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New markers for monitoring “fresh mushroom aro ma” in wine: A dual approach using microbiological and chemical tools from the vineyard to winery–A synthesis of recent research advances

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Abstract. The “fresh mushroom aroma” has been recognized by the wine industry as an emerging defect since the 2000s. For many years, this off-flavour was not specifically characterized and rather grouped under “earthy” and “musty” taints. However, it has become increasingly problematic due to its rising prevalence. After five years of collaborative research, new markers have been identified for monitoring this novel risk. These findings enhance our understanding of the “fresh mushroom off-flavour” throughout the entire winemaking process, from vineyard cultivation to wine production. The first part focused on determining mycobiota dynamics from vine to wine and identified members of the *Penicillium* genus as specifically associated with this off-flavour in wines. The second part provided practical benefits for oenologists as we identified a new chemical marker most likely associated with the early stages of this defect development. 1- hydroxyoctan-3-one was identified for the first time as a significant volatile marker involved in “fresh mushroom aroma”. Combined together, these research advances expand our current knowledge, and provide novel solutions and tools for effectively preventing “fresh mushroom aroma” off-flavour in wines.

1. Introduction

The “fresh mushroom aroma” (FMA) was first identified as an emerging problematic for winemakers and vine growers as early as 2005 and then again in 2010-2011 [1]. Initially included in the generic family of “earthy musty aromas”, this defect seems to be associated with rainy climatic conditions towards the end of the grape ripening period, especially in the presence of *Botrytis cinerea* and other fungal species on grape clusters [2].

From 2010 to 2016, scientific researches clarified the “earthy musty aroma” defect by identifying a marker molecule, namely geosmin. The latter is specifically produced on grape jus when there is a microbial interaction between *Botrytis cinerea* and *Penicillium expansum* [3] [4]. However, these studies did not resolve the FMA topic as neither the entirety of the involved molecules [5] nor the understanding of the causes of this defect were determined. Since 2017, this defect continues to create challenges in vineyards [6].

Considering this ongoing situation, the new research presented in this synthesis highlights recent results to better understand the FMA defect. Over the past four years, comprehensive research has been conducted on all components related to the FMA in wines. This research covered the ecology of grape berries and musts to identify the microorganisms and specific chemical molecules responsible for this defect.

2. Advances in understanding the microbiota potentially involved in the FMA defect

The data presented are derived from published research conducted in collaboration between the Robert-Jean de Vogüé Research Center and the Biodiversity and Microbial Ecology Laboratory in Plouzané [7].

2.1. A methodology to trace microbiota from vine to wine

Over two vintages (2021 – 2022), 31 vineyards plots growing Pinot Noir and Meunier grape varieties were monitored in the Champagne region. This sampling strategy encompassed all production areas and included a balanced number of Pinot Noir and Meunier grape variety plots from the selected sectors. Analytical monitoring of each plot was carried out during berry maturation up until harvest. At this stage, 40 kg of grapes, unsorted, were collected from each plot, pressed (using a 40 kg Speinel press), and microvinified in 2-liter flasks. The resulting wines were analysed (physicochemical analyses and specific quantification of 1-octen-3-one) and tasted. This standardized tasting criterion, conducted by two panels - a trained panel and an operational oenologist one - that determined whether wines from the 2021 vintage were categorized as "FMA defect", "Other defects", or "Without defects". Wines from the 2022 vintage exhibited none of the sought-after defects. Concurrently, two methodologies were used to track the microbiota from berry to wine: a cultural approach and a metagenetic approach targeting bacteria (using the 16S rRNA region) and fungi (targeting the ITS2 region). The methodology is summarized in Figure 1.

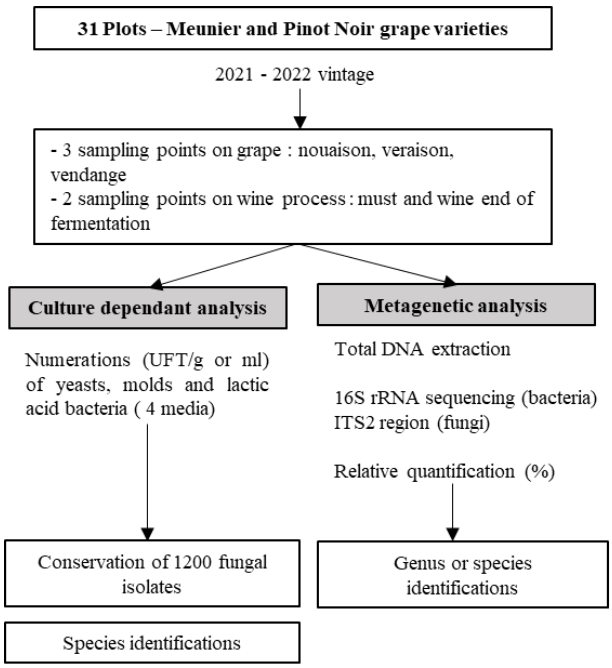

Figure 1 : Experimental design

2.2. First dynamic description of the mycobiota from Champagne region grape berries

For the 2021 vintage, two types of data were obtained for the 31 plots: quantitative changes in mycobiota counts and composition during grape berry maturation, in musts and in wines, as well as the link between this mycobiota and the sensorial properties of the corresponding small-scale wines that were produced.

During berry maturation, total fungal counts increased from an average of 4 log CFU/g at fruit set to 5.3 log CFU/g at harvest. More specifically, at fruit set and veraison stages, *Aureobasidium pullulans* (mean relative abundance of 57% and 58%, respectively) and *Cladosporium cladosporioides* (17% and 14%, respectively) were predominant. For the other identified genera and species, no clear dynamics emerged at these early stages. At harvest, *Botrytis cinerea* was significantly present in 9 parcels representing up to 28% relative abundance (culture dependant method).

At this stage, the *Penicillium* genus was also present with *P. brevicompactum* detected in 8 parcels and *P. bialowiezense* in 6 parcels. Furthermore, *Penicillium* spp. were not uniformly represented as they accounted for 2 to 40% of the total fungal abundance among the plots where *Penicillium* was detected. Based on these cultural data, no significant differences were observed between the two studied grape varieties (Pinot Noir and Meunier) at veraison and harvest stages. However, at fruit set, fungal abundances were significantly higher for Meunier grapes than for Pinot Noir grapes with, in particular, *A. pullulans* being more abundant on Meunier grapes.

To further determine fungal diversity and dynamics, a complementary metagenetics approach was also used. The results again showed that the most dominant genera in the early stages were *Aureobasidium* (mean relative abundance: 63% at fruit set, 58% at veraison, 36% at harvest) and *Cladosporium* (mean relative abundance: 29% at fruit set, 24% at veraison, 17% at harvest) but also *Vishniacozyma* (mean relative abundance: 6% at fruit set, 2% at veraison, 5% at harvest). Between veraison and harvest, *Botrytis* and *Penicillium* relative abundances increased according to the considered sample (*Botrytis* mean relative abundance: 16% at veraison versus 39% at harvest; *Penicillium* mean relative abundance: 0.2% at veraison versus 1.8% at harvest) while in musts yeast dominated (*i.e. Starmerella*, *Metschnikowia*, *Pichia* and *Hanseniaspora*).

The results presented for the 2021 vintage for two Champagne grape varieties, Pinot Noir and Meunier, are the first for this viticultural area. They are well correlated to previously published data described in various vineyards, for different grape berry varieties, at various maturity stages and within diverse pedoclimatic contexts [8], [9], [10]. In these studies, the main genera included *Alternaria*, *Acremonium*, *Aspergillus*, *Cladosporium*, *Fusarium*, *Penicillium*, and *Rhizopus*. Nevertheless, their proportions vary depending on climate and geographical location: *Alternaria* (2.8% - 80%), *Acremonium* (0.3% - 0.8%), *Aspergillus* (1% - 79.7%), *Cladosporium* (4.4% - 92.2%), *Fusarium* (0.5% - 18%), *Penicillium* (2.3% - 31%) and *Rhizopus* (0.8% - 2.4%) [11].

This segmentation to the genus level is the most common denominator found in different studies. Segmentation to the species level has already been shown to vary between two vintages of the same grape variety and within the same geographic area [12]. Over the last five years, studies have shown the necessity to situate research on grape bunch mycobiota within a given geographical

perimeter and contextualize it by vintage (climate), by varietal and by "viticultural coherence zone" (practices, soil type, exposure, etc.) [13], [14], [15]. Thus, data acquired in the Champagne region could feed future work in this viticultural zone.

2.3. The must stage, a key step to correlate mycobiota with the FMA defect in wine

Micro-vinifications obtained from sampling at each vineyard, using a 2 liters scale, facilitated studying mycobiota continuum throughout the winemaking process. As mentioned, three sensory classes of wines were defined ("Without defects", "FMA defect", and "Other defects") to further investigate the link between mycobiota composition, and the presence of specific genera and/or species from grape to transformation and the "fresh mushroom aroma" defect identified in some wines.

Based on cultural data, total fungal abundance was similar between samples from each sensory class from fruit set to harvest (around 10^4 CFU/ml at fruit set to 10^5 CFU/ml at harvest) (Figure 2). However, a distinct difference was observed between sensory classes during the winemaking process. Samples belonging to both "FMA defect" and "Other defects" retained high total counts in musts (around 10^3 to 10^5 CFU/ml), while these counts decreased to levels below 10^2 CFU/ml for samples from the "Without defects" class.

To better understand the main differences explaining these results and based on cultural data, *Penicillium* genus was identified as significantly more prevalent in musts that produced FMA wines than those belonging to the "Without defects" or "Other defects" wines. This genus accounted for 19% of the total fungal abundance in "FMA defect" class musts. Overall, based our results, *Penicillium* genus persisted between the "harvest" and "must" stages (only 1 log CFU/ml difference) while most other fungal genera decreased by about 4 log CFU/ml. At both "harvest" and "must" stages, some yeasts were also present (*A. pullulans)* or more frequently isolated (*Vishniacozyma carnescens* and *Hanseniaspora uvarum)*. Metagenetic data provided a different perspective on the results, with mainly significant differences between relative abundances for specific genera associated with the "FMA defect" and "Without defects" classes on one hand, and "Other defects" on the other. These differences were linked to the genera *Starmerella*, *Aureobasidium*, *Cladosporium*, *Hanseniaspora*, *Vishniacozyma*, and *Penicillium*. Compared to the "FMA defect" and "without defects" categories, the "Other defects" category exhibited a higher relative abundance of *Starmerella* and a lower relative abundance of *Aureobasidium* and *Cladosporium*. The relative abundance of *Vishniacozyma* was significantly lower in the "Other defects" category compared to that in the "Without defects" category. Lastly, the genera *Penicillium* and *Hanseniaspora* presented slightly higher relative abundances in the "Without defects" category.

These results highlighted that the "must" stage exhibited clear differences in mycobiota composition among the "FMA defect", "Other defects, and "without defects" sensory classes. In musts producing wines with the FMA defect, total fungal counts remained at high levels and the predominant genus was *Penicillium.* Species level analyses identified multiple species including *P. corylophilum*, *P. citrinum*, *P. crocicola*, *P. bialowiezense*, *P. scabrosum* and *P. expansum*. These species have been previously described on Pinot noir [12] and Chardonnay [16] grape berries. In a study by Vacher *et al*. [2], *Penicillium* was also described but they concluded that no link could be established between the FMA defect in wine and any given genus.

3. Progress in identifying molecules involved in the FMA defect:

In an oenological matrix, molecules generally associated with the FMA defect are polyfunctional compounds with seven, eight or nine carbon atoms. Research from the 2000s on this topic identified various alcohols and ketones responsible for fungal odours in different grape varieties (Cabernet Sauvignon, Merlot, Gamay, Pinot Noir, Sauvignon, and Sémillon), namely 1-octanol, 2- octanol, 3-octanol, 1-octen-3-ol, 2-octen-1-ol, octan-3- one, 5-octadiene-3-ol, and 2-heptanol [17], [5].

The new data presented herein originates from research conducted in collaboration between the Comité Champagne, the Science Pour l'Oenologie laboratory, and the Robert-Jean de Vogüé Research Center, which has already been published [18] [19] [20].

3.1. A new chemical marker linked to "fresh mushroom aroma" wine defect

The first significant advancement was the identification and characterization of a new marker to assess the FMA defect in wine. With higher levels compared to other volatile FMA compounds, 1- hydroxyoctan-3-one, identified by Delcros *et al*. [19], appears to be a reliable marker for wine screening. Two experimental phases on characterizing the molecule both chemically and sensorially ensured its identification and its connection with the FMA defect in wine.

Firstly, the experiment focused on a model system using the *Crustomyces subabruptus* fungus (strain CICV 02RE14). Two musts from Pinot Noir and Meunier grapes (Champagne region) were obtained from pressing 160 kg of healthy bunches. The resulting musts were contaminated according to the protocol by Meistermann *et al*. [21] and then vinified. The volatile compounds were analyzed by GC-MS (gas chromatography – mass spectrometry and revealed that both 3-octanol and 1-octen-3-ol were found in both contaminated and healthy musts while 1-octen-3-one was only found in contaminated musts. Lastly, 1-hydroxyoctan-3-one was only detected and quantified in contaminated musts, which suggests a link between *C. subabruptus* contaminated musts and the presence of this chemical marker. After fermentation, wines derived from contaminated musts also displayed significant levels of 1- hydroxyoctan-3-one

(ranging from 16 ± 6 µg/L to 120 ± 14 µg/L equivalent of 4-nonanol), indicating its likely involvement in the FMA defect in wine.

These data, based on voluntary inoculation, were then verified on a collection of wines (2017) that exhibited a range of FMA defect intensities. The wines were stored as still wines in a cellar (13°C) and were then analyzed after 4 years of storage. Only four out of the 16 analyzed wine samples had quantities of 1-octen-3-one above the sensory detection threshold. However, the measured concentrations of 1-hydroxyoctan-3-one were correlated with the sensory perception of the FMA defect in wines (Spearman test ($\rho = 0.9226$)). These findings indicate that 1-hydroxyoctan-3-one could be a new and reliable marker for FMA defect in wine.

3.2. Identification of glycosylated precursors and fate of FMA compounds during fermentation

To our knowledge, up until the research conducted from 2020 [18], no literature mentioned a link between glycosylated precursors and fresh mushroom aroma in the field of oenology. The odourless nature of musts as well as the fluctuating aromatic character on wine led to the assumption that this hypothesis needed investigation.

Firstly, it has been shown that glycosidic precursors of 1-octen-3-one, 3-octanol, and 1-octen-3-ol are present in healthy Pinot Noir and Meunier musts (respectively 0.492 ± 0.008, 0.047 ± 0.009, and 0.044 ± 0.009 µg/L 4-nonanol equivalents); however, the glycosidic form of 1-hydroxyoctan-3-one was not identified in these healthy musts. On the other hand, in musts contaminated either by *C. subabruptus* or naturally contaminated by gray rot, the levels of glycosylated forms of these molecules are present in greater quantities. More specifically, in the *C. subabruptus* contamination model on Pinot Noir, glycosidic precursors of 1-octen-3-one and 1-hydroxyoctan-3-one increased significantly between healthy musts and contaminated musts [18]. However, in the samples naturally contaminated by gray rot (collected in 2021) only the glycosylated form of 1-octen-3-ol increased [20]. The profile of glycosidic precursors is likely dependent on the type of fungal contamination.

In a second step, these various compounds (1-octen-3-ol, 1-hydroxyoctan-3-one, 3-octanone, and 3-octanol) were monitored during controlled fermentations in their bound and free forms on both healthy musts and contaminated musts. At the beginning of the experiment, no significant difference was found between the aglycones of 3-octanol, 2-octanol, and 1-octen-3-ol between healthy musts and contaminated musts. However, the aglycone of 1-hydroxyoctan-3-one was only found in contaminated musts (contents between 0.175 and 0.743 µg/L of 4-nonanol equivalents).

After fermentation, wines produced from healthy matrices exhibit the same residual levels of aglycones for 3-octanol, 2-octanol, and 1-octen-3-ol. The 1-hydroxyoctan-3-one aglycone was also measured in wines from a healthy matrix despite its absence in musts (2 out of 3 replicates). This result suggests that the technological yeast added for fermentation could produce this type of glycoside during the fermentation step from healthy matrix.

Regarding volatile compounds, 1-octen-3-one, 3-octanol, and octan-3-one were detected in both healthy musts and contaminated musts at variable levels. As expected by the chosen protocol, levels of 1-octen-3-one were elevated in contaminated musts (0.44 ± 0.19 to 3.35 ± 0.56 µg/L). Similarly, 1-hydroxyoctan-3-one was only measured in contaminated musts between 184 ± 68 and 452 ± 128 µg/L.

After fermentation, wines derived from healthy must showed an increase in concentrations of 3-octanol (which could be related to yeast metabolism), constant levels of 3-octanone between wine and must, and very heterogeneous levels of 1-octen-3-one according to the considered replicate. Conversely, 1-hydroxyoctan-3-one was initially absent in must but present at concentrations between 67 to 103 ± 26 µg/L in healthy wine. 3-octanol and 3-octanone exhibited the same behaviour during fermentation in contaminated matrices as they did in healthy ones. However, the concentration of 1-octen-3- one behaviour varied according to the considered experimental replicate.

Lastly, the concentration of 1-hydroxyoctane-3-one decreased between must and wine to reach final levels in wine around 145 to 243 ± 34 µg/L.

To conclude, 1-hydroxyoctan-3-one appears to be produced during fermentation from a healthy matrix and consumed or transformed during fermentation from a contaminated matrix. Further research is necessary to understand the seemingly pivotal role played by this metabolite.

4. Conclusion

The goal of this synthesis was to provide an overview of the most recent research on “Fresh Mushroom Aroma” in wine and to highlight new markers for assessing this type of risk. From a microbiological perspective, the members of *Penicillium* genus appears to be more abundant in musts that produce FMA wines. Chemically, a newly described molecule - 1-hydroxyoctan-3-one - has been identified as an early and stable marker in FMA wines with. Both microbiological and chemical markers could serve as new research targets for enhancing our understanding of FMA defects in wine.

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