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Microbiota, disease-resistant varieties and wine quality

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Abstract. The development of interspecific hybrid varieties (IHVs) resistant to diseases such as powdery mildew and downy mildew is enabling a reduction in the use of inputs in viticulture. These IHVs respond to societal demands for reduced environmental impact and are increasingly being adopted. Meanwhile, wines produced from spontaneous fermentations, based on indigenous flora, are gaining popularity. Opting for spontaneous fermentations, which aim to utilize and preserve natural biodiversity, requires an in-depth understanding of microbial communities and their impact on fermentation and wine quality. This project aims to characterize the diversity of the microbiota on IHV grapes and the biotic and abiotic factors influencing it. Fifteen varieties, including IHVs, were collected from four vineyards in the Languedoc region of France, and their microbiota was analyzed using metabarcoding. Variations related to the region, agro-ecological environment, and variety were studied. The impact of the microbiota on the aromatic quality of wines made from indigenous fermentations was also assessed through microfermentations using the microbiota of Carignan (a traditional grape variety) and Artaban (an IHV).

1. Introduction

The surface microbiota of grape berries is made up of yeasts, filamentous fungi and bacteria. [1]. The composition of the microbiota, particularly yeasts, has an impact on the aromatic complexity of wines [2]. Yeasts have the ability to modulate ethanol production, release mannoproteins or express metabolic pathways that release aromas [3,4,5]. Fermentative yeasts can be divided into two main groups, *Saccharomyces* and non-*Saccharomyces*. *Saccharomyces* are the microorganisms with the highest fermentation capacity. For this reason, they are the first to be used as starters to guarantee the start and completion of alcoholic fermentation [6]. However, the use of *Saccharomyces* as a starter leads to homogenisation of the final product, with a loss of aromatic complexity in the wine [7]. Non- *Saccharomyces* species, on the other hand, are linked to the aromatic complexity of wine, a demand from consumers that is growing all the time. Wine producers will therefore benefit from a better understanding of indigenous fermentation, i.e., fermentation without the addition of starter strains. In this context, several studies on indigenous fermentation have been carried out to characterize indigenous yeasts and their role in fermentation [5].

At the same time as consumers are looking for a wine with greater aromatic complexity, there is a societal demand for a reduction in the use of pesticides. Meanwhile, a number of public policies are being implemented in Europe to promote a transition to more sustainable agriculture [8]. It is in this context that the use of varieties resistant to vine diseases, in particular powdery mildew and downy mildew, is used as an alternative to reduce the need for fungicides [9]. These varieties, known as interspecific hybrid varieties (IHVs), have been bred for several decades following crosses between traditional *Vitis vinifera* varieties and wild *Vitis* species [10,11]. Until now, there have been no studies on the microbiota of IHVs. The aim of this study is to determine the factors that modulate the microbiota of IHV grape berries and their impact on the quality of wine produced by indigenous fermentation over one vintage (2023).

2. Materials and methods

2.1. Diversity of grape berry microbiota

In this study, 15 varieties were studied, including French and Mediterranean grapes, disease-resistant varieties and a

direct-producer hybrid, Isabelle. These varieties were collected from four vineyards in the Languedoc-Roussillon region: the Pech Rouge experimental unit (PR) and the Cazes, Vassal and Chapitre estates. Grapes were harvested between August 22 and September 18, 2023. For the study of the locality effect, the G14 variety was harvested at Pech Rouge, Cazes and Vassal; Syrah and Artaban at Pech Rouge, Chapitre, Cazes and Vassal. To study the variety effect, several varieties were harvested in the same vineyard, and if possible in the same parcel. On the Cazes estate, the varieties harvested were Artaban, Syrah, G14, Petit Verdot, Fer Servadou, Touriga National, Niellucio, Pinotage, Vidoc, Monarch, Cabernet Cantor, Merlot Khorus and Pinotin. At Pech Rouge, the varieties sampled were Artaban, Carignan, G14 and Syrah. At Vassal, samples were taken from Artaban, G14, Isabelle and Syrah. To study the impact of the agro-ecological environment, the SALSA system from the Domaine du Chapitre was used. The resistant Artaban variety was subjected to three different cropping systems: a conventional reference system (TViti), and two agro-ecological viticultural systems (AViti and DViti). AViti included grass between the rows, while DViti included trees such as fig and pomegranate. A Syrah control was also considered as a conventional cultivation modality. Each system is divided into three blocks.

Each grape variety was collected in three 500 g batches for microbiota characterization. The grape varieties were subjected to a general protocol for the recovery of microbiota by berry rinsing, for the physico-chemical characterization of the initial musts (assimilable nitrogen, sugars, pH, total acidity, malic acid) and for monitoring vinification. For berry rinsing and recovery of microorganisms present on the berry surface, grapes were rinsed in a buffer containing 0.9% w/v NaCl and Tween [12]. The rinse buffer was then centrifuged and the pellet containing the microbiota was frozen at -20°C. DNA from the pellet was extracted using the powersoil Pro kit (Qiagen, France), then used for metabarcoding analysis with ITS1 as the taxonomic marker. Primers used were ITS1F (5′-TCGTCGGCAGC GTCAGATGTGTATAAGAGACAG-3′) and ITS2 (5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACA G-3′). Amplicons were sequenced by Illumina using a Miseq system.

2.2. Impact of microbiota on fermentation kinetics and the aromatic quality of wines

For this experiment, the Carignan and Artaban varieties were harvested at Pech Rouge.

The fermentations were carried out on the fermentation platform (automatic fermenter, weight taken every 20 min) at INRAE-SPO, with a flash pasteurized Merlot must (259 g/L sugar, assimilable nitrogen 41 mg/L adjusted to 160 mg/L, pH 3.3 and total acidity 3.23). The inoculated musts were spread on Petri dishes to enumerate cultivable yeasts present at T0 (initial time).

To prepare the Pied-de-Cuve, Artaban and Carignan grape musts were fermented until 10 g/L of CO_2 was released. The yeast concentration of the fermenting musts from the Pied-de-Cuve was estimated using a coulter and their microbiota was recovered by centrifugation. The centrifugation pellet was then resuspended in an equivalent volume of the sterile Merlot must to inoculate three fermenters at concentrations of 2.10^6 cells/mL. At the same time, control fermentations of the same Merlot must were inoculated with *S. cerevisiae* strain K1 at a concentration of 2.10^6 cells/mL. During fermentation, samples were taken at various time points for physico-chemical analysis and metabarcoding analysis with ITS1 as the taxonomic marker. At the end of fermentation, quantification of volatile compounds was performed by GC-MS (gas chromatography-mass spectrometry). Analyses were performed in triplicate.

2.3. Analysis of aromatic components by gas chromatography-mass spectrometry

The methodology used to prepare the samples was previously described [13]. This methodology allowed identification of 33 volatile compounds grouped into four families: higher alcohols, acids, acetate esters and ethyl esters. Samples were analyzed using an Agilent 8860 gas chromatograph equipped with an Agilent 7693A autosampler and coupled to an Agilent 59778 mass spectrometer (Agilent Technologies, Santa Clara, California, USA). Data were acquired and processed using OpenLab CDS 2 software (Agilent Technologies, Santa Clara, California, USA). The gas chromatograph was equipped with a ZB-WAX 30 m × 0.25 mm × 0.25 µm fused silica capillary column (Phenomenex, Torrance, California, USA). Helium was used as the carrier gas (Air Products, Allentown, Pennsylvania, USA) at a constant flow rate of 1 mL/min. The oven parameters used for this analysis were as follows: the initial temperature was 40°C held for 3 minutes, followed by an increase at a rate of 4°C/min to 160°C, then an increase at a rate of 15°C/min to 220°C, and finally an increase at a rate of 20°C/min to 240°C, held at this temperature for 10 minutes. The injector was set at 240°C, the autosampler at 8°C, and the sample volume injected was 2 µL in split mode with a split ratio of 10:1. The temperature of the mass spectrometer quadrupole was set at 150°C, the ion source at 230°C and the transfer line at 240°C. For quantification, mass spectra were acquired in Selected Ion Monitoring (SIM) mode with an electron impact energy of 70 eV.

2.4. Statistical Analysis

To analyze the metabarcoding data, amplicon sequence variants (ASVs) were constructed from the raw reads using the FROGS v.5.0.0 pipeline. The process begins with a cleaning step. Firstly, reads containing indeterminate bases (N) were eliminated from the FASTQ files and primers were removed using Cutadapt. Next, the reads were denoised using the DADA2 algorithm [14]. The sequences of the R1 and R2 reads were then aligned (overlapped) where possible using PEAR [15]; where alignment was not possible, the R1 and R2 reads were kept separate. Primers were then removed from the sequences using Cutadapt.

The remaining sequences were filtered for length (min: 50 bp) before dereplication. Chimera were removed with vsearch [16] and low-abundant ASVs (less than 10,000) were also discarded. ITSx [17] was applied to remove non-ITS ASVs and remaining ASVs were taxonomically affiliated using UNITE v9.0 [18] completed with manually curated sequences [19]. Analyses were then performed using the Easy16S tool [20]. For the study of β-diversity between the microbiota of different samples, the statistical test used was a Permutational Multivariate ANOVA (PERMANOVA) with 999 permutations. Significance was considered when $p<0.05$.

The other data analyses (ANOVA and Tukey tests) were performed using Rstudio (version 2024.04.2+764). Significance was considered when $p<0.05$.

3. Results and discussion

3.1. Diversity of grape berry microbiota

3.1.1. The effect of vineyard location

Based on the metabarcoding results, it is possible to study the effect of the geographical location of vineyards on the microbiota of grape berries. The following Principal Coordinate Analysis (PCoA) was generated using the β-diversity data of the microbiota.

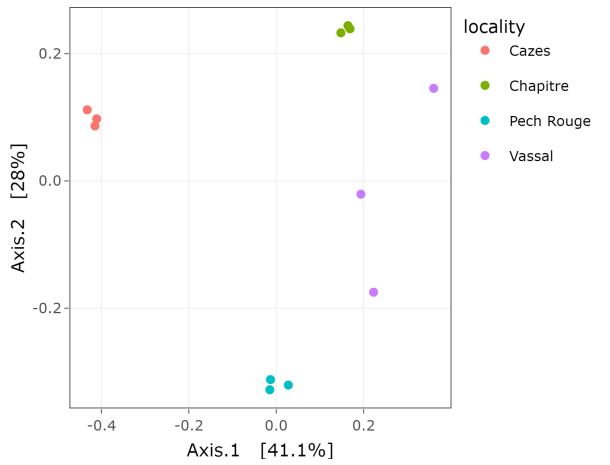

Figure 1. Effect of vineyard location on the microbiota of Syrah berries.

Figure 1 shows a PCoA using a Bray-Curtis distance for the locality effect for the Syrah variety. In the case of Syrah, the PCoA explains the variability of about79% of the data. For the β-diversity analysis, PERMANOVA analyses were also performed taking into account the location factor. The location explained 82% of the data, with a significant p-value ($p<0.0001$).

Location was also shown to be an important explanatory factor for the microbiota of the varieties Artaban (sampled at Pech Rouge, Chapitre, Cazes and Vassal) and G14 (Pech Rouge, Cazes and Vassal). For Artaban, a PERMANOVA analysis showed that location explained 87% of the data with a significant p-value ($p<0.001$). For G14, the location effect explained 90% of the microbiota data, with a p-value <0.01. The localisation effect on microbiota composition shown for three varieties in our study confirms data in the literature [21,22].

3.1.2. The effect of variety

It has been described in the literature that for non-HIV, variety is a factor impacting the composition of grape berry microbiota [23, 24, 25]. We were able to verify the effect of variety on the composition of the microbiota for samples collected at Vassal. The PCoA carried out on the Artaban, G14, Isabelle and Syrah varieties showed a grouping of samples according to variety (Figure 2).

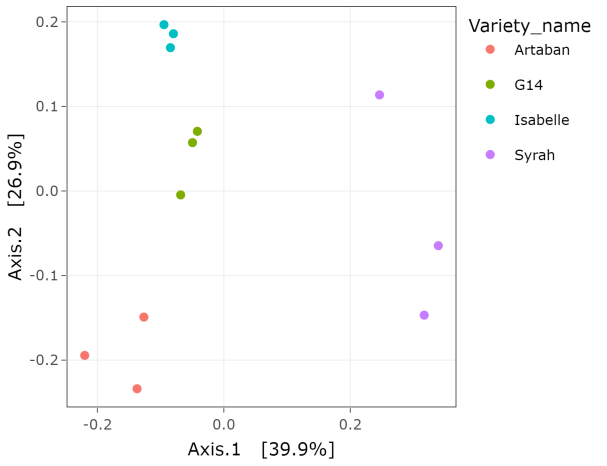

Figure 2. PCoA (Bray-Curtis distance) of grape berry microbiota for the Artaban, G14, Isabelle and Syrah varieties at the Vassal estate.

The PERMANOVA test of β-diversity also showed a significant difference between the microbiota of the samples according to the different varieties collected at Cazes ($p<0.0001$). Similar results were found for samples taken at Pech Rouge, with a PERMANOVA test showing that variety explained 75% of the microbiota data with a significant p-value ($p<0.001$).

3.1.3. Effect of the agro-ecological environment

The SALSA device at the Domaine du Chapitre presents three different agro-ecological systems coupled with two cultivation methods.

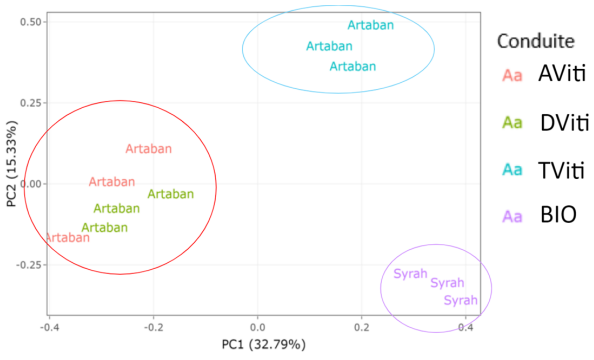

Figure 3. PCA of the microbiota of the Syrah and Artaban varieties from the Domaine du Châpitre according to the different cultivation methods.

Figure 3 shows a Principal Component Analysis (PCA), which compares the microbiota of the Syrah and Artaban varieties according to the different cultivation methods. It accounts for 48% of sample diversity and shows three distinct groups of samples: a first group comprising the control triplicates of Artaban (TViti); a second group including the AViti and DViti cultivation methods; a third group comprising samples of the Syrah variety. The results of PERMANOVA tests show marginally significant differences between the AViti and TViti methods, as well as between DViti and TViti, with a p-value < 0.1 for both comparisons. Similar differences were observed when comparing the AViti or DViti modalities with the Syrah variety (p-value < 0.1). The observation of these differences with the microbiota of the Syrah variety was expected due to the variety effect, but was not expected between the three modalities of Artaban. However, the average brix (sugar content) for the AViti and Dviti modalities was 21 while it was 18 for TViti suggesting that their maturity was different. Maturity also has a major impact on microbiota [26]. It is therefore not clear whether the difference in microbiota between the two groups formed by the Artaban modalities is due to agroecology or to maturity.

The fact that the composition of the microbiota in the AViti and DViti modalities was not significantly different could be explained by the date when the plots were planted. The Pomegranate and fig trees, planted in 2018 in the DViti modality, have not yet had time to grow sufficiently to exert a distinct environmental pressure compared to the weeds present in the AViti modality. It is possible that more time is needed for the trees to have a significant impact on the microbiota, which highlights the importance of study duration in observing long-term agroecological effects.

4. Impact of microbiota on fermentation kinetics and final wine quality

4.1. Fermentation kinetics

To show the impact of microbiota on fermentation kinetics and wine quality, the microbiota from the Pied-de-Cuve of Artaban and Carignan varieties was transferred to a flash-pasteurized Merlot must. Figure 4 shows fermentation kinetics curves for the different microbiota transplantation modalities, i.e., Pied-de-Cuve from Artaban and Carignan, and *S. cerevisiae*.

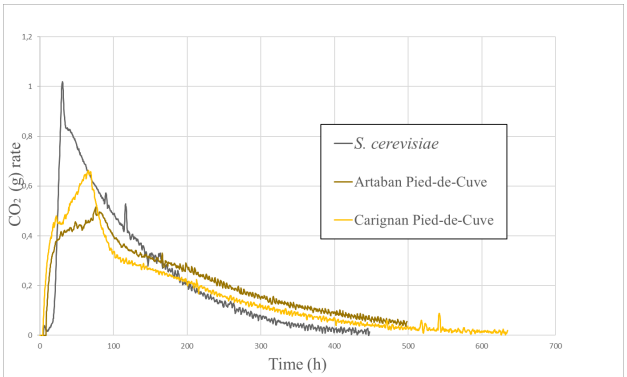

Figure 4. Fermentation kinetics comparing the modalities of Pied-de-Cuve inoculation with Artaban and Carignan microbiota with that of *S. cerevisiae* inoculation.

The distinct kinetic behaviors observed can be attributed to the varying inoculation modalities. Four parameters have been extracted from the presented curves (Table 1). Lag time is defined as the time required to reach 1 g/L of CO_2 released. Max flow rate represents the flow rate at maximum CO_2 release. Vmax time corresponds to the time when the maximum CO_2 release rate is observed. Total time is the time required to achieve fermentation, considered at a flow rate lower than 0.05 g/L/h. The letters in Table 1 represent different Tukey test groups.

Table 1. Mean values and standard deviations for key parameters of the fermentation kinetics.

Modalité	Lag time (h)	Vmax time (h)	Max flow rate (g/L/h)	Total time (h)
Pied-de-Cuve Carignan	9,8933 ± 2,69 c	66,56 ± 0,38c	0,7 ± 0,06 c	559,39 ± 68,61 ab
Pied-de-Cuve Artaban	14,72 ± 1,96 b	78,6 ± 4,52b	0,49 ± 0,02 b	596,71 ±85,34 b
S. cerevisiae	20,75 ± 1,82 a	29,30 ± 1,65a	0,96 ± 0,1 a	495,93 ± 41,38 a

A significant difference in ANOVA was observed for all the parameters. Tukey's test confirmed significant differences ($p<0.05$) between the means of all inoculation modalities for the latency time, Vmax time and max flow rate parameters. For the total time of fermentation only differences between Pied-de-Cuve Artaban and *S. cerevisiae* were found. The modality inoculated with Carignan Pied-de-Cuve started faster than the others, followed by Artaban pied-de-cuve and the *S. cerevisiae* modality. The faster start-up of the Pied-de-Cuve can be explained by the fact that, at the time of inoculation, the Pied-de-Cuve microbiota had already adapted to the must, unlike the *S. cerevisiae* modality inoculated with active dry yeast, which required more time to adapt.

4.2. Fermentative aromas

The volatile aromatic compounds present in wines at the end of the fermentation process were subjected to analysis

via GC-MS. The data generated for the 33 compounds under investigation were initially subjected to a PCA (Figure 5).

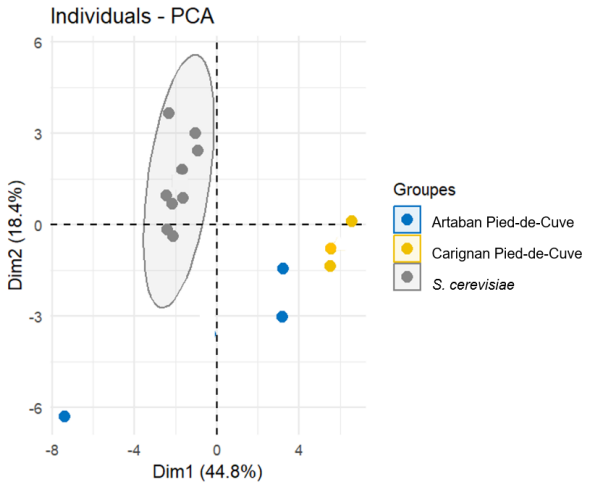

Figure 5. PCA generated from the results of 33 fermentative aromas obtained through the use of GC-MS.

The PCA explains 63.2% of the total variance in the data set (Dim1 + Dim2). A clustering of aromas is observed according to the modality of microbiota inoculation, with the exception of a replicate of Artaban Pied-de-Cuve. This clearly confirms that microbiota has an impact on volatile aromas.

Volatile compounds can be classified into four primary categories based on their fermentation characteristics: higher alcohols, acids, ethyl acetates, and acetate esters. The total concentrations of these four families of aroma components are illustrated in histograms in Figures 6 and 7, according to the three microbiota inoculation modalities. The data demonstrate that the wines produced via inoculation with the Carignan Pied-de-Cuve exhibit a higher concentration of acids and ethyl esters, while displaying a lower concentration of higher alcohols.

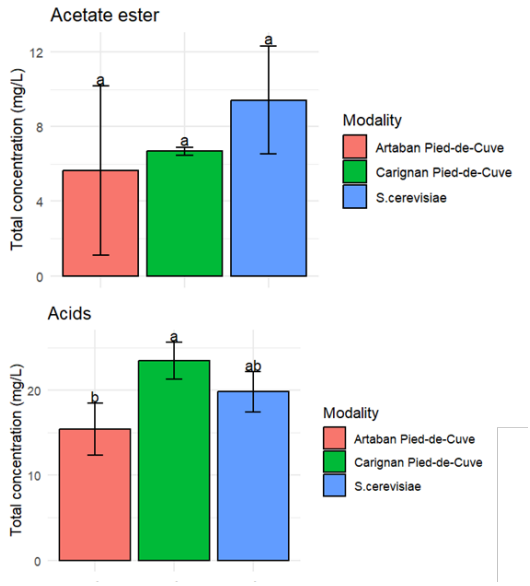

Figure 6. Histograms of total concentrations of acetate acids and acetate esters.

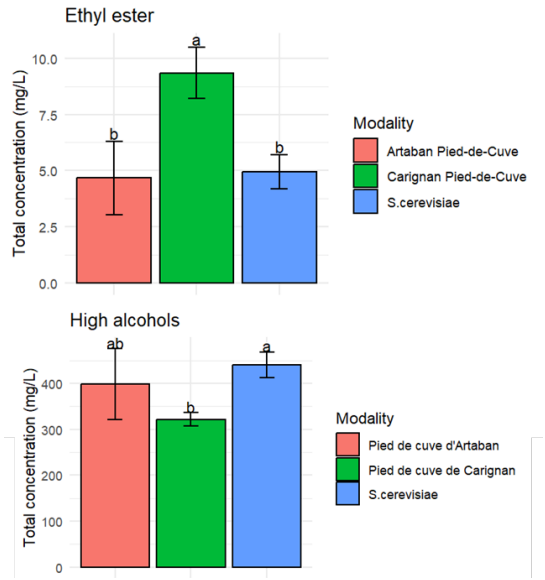

Figure 7. Histograms of total concentrations of ethyl ester and higher alcohols.

To explain the differences in the production of volatile aromas, it is essential to determine the composition of the microbiota at the inoculation step. Figure 8 illustrates the microbiota composition of each microbiota transplantation modality in the inoculated must, with three replicates per modality.

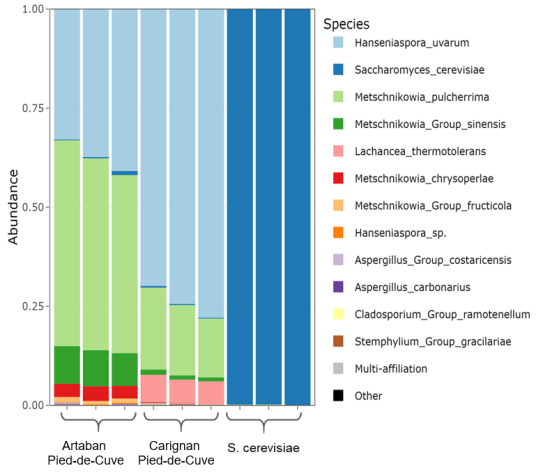

Figure 8. Microbiota composition of flash-pasteurized must after inoculation with different microbiota: Artaban Pied-de-Cuve, Carignan Pied-de-Cuve and *S. cerevisiae*.

The yeast species *Metschnikowia fructicola* and *Metschnikowia chrysoperlae* were only identified in the Artaban Pied-de-Cuve sample, while *Lachancea thermotolerans* was only detected in the Carignan Pied-de-Cuve sample. This latter yeast is widely known for its capacity to produce wine with a reduced alcohol content compared to controls inoculated with *S. cerevisiae* [27]. As illustrated in Figure 7 (histogram of higher alcohols), the total quantity of higher alcohols produced by the Pied-de-Cuve of Carignan is smaller than that produced by *S. cerevisiae*. Additionally, it is established that *L. thermotolerans* produces a higher total quantity of esters than *S. cerevisiae*, which imparts fruity aromas to the wine [28,29]. In our study, a higher quantity of total ethyl esters

was observed in Carignan Pied-de-Cuve, yet no significant difference was identified in the total acetate esters in comparison to *S. cerevisiae* (Figures 6 and 7).

The genus *Hanseniaspora* has been found to produce a significant amount of acetate esters [30]. Nevertheless, an elevated *H. uvarum* population in Carignan Pied-de-Cuve did not result in augmented total acetate ester production in comparison to other inoculations (Figure 6).

The presence of *M. pulcherrima* is associated with an increase in total esters in comparison to *S. cerevisiae* [31, 32]. In this study, approximately 50% of the reads correspond to *M. pulcherrima* in the Artaban Pied-de-Cuve modality while in the Carignan modality, this species represents about 17% of the reads. The results showed that, contrary to expectations, the Artaban modality did not produce a higher total ester content than the other modalities. In fact, the data revealed a higher production of total ethyl esters for Carignan Pied de Cuve, with a significant difference. Conversely, there was no significant difference in acetate ester content across all modalities.

It is important to note that different yeast species interact with one another, influencing and modifying metabolic pathways and impacting flavor production [33]. Similarly, strains of the same species can exhibit different behaviors, which impact the production of volatile compounds in varying ways [34]. In this study, it was not feasible to attribute the production of an aromatic component to the presence or absence of a species due to the simultaneous presence of several strains and species.

5. Conclusion

The study emphasized the impact of variety and geographical location on grape berry microbiota diversity for a specific vintage, with a particular focus on IHV. In contrast, the agro-ecological modalities of the SALSA device did not show any difference in grape berry microbiota during the 2023 vintage. It will likely be several years before the specificities of each modality are more pronounced in terms of the landscape. The results demonstrate that each factor exerts a significant impact on microbial composition, indicating the presence of intricate interactions between the plant, its surrounding environment, and associated microbial communities.

Furthermore, the influence of microbiota on fermentation kinetics and wine organoleptic characteristics was investigated in greater detail by carrying out microbiota transplantation experiments. Using flash-pasteurized must, the microbiota of two distinct varieties, Artaban and Carignan, were transferred, enabling direct analysis and comparison of the effect of variety on microbial dynamics. This experimental approach yielded valuable information on the specific interactions between microbiota and variety, paving the way for a better understanding of the mechanisms underlying microbial diversity in vineyards and their potential impact on wine quality.

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