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Review

# Strategies for microbiological control of the alcoholic fermentation in wines by exploiting the microbial *terroir* complexity: A mini-review

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ABSTRACT

A complex consortium of yeasts is the principal responsible of wine fermentation, being *Saccharomyces cerevisiae* the main driver. The use of selected yeast, beginning with *Saccharomyces* strains, is one of the main achievements in microbiological control in the wine industry. However, the use of single strain starters of *S. cerevisiae* and the limited variability of strains has increased the objections to its use due to the production of wines with a homogeneous profile. New tendencies in winemaking have emphasized the microbiological *terroir* and challenged the use of selected starters from different areas, including Non-*Saccharomyces* yeast or multi-strain starters in order to add complexity to the biochemical composition of wines. Nevertheless, these strategies also harbor their own challenges. In the present mini-review, we focus on the alternatives to current commercial yeast starters mainly based on the local multispecies starters or controlled spontaneous fermentations, considering the advantages and limitations of each strategy. Also, we present an ancestral technique based on the use of pre-fermented must (*Pied-de-Cuve*) because it provides certain microbial control to the alcoholic fermentation while allows the development of autochthonous microorganisms that might confer microbial typicity to the produced wines. Nevertheless, the latest strategy needs further research to establish a scientific background for the selection of the best *Pied-de-Cuve* development. Finally, the tendencies in winemaking should find a commitment between microbial control to guarantee alcoholic fermentation fulfillment and the production of wines reflecting microbial typicity to respond to consumer demands, without forgetting the scaling up in the cellars.

## 1. Microbiological control of alcoholic fermentation by Active Dry Yeast

Wine making is characterized from the microbiological point of view as a succession of microorganisms. This succession starts with some non-*Saccharomyces* yeasts originated mostly from the grape surface, which are displaced by *Saccharomyces* species (mainly *S. cerevisiae*), which conduct the successful alcoholic fermentations until sugar from the grapes is practically consumed (Fleet, 2003). Although the presence of *S. cerevisiae* on the grapes has been demonstrated and its isolation is possible starting from the vineyard without contamination from the cellar (Clavijo et al., 2010; Renouf et al., 2005), in absence of inoculation, the main source of *S. cerevisiae* is the cellar and cellar equipment where eventually they become cellar-residents (Beltran et al., 2002). Currently, the imposition of the *Saccharomyces* species during alcoholic fermentation is mostly due to its controlled inoculation and certain enological practices that favor its takeover. From the different cellar strategies to achieve this imposition, it should be emphasized the use of $SO_2$ given that *S. cerevisiae* is especially resistant to this compound as the result of its domestication process (Pérez-Ortín et al., 2002), which facilitates the conduction of alcoholic fermentation by this species (Constantí, 1998). Nevertheless, the main breakthrough of the microbiological control was the development of wine yeast starters during the 20th century, especially the development of commercial presentations that were cellar-friendly, such as the Active Dry Wine Yeast (ADWY). Although the acquisition of commercial ADWY represents an additional expense for the cellar, the microbial control and reproducibility of the alcoholic fermentation mostly compensate the economic investment. The appropriate wine yeast starters should present adequate biotechnological properties as good fermentation kinetics, tolerance to osmotic and ethanol stress and efficiency to use nitrogen sources (Varela et al., 2004). Other properties often considered are the killer phenotype, tolerance to $SO_2$ and optimal growth temperature depending on the application. In addition, is also desirable that the

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**Table 1**
Comparison of different strategies to perform the alcoholic fermentation for winemaking.

| Strategies | Advantages | Limitations |
|---|---|---|
| Spontaneous | Cheaper than using commercial strains | Longer fermentation kinetics |
| | Contribution to wine chemical composition by autochthonous microorganisms that could reflect the *terroir* of each area | Lack of microbial control which could lead to off-flavor compounds and sluggish or stuck fermentations |
| | Original wines | Lack of reproducibility of the process and product |
| *Saccharomyces* starters | Optimal fermentation kinetics and reproducibility of both the process and product | Lack of genetic and phenotypic diversity leading to uniformization of wines |
| | Tolerance to fermentation stresses | More expensive when commercial starters are used |
| | Out competition of autochthonous yeasts | Prevent the microbial *terroir* footprint |
| Non-*Saccharomyces* starters (usually, combined with *Saccharomyces*) | Wider genetic and phenotypic diversity | Lower tolerance to ethanol and fermentation stresses |
| | Increase of wine complexity or acidity | Less competitive than *Saccharomyces* |
| | Some non-Saccharomyces are able to complete the alcoholic fermentation | Usually, sequential inoculation is required to allow the development of Non-Saccharomyces |
| | Biocontrol of non-desired microorganisms | Risk of nutrient depletion and stuck fermentation |
| Multi-strain starters | Contribution to wine regional typicity when autochthonous selected yeast are used | Sequential inoculation of *Saccharomyces* is needed |
| | Increase of wine complexity | Complexity to handle and propagate at the laboratory: not available for small cellars |
| | Higher microbial control than spontaneous fermentation | Difficulties to control and reproduce the alcoholic fermentation |
| Pied de Cuve | Increase of microbial control by using a pre-fermenting must | Minimal laboratory requirements |
| | Good fermentation kinetics | Selection and optimization of PdC is required and lack of scientific background |
| | Contribution to wine regional typicity when spontaneous | Uniformization of wines when inoculated |

starter does not contribute with any off-flavor or compounds that might impact the wine perception in a negative way.

One of the main characteristics of the alcoholic fermentations led by ADWY was the quick takeover of the fermentation by the inoculated yeast which resulted in wines that were reproducible and presented homogeneous sensory profiles (Fleet, 2003). However, these profiles were quickly standardized, and some winemakers started looking for some alternatives (Table 1). Consequently, as few selected yeasts took over the market, soon appeared the pressure to select "local" or "wild" strains that could be more adapted to the musts of the different viticultural areas and protected the local character of the wines (reviewed in Molinet and Cubillos, 2020). Each strain of *S. cerevisiae* can contribute to the sensory profile of the wine in a different way but not all of them are well adapted to all kind of fermentations processes and the lack of genetic and phenotypic diversity of those strains prevents the differentiation of the product, as demonstrated by studies in the last years (Alperstein et al., 2020; Kang et al., 2019).

Thus, the main challenge appeared: the uniformization of the chemical characteristics of the wines produced with the same selected strains. After the consideration that some local characteristics could derive from the presence of some autochthonous microbiota and the knowledge that some non-*Saccharomyces* yeast could provide certain positive characters, several approaches tackled this demand (Benito et al., 2019; Ciani and Comitini, 2011; Jolly et al., 2014). One of the first proposals was the delay in the inoculation of the selected yeast of *S. cerevisiae*, which would allow the development of some local characteristics from the autochthonous non-*Saccharomyces* yeast (Fleet, 2003). Although this practice is probably one of the easiest to implement in the cellar, it is not exempt of risks: the autochthonous non-*Saccharomyces* yeasts may deplete the main nutrients and they can produce unwanted compounds that can jeopardize the quality of the final wine (Andorrà et al., 2012). Furthermore, it has been demonstrated that delays of more than 3 days in the inoculation process can risk the fermentation takeover by *S. cerevisiae* and derive in sluggish or stuck fermentations (Lleixà et al., 2016a). A much better alternative was the use of selected non-*Saccharomyces* yeast as starters (Jolly et al., 2014). Several non-*Saccharomyces* have been already selected and commercialized based on the aromatic compounds specifically produced or generated in given grape varieties, and nowadays there are many species of non-*Saccharomyces* yeasts that are available in cellar-friendly commercial presentations.

## 2. Non-*Saccharomyces* starters to avoid wine uniformization

Currently, more than 40 commercially available presentations of *non-Saccharomyces* can be found currently in the market of which more than 75% correspond to species of *Torulaspora delbrueckii*, *Metschnikowia pulcherrima* and *Lachancea thermotolerans* or combinations thereof (Roudil et al., 2020). Most of the wine yeast companies offer a commercial presentation of one of them. The main target is the increase of complexity of the final wines from the aromatic point of view, although some of them include also acidification of wines (Table 1). The other species including commercial presentations are *Metschnikowia fructicola*, *Schizosaccharomyces pombe*, *Pichia kluyveri*, *Zygosaccharomyces bailii*, *Zygosaccharomyces parabailii*, *Starmerella bacillaris*, *Wickerhamomyces anomalus* and *Kluyveromyces wickerhamii* (Roudil et al., 2020). Thorough reviews of these species and the main characteristics that can be developed in the resulting wines can be found (Benito et al., 2019; Jolly et al., 2014; Roudil et al., 2020; Vejarano and Gil-Calderón, 2021). A different species that is worth to mention here is *Hanseniaspora vineae*, which was isolated in Uruguay vineyards (Medina et al., 2013) and is very different from the typical *Hanseniaspora uvarum* that is commonly found. Although *H. uvarum* is the main non-*Saccharomyces* species found in many parts of the world, their use in wine making is limited due to the production of ethyl acetate and acetic acid, even if its initial presence during fermentation has been considered positive (Tristezza et al., 2016). However, it is not tolerant to ethanol and in presence of *S. cerevisiae* soon disappears (Padilla et al., 2017; Wang et al., 2015b). On the contrary, *H. vineae* is able to support high concentrations of ethanol and even conduct the alcoholic fermentation to the end (Lleixà et al., 2016b) yielding the final wines with a distinctive character and very pleasant fruity aromas (acetate esters), terpenes and benzenoids (Martin et al., 2018).

Some commercial presentations include a mixture of species, mostly a combination of one of the main non-*Saccharomyces* together with *S. cerevisiae*, which could be considered as a coinoculation. In addition, some commercial starters include two non-*Saccharomyces* (*Torulaspora delbrueckii* and *Lachancea thermotolerans*) together with *S. cerevisiae* (Roudil et al., 2020). The main reason of including *S. cerevisiae* is that it is basically the only species able to finish the alcoholic fermentation consuming all the sugars. Nevertheless, no all the mixed presentations include *S. cerevisiae,* as currently there are commercial presentations that are combinations of two non-*Saccharomyces* (Vejarano and Gil-Calderón, 2021). These mixed presentations of commercial starters raise another relevant aspect for the evolution of the microbiota during the

alcoholic fermentation and the final composition of the wines: the interaction between *S. cerevisiae* and the non-*Saccharomyces* yeasts, which are influenced by several factors (recently reviewed by Mencher et al., 2021).

The complexity of interactions between yeasts decides the fate of the alcoholic fermentation. Previous studies have focused mainly on the effect of physicochemical factors such as $SO_2$ addition, pH, osmotic pressure, temperature or nutrient availability either on single or mixed fermentations (Alonso-del-Real et al., 2017; Bokulich et al., 2015; Ciani et al., 2006; García-Ríos et al., 2014; Maturano et al., 2018; S.C. Morgan et al., 2017; Padilla et al., 2017). However, recent results suggest that, besides the physicochemical conditions, the ecological interactions play an important role in determining the contribution of individual species to the result of the alcoholic fermentation (Bagheri et al., 2020; Wang et al., 2015b).

The usual interaction between yeasts is normally negative and aggressive and from those interactions derives the final characteristics of the wine (Comitini et al., 2021; Zilelidou and Nisiotou, 2021). Species competing for the same niche might induce the elimination of their competitor by substrate uptake (Melvydas et al., 2020; Sgouros et al., 2020), a broad array of toxic compounds such as killer factors (Branco et al., 2014; Peña et al., 2020), small peptides derived from glycolytic proteins with specific interactions (Kemsawasd et al., 2015) or toxic fatty acids (Li et al., 2020). During wine production, *S. cerevisiae* might use these strategies along with an optimized metabolic setup for alcoholic fermentation, the alcohol production and a human help in form of $SO_2$ addition to take over completely the fermentation and eliminate or at least reduce the growth of competitors (Fleet, 2003; Wang et al., 2015b). However, the other non-*Saccharomyces* yeast species have also a complete warfare defense mechanisms and some of them are able to compete efficiently with *S. cerevisiae* if, for example, $SO_2$ is not provided (Navarro et al., 2020). In fact, some non-*Saccharomyces* are considered as "friendly" starters because they allow the development of the autochthonous microbiota at the beginning of the alcoholic and might contribute positively to wine flavor, as it is the case of *H. vineae* (Carrau and Henschke, 2021). Finally, yeast interactions have been shown to be not only species-specific, but also very dependent on the strains or, in other terms, they are also strain specific (Onetto et al., 2021; Wang et al., 2016). The possible interactions among the different wine yeast species (and strains) open up a whole new world of research and reveal the different yeast species as not only actors and producers of fermentation products but also as biocontrol agents (Morata et al., 2021). In fact, the biological control through inoculation of non-*Saccharomyces* starters limiting the growth of unwanted microorganisms is currently being explored as an effective way of bioprotection and to decrease the use of chemical preservatives as $SO_2$, considered negative for human health (Di Gianvito et al., 2022; Morata et al., 2021).

Although the use of commercial Non-*Saccharomyces* species can increase the complexity of the wines and, thus, it can help differentiation of the wines from those produced with only *S. cerevisiae*, still there is no response to the search of the "typicity" that might reflect the microbial *terroir* (Table 1).

## 3. Selection of local multispecies starters to reproduce the microbial *terroir*

The *terroir* refers mainly to the climate, soil, and human management within a geographic area that contribute to the typicity of wines. Nowadays, due to the importance of the vineyard microbiome composition over the regional wine typicity, the term "microbial *terroir*" is gaining relevance (reviewed by Belda et al., 2017). By grapevine microbiome we understand the whole community of microorganisms that interact with the plant and play a key role in its growth and health (Vacher et al., 2016). Besides, grape microorganisms can be transported to the winery and enter in contact with wine, affecting its chemical composition and influence its quality (Belda et al., 2017; Bokulich et al., 2016; H.H. Morgan et al., 2017). The grape microbiome is influenced by several factors like climate, region, and grape cultivar. Therefore, those factors are responsible of a nonrandom distribution of the microbial community at each *terroir* (Bokulich et al., 2014; Burns et al., 2016; Gilbert et al., 2014; Kioroglou et al., 2019; Morrison-Whittle et al., 2017; Pinto et al., 2014; Portillo et al., 2016; Taylor et al., 2014).

Some winemakers defend the need of spontaneous fermentations to maintain the *terroir* concept in wines. However, they might be slow and with uncertain outcome, because of their dependence on the quality and quantity of the microbiota present and the capability to take over a successful alcoholic fermentation (Table 1). Furthermore, there is a resident microbiota in the winery that might alter the development of the *terroir* microbiota (Beltran et al., 2002). However, this resident microbiota could also be considered as part of the specific *terroir* of each cellar if its origin was the corresponding vineyard. It has to be emphasized, though, that if the cellar has used some commercial strains, those could become also resident and take over the fermentations, losing the goal of the spontaneous fermentations (Beltran et al., 2002; Mercado et al., 2007; Santamaria et al., 2005). Recently, the opposite case was reported when the indigenous biotypes showed better performances than those reported for commercial strains, highlighting the low dominance or prevalence of some commercial starter cultures during the fermentation (Aponte et al., 2020).

The microbial control of the fermentation to become successful and with a predictable product outcome is lost in the spontaneous fermentations. An approach to a controlled "spontaneous" fermentation could be the selection of indigenous yeast strains for industrial development, which has been already the subject of different studies (Garofalo et al., 2018; Settanni et al., 2012; Tofalo et al., 2014). This was also the goal of the European project WILDWINE (2012–2015, http://www.wildwine.eu/en/). In this project, the selection of several strains and species from each participating *terroir* was aimed to produce a multi-species starter to maintain the local character of the wines. Somehow it could be abbreviated as trying to reproduce the microbiota present in the vineyard in a starter that could maintain the same species and strains usually present in each vineyard for being used in the wine production. During that project, Padilla et al. (2016) designed a multistarter that consisted of 4 strains of different species that were isolated from the vineyards in the DOQ Priorat (Tarragona, Spain). These species were *Hanseniaspora uvarum* (that was present in large quantities), *Starmerella bacillaris*, *T. delbrueckii* and *Metschnikowia pulcherrima* and they were mixed in similar proportions to those found in the vineyard (Padilla et al., 2016; Portillo et al., 2016; Wang et al., 2015a). They were inoculated in the grape must in both laboratory and industrial conditions after the pressing, reproducing the first stage of the Non-*Saccharomyces* predominance. Another multi-starter of three different strains of *S. cerevisiae*, also isolated and selected from the same local vineyards, was sequentially inoculated. The duration of the alcoholic fermentation using the sequential inoculation of the multistarter was comparable to that inoculated with the commercial ADWY regularly used by the cellar. Additionally, the inoculated *S. cerevisiae* strains were detected at the end of both fermentations while the inoculated non-*Saccharomyces* were recovered at mid fermentation of the multistarter batch. On the contrary, at the fermentation driven by the commercial strain, this one was the only yeast recovered at the end of fermentation (Padilla et al., 2017). Furthermore, in the same project, several regional strains of the lactic acid bacteria *Oenococcus oeni* were selected and used for the malolactic fermentation of red wines (Franquès et al., 2017).

The selection of autochthonous species is a suitable alternative to spontaneous fermentation, although the complexity of handling these multispecies starters is far beyond most of the cellars that do not have an appropriate microbiological laboratory to perform the propagation of the selected strains in a sterile environment. Besides, the control and reproducibility of multi-cultures fermentation represent a challenge even at laboratory level (Table 1). Therefore, new developments and guidelines are needed to improve the biotechnological processes by

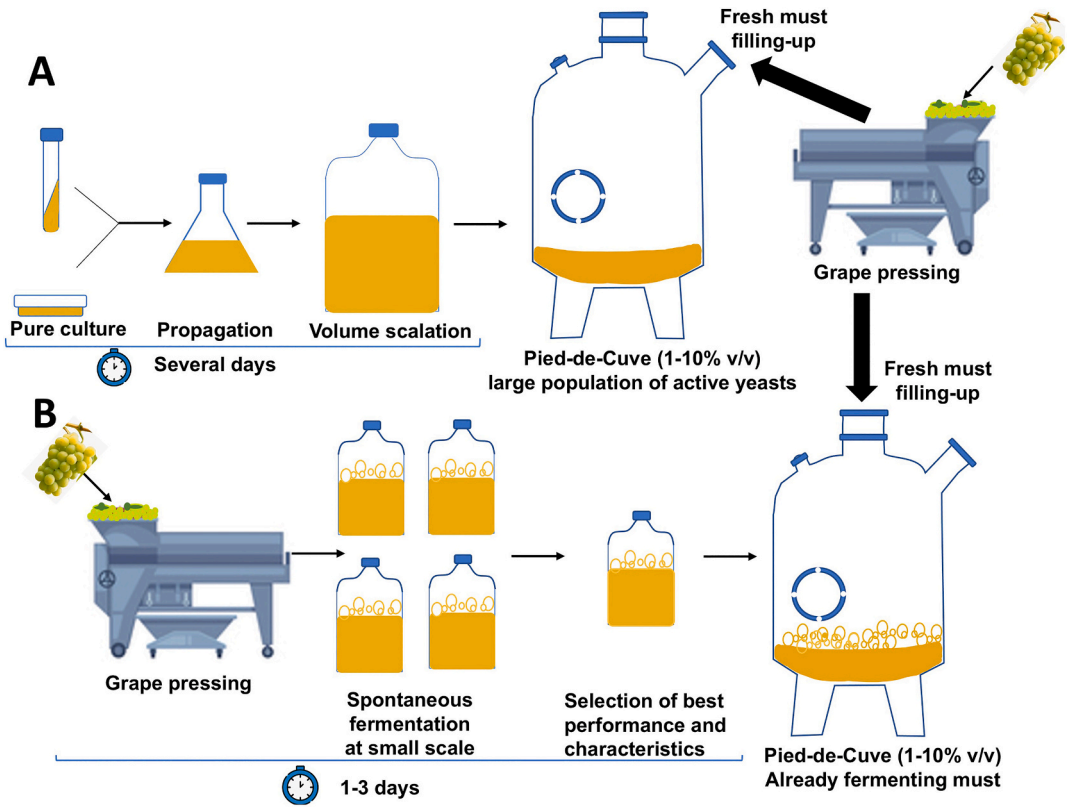


**Fig. 1.** Schematic representation of the steps to elaborate the inoculated (A) and non-inoculated (B) pied-de-cuve.

taking advantage of indigenous microorganisms at cellar scale.

## 4. Pied-de-cuve

The pied-de-cuve (PdC) is a traditional protocol to introduce a selected yeast or initiate fermentation from an already fermenting must (Fig. 1). The name comes from the "bottom of the deposit" in French and it means that a small volume (often 5–10%) is deposited at the bottom. An enrichment in yeasts with high fermenting activity take place by pre-fermenting the must at the bottom of the deposit and then the rest of the deposit is filled up with fresh must. The initial fermenting must should have very high concentration of active yeast (populations between $10^7$ and $10^8$ cells/ml) to easily lead the fermentation of the new must. This technique is traditionally applied by some wineries and guarantees a quick fermentation start and a good rate (Clavijo et al., 2011; Li et al., 2012; Moschetti et al., 2016). The PdC can be derived from a single yeast culture (generally a laboratory-selected strain) that needs to be propagated in appropriate media to reach enough volume to be used as PdC (Fig. 1.A). The selected strain will take over the fermentation and provide its oenological characteristics to the final wine. The yeast propagation needs a laboratory with minimal sterile conditions and the process may take several days. However, the PdC can also be derived from vats already fermenting spontaneously using, for example, grapes that are harvested earlier in time (Fig. 1.B). In this case, the PdC would be the result of a mixed inoculum and the final wine might reflect better the complexity and the typicity of the microbial *terroir* (Table 1).

In the case of spontaneous fermentations, both the quantity and persistence of any given autochthonous species would influence to the final wine character and aroma.

Nevertheless, scarce studies have reported results on inoculation by PdC. For example, Bely et al. (2005) used different protocols of inoculation with either an isolated yeast collected from a fermented must or pre-cultured yeast and found a decrease of the acetic acid in the resulting wines compared with the direct inoculation of rehydrated ADWY. Also, crushed grapes with the addition of ethanol (denominated fortified PdC) have been successfully used to speed up spontaneous alcoholic fermentation and to improve the sensory profile of red wines (Moschetti et al., 2016). In a recent study, Morgan et al. (2019) described that the results of the use of a PdC prepared with vineyard-derived yeasts was highly dependent on sulfite addition and on the microbial composition of the inoculum. Therefore, the authors recommend winemakers to perform PdC at small-scale fermentations and later blend them in order to increase wine complexity (Morgan et al., 2019). Nevertheless, these studies were mainly performed at laboratory scale and, consequently, the implementation of the PdC method is far from having the necessary scientific background for the wineries and it remains hardly reproducible. Thus, the real impact of PdC on the endogenous yeast community diversity during alcoholic fermentation or in wine quality is mostly unknown. In this sense, it is remarkable that Börlin et al. (2020) analyzed the effect of different modalities of PdC on the diversity of *S. cerevisiae* and wine composition at industrial scale. They found that the use of PdC produced fermentation kinetics and chemical analysis of resulting wines comparable in efficiency to that of ADWY. Also, the authors concluded that use of PdC could represent an improvement respect the spontaneous fermentation (Börlin et al., 2020). However, negative contribution of the PdC or spontaneous fermentation to the malolactic fermentation has also been reported when compared with controlled alcoholic fermentation (Berbegal et al., 2019). These authors found a more heterogeneous population and an incomplete consumption of the malic acid during malolactic fermentation for both PdC and spontaneous fermentation (Berbegal et al., 2019). In consequence, further studies are needed in order to evaluate the implementation of the PdC to control the alcoholic fermentation as well as preserving the typicity and quality of the final wine.

## 5. Conclusions and future perspectives

In order to avoid the standardization of wines by the use of common

commercial strains, different strategies have been evaluated. An initial approach has been the selection of indigenous yeasts as starters or their coinoculation with non-*Saccharomyces* yeast. However, some practices of alternative wine making have questioned the use of ADWY, even if local strains are used. Besides, the requirements at the cellar to the adequate propagation of the ADWY might limit its use, especially in the case of multi-strain starters. When the objective is to simulate the native microflora, a possible practice could be the non-inoculated pied-de-cuve, which can be generated from fermenting early musts. In this case, it should be highly recommended the use of several criteria to select the appropriate pied-de-cuve according to some preestablished variables such as suitable fermentation activity and/or appropriately selected sensory attributes. This procedure might increase the microbiological control during the alcoholic fermentation when compared with the spontaneous fermentation while could preserve the typicity and *terroir* of the final wine. Nevertheless, further information about the real impact of the pied-de-cuve on the microbial community during fermentation and the factors favoring an appropriate development of the microbial population is needed before the consideration of its wide application to cellars. In fact, the responses of the microbial population to different stresses (pH, temperature, dosage of $SO_2$, etc…) could generate different PdC and selective protocols for the development of desired characteristics in final wines. In this sense, further research is mandatory to elucidate the interactions between species and the mechanisms responsible of specific regional characteristics in wine.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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