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## The “pied de cuve” as an alternative way to manage indigenous fermentation: impact on the fermentative process and *Saccharomyces cerevisiae* diversity

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### ABSTRACT

Winemakers are increasingly keen to limit the use of commercial yeasts in order to reduce oenological inputs. The preparation of an indigenous winery-made fermentation starter from grapes called ‘pied de cuve’ (PdC) is becoming popular, especially in organic farming systems. However, the implementation of the PdC method is still empirical and knowledge is lacking regarding the impact of PdC on *S. cerevisiae* diversity during alcoholic fermentation. In this study, the impact of PdC on *S. cerevisiae* genetic diversity and wine composition was evaluated at an industrial scale. Despite very low initial population level of *S. cerevisiae* before inoculation, the use of PdC was as efficient as Active Dry Yeast in terms of fermentation kinetics and chemical analyses on the resulting wines, except for one modality. At mid-fermentation, the diversity of *S. cerevisiae* strains was different depending on the PdC used, and was also different from that in the spontaneous fermentation with, in some cases, clonal expansion. Our results provide evidence that the use of PdC could secure the fermentation process more efficiently than spontaneous fermentation.

### KEYWORDS

pied de cuve, diversity, *Saccharomyces cerevisiae*, microsatellites, wine

Supplementary data can be downloaded through: <https://oenone.eu/article/view/3105>

## INTRODUCTION

Winemaking implies the presence of various and successive populations of microorganisms, like fermentative yeasts, to conduct alcoholic fermentation. The use of Active Dry Yeast (ADY) as a fermentation starter is widely recommended to secure the process and avoid sluggish or stuck fermentation. In an opposite direction, there is an increasing demand, mainly from organic wine producers, to use indigenous *S. cerevisiae* strains from their own vineyards with the aim of limiting oenological inputs during the process and to try to increase specificities associated with the local “terroir”. Indeed, the selection of yeast strains from indigenous populations for industrial development has been the subject of different studies (Di Maro *et al.*, 2007; Settanni *et al.*, 2012; Tofalo *et al.*, 2009; Tofalo *et al.*, 2014; Garofalo *et al.*, 2018a; Garofalo *et al.*, 2018b). However, in some cases, spontaneous fermentations could lead to irregular wine quality due to lack of alcoholic fermentation control, thus resulting in potential aromatic deviations or alterations to the final products. Thus, there is a need for new developments and guidelines to improve technological processes for exploiting indigenous microorganisms.

Besides the classical method of directly using ADY, some wineries traditionally elaborate wines with “pied de cuve” (PdC) (Clavijo *et al.*, 2011; Li *et al.*, 2012; Moschetti *et al.*, 2016). By promoting the growth of fermentative strains in a small volume, this method is used to trigger alcoholic fermentation and control its progression, especially for the first vats filled at the beginning of the harvest period. The PdC is based on a small volume of fermented musts prepared a few days before harvest and used to inoculate the subsequent batches of grape juice. The technique consists in collecting grapes a few days before harvest and obtaining, after grape crushing, a fermenting fresh juice that will be subsequently used as a pre-culture. To avoid possible off-flavours, several grapes are harvested and processed separately in order to select the lot with the best fermentation kinetics and without sensory defects, such as acetic acid or ethyl acetate production. The implementation of PdC can then continue using must from fermenting tanks to inoculate the next batch. This process permits an already adapted yeast population to enter the fresh grape must. Till now, very few studies have reported results on PdC inoculation. Fortified PdC (crushed grapes with the addition of ethanol) has been successfully used to accelerate spontaneous alcoholic fermentation

and to influence the sensory profile of red wines (Moschetti *et al.* 2016). In the case of high sugar concentration fermentation, different inoculum protocols using yeast collected from a fermented must or pre-culture yeast were shown to limit the amount of acetic acid of the resulting wines (Bely *et al.*, 2005). In a recent study, the successful use of a PdC made with vineyard-derived yeasts was shown to be highly dependent on SO<sub>2</sub> addition and on the microbial composition of the inoculum (Morgan *et al.*, 2019). The authors recommend that the winemakers perform PdC on a small subset of fermentation that can later be used for blending in order to increase wine complexity. Thus, the implementation of the PdC method is still empirical in wineries and knowledge is lacking regarding the impact of PdC on *S. cerevisiae* diversity during alcoholic fermentation. The main objective of this study was to evaluate the impact of PdC on the microbial, chemical and sensory characteristics of the wine.

## MATERIALS AND METHODS

### 1. PdC protocol preparation

Two PdC were managed from Ugni blanc and Sauvignon blanc grape varieties in the wine estate Du. Undamaged grapes (15 kg) were manually harvested for each variety 4 days before harvest. The grapes were crushed in clean containers giving for each variety two lots of around 12 litres of must. The lots were kept in the white-wine cellar at a temperature of between 18 and 20 °C. After 2 days of alcoholic fermentation, ammonium phosphate and thiamine were added to reach the level of 200 mg l<sup>-1</sup> (Bely *et al.*, 1990) and 0.60 mg l<sup>-1</sup> respectively. The density and temperature of each PdC were monitored daily. The PdC were then kept until around 30-50 % of the alcoholic fermentation was reached. Finally, the PdC were subjected to sensory (panel of three tasters) and chemical analyses to evaluate possible off-flavour deviations and acetic acid content (analytical method described hereafter). If one of the PdC had failed one of these analyses, it would not have been retained for this project.

### 2. Analytical and population counting analyses

Total acidity (TA), reducing sugars (g l<sup>-1</sup>), turbidity (NTU - Nephelometric Turbidity Unit) and assimilable nitrogen were measured for each PdC before fermentation and for Sauvignon gris must. Sugar (g l<sup>-1</sup>), acetic acid (g l<sup>-1</sup>) and ethanol concentrations (% vol.) were measured by infrared reflectance (Wine Scan™, FOSS,

Hillerod, Denmark) at 30-50 % of fermentation of the PdC modalities and at the end of alcoholic fermentation in barrels. The volatile acidity (expressed in g l<sup>-1</sup> acetic acid) was determined chemically by colorimetry (A460 nm) in continuous flux (Sanimat, Montauban, France). The analyses were performed in accordance with the official methods described by the European Commission (1990). The amount of indigenous yeast population (CFU l<sup>-1</sup>- Colony-Forming Unit) was calculated by YPD-plate counting in the PdC before and at 30-50 % of fermentation.

### 3. Barrel inoculation

The PdC were transferred into four 225 litre barrels which were filled with Sauvignon gris “cold settled” must (Sugars 231 g l<sup>-1</sup>, pH 3.35, Total acidity 6.1, Yeast Assimilable Nitrogen 133 mg l<sup>-1</sup>, Turbidity 51 NTU). Yeast Assimilable Nitrogen level was adjusted to 200 mg l<sup>-1</sup> (Bely *et al.*, 1990). Initial colony counts in Sauvignon gris were 4.10<sup>6</sup> and 9.10<sup>5</sup> UFC ml<sup>-1</sup> for total yeast and non-*Saccharomyces* yeast respectively. PdC inoculum volume ratio was 1/20 of Sauvignon gris must. Two additional barrels were prepared as controls: one was inoculated with the commercial strains Zymaflore X5 at 200 mg l<sup>-1</sup> following the industrial recommendations, and the other was spontaneously fermented. The density and temperature of each barrel was monitored every day. At the end of the alcoholic fermentation, samples were taken for chemical analyses and each barrel was judged for tasting criteria.

### 4. Microbial sampling

Samples were taken from the PdC must at around 30-50 % of the alcoholic fermentation, just before the barrel's inoculation. They were directly plated onto the media of Total Yeast (TY) and Non-*Saccharomyces* (NS) at different serial dilutions (10<sup>-4</sup>, 10<sup>-5</sup> and 10<sup>-6</sup>), and kept at 26 °C on these two media for 5 and 10 days respectively. The TY and NS media were based on YPD (yeast extract, 1 % w/v, peptone, 1 % w/v, glucose, 2 % w/v, agar 2 % w/v), with 100 µg ml<sup>-1</sup> of chloramphenicol and 150 µg ml<sup>-1</sup> of biphenyl to delay bacterial growth for the TY medium, and an additional 500 µg l<sup>-1</sup> of cycloheximide for the NS medium. Microbial sampling was carried out on the Sauvignon gris must before alcoholic fermentation and on the 6 fermented barrels when they reached 75 % of the fermentation. For PdC just before inoculation, 30 randomly chosen colonies were collected. For barrels at 75 %, 10 colonies were collected.

After two sub-cloning on YPD plates, each colony was stored in (30 %, v/v) glycerol at -80 °C. In addition to those fermentation samples, and as a referenced out-group, 33 commercial wine strains (Supplementary data, Table S1) which are widely used as yeast starters were added to the data set.

### 5. Molecular methods

For all clones, a small amount of fresh colony was suspended in 50 µl of MilliQ water and 7 µl of this suspension was dropped on a Sigma-Aldrich Whatman® FTA® card for DNA preservation.

The clones were first analysed with PCR amplification of the ITS region with primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (Esteve-Zarazoso *et al.*, 1999) to differentiate *Saccharomyces* from non-*Saccharomyces*. The PCR reaction was made for 25 µL of final mix with 5 µL of Taq-&GO™ Ready-to-use PCR Mix from MP, 0.5 µL of each primer, 19 µL of MilliQ water and the DNA disc. The following routine PCR programme was used: initial denaturation at 95 °C for 5 minutes, followed by 35 cycles at 94 °C for 1 minute, 55.5 °C for 2 minutes, 72 °C for 2 minutes and a final extension at 72 °C for 10 minutes. PCR products were sized on the Shimadzu Microchip Electrophoresis System for DNA/RNA Analysis MCE®-202 MultiNA using the Shimadzu DNA-1000 reagent kit.

The selected *Saccharomyces* isolates were then analysed using microsatellites markers. Isolates were genotyped using 2 multiplex PCR reactions of 8 and 7 microsatellites loci for mixes 1 and 2 respectively (Supplementary data, Table S2) (Börlin *et al.*, 2016). At least 8 samples were prepared for a volume of 100 µl with 50 µl of 2X QIAGEN Multiplex PCR Master Mix in both mixes. Mix 1 had 8 multiplexed primers and mix 2 the 7 others, each having a specific concentration (specified in Table S2). The PCRs were run in a final volume of 12 µl containing 2 µl of cell suspension. The following routine PCR programme was used: initial denaturation at 95 °C for 15 minutes followed by 35 cycles of 95 °C for 30 seconds, 57 °C for 2 minutes, 72 °C for 1 minute and a final extension at 60 °C for 30 minutes. PCR products were sized on a capillary electrophoresis ABI3730 (Applied Biosystems) using size standard 600LIZ® (GeneScan™).

### 6. Data analyses

ABI3730 genotyping results were read using GeneMarker (V2.4.0, Demo). The presence of

a missing value was allowed to a maximum of 4 markers per isolate. GenClone (V2.0) software was used to remove from our dataset strains with exactly the same profiles resulting from potential clonal expansion. Dendrogram was constructed using Bruvo's distance (Bruvo *et al.*, 2004) and neighbour-joining clustering by means of the R programme (V3.2.5) with the following packages: ape (V3.2.5) and poppr (V2.8.0).

Shannon Index ( $H'$ ) was determined using the following equation, with  $n_i$  as the number of individuals for genotype  $i$  and  $N$  the total of unique genotypes:

$$H' = - \sum \left[ \left( \frac{n_i}{N} \right) \ln \left( \frac{n_i}{N} \right) \right]$$

## RESULTS

### 1. PdC implementation

Sauvignon blanc (SB) and Ugni blanc (UB) (late and early ripening grape varieties respectively) were collected four days before harvest to

produce two PdC, with the aim of obtaining PdC from two grape samples with contrasted maturity levels. Indeed, chemical analyses of the juice just after grape crushing showed different maturity levels: sugar content was 182 g l<sup>-1</sup> and 135 g l<sup>-1</sup> for SB and UB respectively, and total acidity was 5.9 g l<sup>-1</sup> and 8.6 g l<sup>-1</sup> for SB and UB respectively. The colonies count on both media were higher in Sauvignon blanc juice than in Ugni blanc: 1.10<sup>5</sup> and 1.10<sup>4</sup> UFC/mL respectively for total yeast, and 8.10<sup>4</sup> and 7.10<sup>3</sup> UFC/mL for non-*Saccharomyces* yeast.

Chemical and microbial analyses were then performed just before the inoculation of the barrels to evaluate the fermentation stage, and to infer if the PdC were good potential starters (Table 1). The sugar consumption of the Sauvignon blanc PdC was between 34 and 38 %, whereas it was between 42 and 50 % for the Ugni blanc PdC. The acetic acid content was similar whatever the modality considered, ranging from 0.35 to 0.40 g l<sup>-1</sup>. Just before the inoculation of the barrels, the different PdC modalities had similar population levels, from 2.10<sup>7</sup> to 6.10<sup>7</sup> UFC/mL for TY

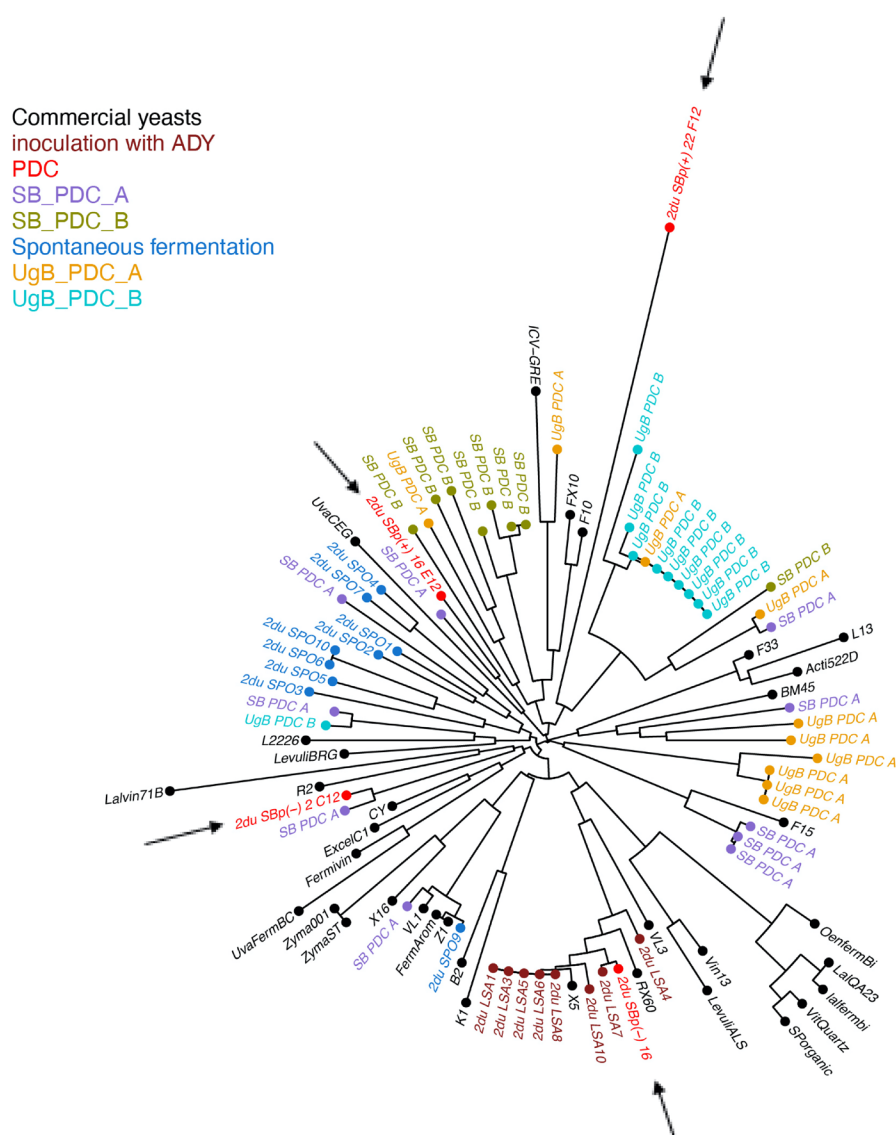
**TABLE 1.** Chemical and microbial analyses of PdC musts before barrels inoculation. SB and UB meant respectively Sauvignon blanc and Ugni blanc and PcA and PcB represented the duplicats. For yeast population counts, values in brackets represent standard deviation (n=3).

	Total yeast counts (ufc.mL <sup>-1</sup> )	Non- <i>Saccharomyces</i> count (ufc.mL <sup>-1</sup> )	Sugar g l <sup>-1</sup>	Volatile acidity g l <sup>-1</sup> of acetic acid
SB PcA	2.10 <sup>7</sup> (± 1.10 <sup>3</sup> )	1.10 <sup>7</sup> (± 2.10 <sup>6</sup> )	120	0.48
SB PcB	6.10 <sup>7</sup> (± 2.10 <sup>6</sup> )	3.10 <sup>7</sup> (± 1.10 <sup>7</sup> )	113	0.43
UB PcA	4.10 <sup>7</sup> (±0)	2.10 <sup>7</sup> (± 3.10 <sup>6</sup> )	78	0.43
UB PcB	3.10 <sup>7</sup> (± 4.10 <sup>6</sup> )	2.10 <sup>7</sup> (± 8.10 <sup>6</sup> )	67	0.49

**TABLE 2.** Summary of samples collected with PdC or barrel origin, indication of the samples code, number of yeast isolates analyzed by ITS PCR, microsatellites, final number of *S. cerevisiae* profiles after removing all strains with more than 3 missing markers values and Shannon index ( $H'$ ).

Origin	Samples	Number of isolates analyzed by ITS	Number of <i>S. cerevisiae</i> isolates analyzed by microsatellites	Number of strains with less than 4 missing markers	Shannon Index $H'$
PdC	SB PA	30	3	2	
	SB PB	30	2	2	
	UB PA	30	0	-	
	UB PB	30	0	-	
Barrels	SG/SB_PDC_A	10	10	8	1.74
	SG/SB_PDC_B	10	10	10	1.90
	SG/UB_PDC_A	10	10	10	1.83
	SG/UB_PDC_B	10	10	10	1.00
	SG Spontaneous fermentation	10	10	9	2.04
	SG inoculation with ADY	10	10	8	-
Total		180	65	59	





**FIGURE 1.** Neighbor joining tree clustering 59 strains from PdC and barrels fermentations and 33 commercial strains. The tree was constructed from Bruvo's distance between strains based on the polymorphism at 15 loci.

Color code: black; commercial strains, red; *S. cerevisiae* isolated from PdC, purple and green; isolates from Sauvignon blanc PdC barrels, orange and light blue; isolates from Ugni blanc PdC barrels, brown; barrels inoculated with commercial strain X5, blue; isolates from barrels from spontaneous fermentation. Black narrow bars indicated *S. cerevisiae* isolates collected from PdC.

and  $1.10^7$  to  $3.10^7$  for NS, thus suggesting that the non-*Saccharomyces* yeast population was the dominant one.

The possibility of using the PdC to inoculate the barrels was further confirmed by sensory analysis, since no defect was detected.

From the TY medium, 30 yeast isolates obtained from the 4 fermented PdC before inoculation and 10 isolates at 75 % of alcoholic fermentation of the 6 barrels were analysed by ITS-PCR-sequencing

(Table 2). From the different PdC, only five isolates out of 120 were identified as *S. cerevisiae*, whereas the remaining 115 isolates were identified as *Hanseniaspora uvarum*. *S. cerevisiae* was only isolated from the SB PdC (Table 2). Isolates collected from the six barrels were all identified as *S. cerevisiae*, thus resulting in a total number of 65 *Saccharomyces* isolates analysed through microsatellites markers. Only strains with less than 4 missing values were retained in the data set, which resulted in 59 *S. cerevisiae* isolates for further analysis (Table 2).

The genetic diversity of the 59 *S. cerevisiae* isolates was analysed through a neighbour-joining tree, including 33 commercial starters (Figure 1). It was possible to observe that the four *S. cerevisiae* isolates originating from the PdC were spread over the tree (black arrows, Figure 1). Thus, none of the 4 strains isolated from the PdC were recovered at mid-fermentation in the barrels.

Moreover, isolates collected from barrels inoculated with the same PdC variety clustered in different groups. A cluster of almost identical isolates resulting from clonal expansion was observed, especially for the Ugni blanc PdC (UgB\_PDC\_B) grouping 9 out of the 10 strains analyzed with the lower diversity index (Table 2). To a lesser extent, similar clusters of almost identical isolates resulting from clonal expansion can be noticed for strains isolated from PdC Sauvignon blanc (SB\_PDC\_B) and PdC Ugni blanc (UgB\_PDC\_A), grouping 4 out of 8, and 4 out of 10 strains analysed respectively. As expected, 8 isolates collected in the inoculated barrel with the industrial starter X5 clustered with the X5 strain. Surprisingly, one *S. cerevisiae* isolate originated from the PdC (2duSBp(-)16) grouped in the same cluster. No clonal cluster was observed for the 8 isolates of the spontaneous fermentation, this sample showing the higher diversity (Shannon index, Table 2).

## 2. Wine analyses and fermentation time

The chemical analyses and fermentation time of the samples collected at the end of the alcoholic fermentation of the six modalities (four barrels fermented with the PdC, plus two controls; one with spontaneous fermentation and one directly inoculated with X5 commercial strain fermentation) are given in Table 3. The fermentation

kinetics were similar for the inoculated modality and for the Sauvignon blanc PdC (Table 3 and Figure S1). However, fermentation time of the PdC Ugni blanc B modality was longer than for the spontaneous fermentation modality with 18 and 17 days respectively. Volatile acidity level (0.44 to 0.57 g l<sup>-1</sup> acetic acid) was similar for all the modalities, except for SG/UB\_PDC\_B for which volatile acidity was high (0.61 g l<sup>-1</sup> acetic acid), probably due to longer fermentation duration. Finally, a sensory analysis was performed by a panel of 15 professional tasters using black ISO glasses (NF V09-110, 1971), who gave a descriptive analysis of the wines. No significant differences were detected between all the modalities (results not shown).

## DISCUSSION

There is an increased demand, especially for organic wine producers, to limit inputs and to better use the microbiological diversity present in their vineyards. Two methods are possible to achieve this goal: i) the selection of high fermentative *S. cerevisiae* yeasts from the indigenous population, followed by small scale industrial production, following organic production rules in the case of organic producers (Settanni *et al.*, 2012; Liu *et al.*, 2015), or ii) the production of a home-made yeast inoculum from selected grape juice a few days before harvest or from the first spontaneous fermented vat. This latter method has been reported to have some positive impacts on fermentation and the final product (Bely *et al.*, 2005; Santamaría *et al.*, 2005; Börlin *et al.*, 2016).

In this study, modalities using two grape varieties were prepared to evaluate the impact of the PdC on *S. cerevisiae* diversity, fermentation management and wine composition. The use of PdC to

**TABLE 3.** Chemical analyses of the barrel musts at the end of the alcoholic fermentation (or total sugar consumption). SB, UB and SG meant respectively grape varieties Sauvignon blanc, Ugni blanc and Sauvignon gris. OD is for Optical density. Pc, Spo and LSA represented the pied de cuve modalities, spontaneous fermentation and direct inoculated fermentation with X5 commercial strain.

	SG/SB_PDC_A	SG/SB_PDC_B	SG/UB_PDCA	SG/UB_PDC_B	SG Spo	SG ADY
Fermentation time (days)	12	13	13	18	17	11
Final alcohol - % vol.	14.20	14.35	14.20	13.98	14.20	14.00
Total Acidity g l <sup>-1</sup> (of tartaric acid)	6.40	6.12	6.40	6.38	6.28	6.90
pH	3.28	3.31	3.30	3.32	3.24	3.23
Volatile acidity - g l <sup>-1</sup> (of acetic acid)	0.50	0.44	0.57	0.61	0.56	0.54
Total SO <sub>2</sub>	15	9	10	11	38	9
OD 420	0.22	0.20	0.19	0.18	0.16	0.20

manage alcoholic fermentation was compared with the traditional use of ADY and spontaneous fermentation. With the exception of one modality, the fermentation times of PdC inoculated barrels were similar to those observed for the ADY inoculated control fermentation. In this trial, and in terms of alcoholic fermentation kinetics, the use of the PdC method has provided results which are comparable to the classical use of commercial strains. However, more tests are required in order to assess the success rate of such an approach. It is noteworthy that the barrels inoculated with the Ugni blanc PdC and the spontaneous fermentation modality had longer fermentation duration and higher acetic acid content. Except for the two latter modalities the use of the fresh PdC must be as efficient as ADY in terms of chemical and sensory analyses of the resulting wines. However, the grapes with higher maturity level were those leading to a PdC containing higher frequency of *S. cerevisiae*, suggesting that this factor might be important, even though *Saccharomyces* are rarely found in significant numbers on healthy grapes (Mortimer and Polsinelli, 1999). No significant differences were found between the wines as a result of the descriptive sensory analysis. Thus, in our experimental conditions, the use of PdC did not lead to distinct wines according to the origin of the yeast strains in the vineyard.

The microbiological analysis of the PdC revealed surprising results. First of all, the alcoholic fermentation of the PdC mainly occurred through non-*Saccharomyces* yeast; *H. uvarum* and *S. cerevisiae* were particularly rare, especially when using the culture method. It was not possible to isolate *S. cerevisiae* strains in the 2 Ugni blanc PdC, probably due to the lack of ripening for the grapes when they were collected. In our experimental conditions, due to the low *S. cerevisiae* population level in the PdC, more colony analyses, or an estimation of *Saccharomyces* sp. population level through quantitative PCR, would have been required to provide a better estimation of the *S. cerevisiae* frequency. Regarding the barrels, spontaneous fermentation showed high *S. cerevisiae* diversity, indicating that various strains grew leading to polyclonal populations during these fermentations, in contrast to ADY inoculation. For PdC inoculated barrels, clonal expansion of *S. cerevisiae* was highlighted during fermentation, especially for the Ugni blanc PdC, and to a lesser extent for the other modalities, with less than 50 % of the strains clustering together. The use of PdC seemed to enable the winemakers to select indigenous *S. cerevisiae* strains which

differed from one modality to another, and to the *S. cerevisiae* population that occurred during spontaneous fermentation in the cellar. A dominant population, in line with what could be obtained by using ADY, may process alcoholic fermentation. The results seem to be uncertain since one modality failed in terms of fermentation kinetics and a high acetic acid level in the resulting wine. The preparation of different PdC modalities should thus lead to a more secure process.

A question still remains to be answered. It was shown here that the different PdC, apart from one Ugni blanc modality, showed well monitored fermentations with proper final product characteristics similar to the direct ADY inoculation ones. Despite the very low frequency of *S. cerevisiae* yeast identified out of the 30 *S. cerevisiae* analysed for each PdC, the fermentations were completed. It has been shown that *S. cerevisiae* is capable of attaining from 0.1 to 99.9 % of overall yeast population growth in only a few days (Goddard, 2008). *S. cerevisiae* colonises the medium by producing a high amount of alcohol and heat through the Crabtree effect, and this latter trait provides a 7 % fitness advantage over the other members of the community (Goddard, 2008). The fitness advantage of *S. cerevisiae* over non-*Saccharomyces* yeast could explain the short lag-phase and complete fermentation of 3 out of 4 PdC modalities, despite very low *S. cerevisiae* population levels in the PdC before barrel inoculation.

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