addition of SO_2 and ethanol to select representative microbiota in the PdC (Bedoya et al, 2024a). We have observed that specific combinations of these stressors in the PdC favored almost exclusively *S. cerevisiae* strains or a desired combination of non-*Saccharomyces* and *S. cerevisiae* yeasts.

These finding at laboratory trials were validated at the cellar level (Bedoya et al, 2024a) and in multiple trials conducted in Spain and Chile (Bedoya et al, 2024b). We repeated the same conditions in different countries (Spain and Chile), different varieties (Muscat of Alexandria and Sauvignon Blanc) and produced wines that were analyzed at microbiological and sensory level (Bedoya et al, 2024b). As can be observed in Figure 4b, the PdC-inoculated fermentations started always faster than the corresponding controls with ADWY, whereas the spontaneous fermentations (Figure 4a) at the same temperature (16ºC) performed much slower (Bedoya et al, 2024a).

The biodiversity of the different fermentations was analyzed by the Simpson's biodiversity index, as can be seen in Table 1. Fermentations inoculated with PdC showed microbial diversity comparable to spontaneous fermentations, both of which were much higher than the controls inoculated with a commercial ADWY (Bedoya et al, 2024a). This high biodiversity index did not have any variation regarding the conditions to raise the PdC. Furthermore, because of this high biodiversity, non-*Saccharomyces* yeasts were recovered during and at the end of the fermentation with the PdC, as well as several *S. cerevisiae* strains, which ensured a diverse footprint in the final wines. Instead, the wines inoculated with ADWY showed very low biodiversity with total imposition at the end of fermentation, which ensures that the footprint is due to the inoculated strain exclusively.

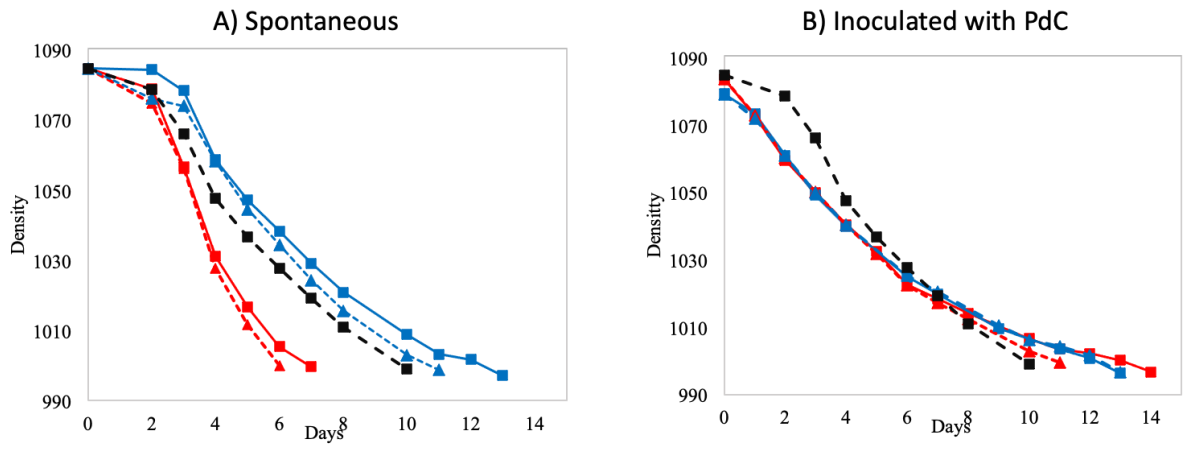

Figure 4: Spontaneous fermentation to raise the PdC (A) and fermentation inoculated with PdC raised in A (B). Continuous black line: control inoculated with commercial ADWY. Blue lines: Spontaneous fermentations performed at 16ºC and inoculated with PdC derived from it. Red lines: Spontaneous fermentations performed at 25ºC and inoculated with PdC derived from it. Continuous lines: without additives, broken lines: with the addition of SO_2 and ethanol.

Table 1: Simpson's diversity index from PdC- and ADWY-inoculated fermentations ranging between 0 and 1, where 1 represents infinite diversity and 0, no diversity. The "Spontaneous F". is the fermentation to raise the PdC sampled before being inoculated into the fresh must. ADWY stands for the inoculated fermentation with the commercial *Saccharomyces cerevisiae* strain. "Begining F"., "Middle F"., and "Final F". were the sample points during the main fermentation based on the must density decrease.

	Conditions	Spontaneous F.	Begining F.	Middle F.	Final F.
PdC1	18ºC	0,76	0,91	0,76	0,98
PdC2	26ºC	0,91	0,76	0,89	0,96
PdC3	SO_2+EtOH, 18ºC	0,96	0,98	0,76	0,98
PdC4	SO_2+EtOH, 26ºC	0,89	0,91	0,96	0,98
ADWY	18ºC	-	0,20	0,51	0,00

Harvest	Inoculation	Descriptive Test						Significance
Muscat	PdC-18-stress	3.2	3	3.2	1.3	2	1.8	
Alexandria	PdC-26-stress	2.8	3.2	2.8	1.5	1.8	1.5	*
2022 Spain	ADWY-18	2.8	2	2.2	2.8	3	2.7	
Sauvignon	PdC-18-stress	3	2.5	3	2.2	3.2	2.2	
blanc 2023	PdC-26-stress	2.7	2	2.3	2.5	2.9	2.2	*
Chile	ADWY-18	3	2.5	2.3	2.5	3.2	2.3	*
Muscat	PdC-18-stress	3.2	2.8	2.8	2.5	2.8	2.8	
Alexandria	SF-18	3.3	3	2.8	2.8	3	2.8	*
2023 Spain	ADWY-18	3.2	3.2	2.8	2.4	3	3	*
		Quality	Terpene	Tropical	Vegetal	Acidity	Bitterness	

Figure 5: Results of the triangle test and descriptive analysis. Green boxes mean positive attributes, red boxes negative attributes, based in the scale below which correlate value and colors. Asterisks mean significant differences between wines; $p<0.05$. Values are the scores in a scale of 1-5.

Figure 5 represents the results from the sensory analysis of the obtained wines. Triangle test confirmed significant sensory differences between the wines produced with PdC and those inoculated with ADWY (Bedoya et al, 2024b). In the descriptive sensory analysis, wines produced with PdC showed a significant difference with higher quality and terpene and lower vegetal and acidity values (Bedoya et al, 2024b). This aligns with the microbial diversity observed in PdC fermentation, which enhances the sensory complexity of the wines.

8. Conclusion

Understanding the microbial dynamics of the alcoholic fermentation is one of the main objectives of winemakers to achieve effective control over the winemaking process and the quality of the final wines. Our research group has devoted more than 25 years to develop tools and expanding knowledge in this area, as highlighted in this review. A significant milestone in the wine industry was the introduction of ADWY technology, which provided reliable control of alcoholic fermentation. However, its widespread use has raised concerns about uniformization and homogenization of wine profiles, prompting some winemakers to revert to spontaneous fermentations, which carry inherent risks. We propose an easy and accessible alternative that could be developed with standard winemaking tools to prepare PdC with appropriate conditions. In our hands, the PdC methodology has shown to be able to have fermentation kinetics comparable to (or better than) those achieved with the commercial inoculum. Furthermore, the wines with PdC have shown to be different than the inoculated controls, with a great microbial diversity at the end of the fermentation. Moreover, wines fermented with PdC exhibited greater microbial diversity and distinctive sensory profiles compared to ADWY-inoculated controls.

The PdC method provides a balanced solution, offering partial microbial control of the alcoholic fermentations with appropriate handling of the PdC while preserving the unique characteristics of spontaneous fermentations. However, the outcome will vary, with different proportions in the yeast population, as the method does not guarantee dominance by any specific strain, unlike the commercial inoculum. Nonetheless, with appropriate handling, it is possible to select a natural yeast population with a proper combination of the common cellar practices.

Importantly, the PdC methodology aligns seamlessly with organic, biodynamic, and natural winemaking principles, offering a sustainable and lower-risk alternative for producing unique wines that maintain microbial diversity and individuality.

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