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Grape berry mycobiota and its contribution to fresh mushroom aroma off-odour in wine

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ABSTRACT

Grape berry mycobiota is a complex and diverse ecosystem that is constantly evolving during the different berry ripening stages of vines, as well as in musts and wines, which can ultimately impact wine sensorial properties and thus overall product quality. To better characterise the role of this mycobiota in wine off-odours, we studied for the first time changes in Pinot Noir and Meunier grape and must mycobiota and carried out sensorial analyses in the resulting wines, with a focus on fresh mushroom aroma. Grapes from three berry ripening stages, fruit set, veraison and harvest, were collected without sorting bunches from 31 parcels, then laboratory-scale grape crushing was performed to obtain musts, followed by micro-winemaking. For all sample types, both culture-dependent and -independent strategies were used to decipher both species and genus level mycobiota composition. After the winemaking process, sensorial analyses detected 9 laboratory wines with fresh mushroom aroma off-odours. Musts that generated these spoiled wines contained higher total fungal counts, especially *Penicillium* spp., compared to other musts. A total of seven *Penicillium* species, including one unidentified *Penicillium* sp., was detected using the culture dependent approach; this genus represented up to 100 % of all fungal counts in musts linked to FMA wines. Fungal interactions inferred using co-occurrence networks revealed positive interactions between *Vishniacozyma, Cladosporium* and *Penicillium* in musts, indicating the possible implication of these three genera in FMA production. Negative interactions were observed in musts between the two yeast genera, *Starmerella* and *Pichia*, with *Penicillium, Botrytis, Vishniacozyma* and *Cladosporium,* as well as between *Metschnikowia* with *Botrytis* and *Vishniacozyma*. *Starmerella, Pichia* and *Metschnikowia* may thus be potential biocontrol candidates for use against *Botrytis* and *Penicillium* grape contamination.

KEYWORDS: Mycobiota, diversity, grapes berries, wine, volatile compounds, co-occurrence networks

INTRODUCTION

From the vineyard to wine, a highly complex and diversified microbial ecosystem is present on grapes, including bacteria, yeasts and filamentous fungi (Diguta, 2010). Focusing on fungal communities, more than 70 fungal genera have been identified on grapes (Rousseaux *et al*., 2014; Martins, 2012), but six predominant genera have been consistently reported, namely: *Botrytis, Cladosporium, Penicillium, Aureobasidium, Alternaria* and *Aspergillus*. These fungi have been commonly described in vineyards in multiple countries worldwide (Portugal, Spain, France, Australia, New Zealand, South Africa, Tunisia, China, Argentina and Chile, etc.) on both red and white grape varieties (Cabernet-Sauvignon, Merlot, Monastrell, Tempranillo, Garnacha, Corvina, Pinot noir, Chardonnay, Sauvignon blanc, Moscatel, Garnatxa Blanca, Garganega, etc.)(Carmichael *et al*., 2017; Diguta, 2010; Kioroglou *et al*., 2019; Liu *et al*., 2020; Scott *et al*., 2022; Zhu *et al*., 2021). The influence of both geographical origins of vines and grape varieties on shaping grape microbiota composition, especially fungi, is evident across these diverse climates. For instance, *Penicillium* spp. are more frequent in cold climates, particularly in northern Europe, while *Aspergillus* spp. are more frequent in warmer and wetter regions (Pitt and Hocking, 2009; Rousseaux *et al*., 2014). *Alternaria* and *Cladosporium* spp. are more frequently isolated from red grapes compared to white grapes (Rousseaux *et al*., 2014).

The presence of specific fungal species and how their diversity evolves on the surface of grape berries is also strongly modulated by the phenological stages of the vine (Zhu *et al*., 2021), from the emergence of the first flower buds to the harvest stage (Lorenz *et al*., 1995). During the early stage of grape berry development, known as fruit set, *Cladosporium* spp. and *Aureobasidium pullulans* are described as predominant (Liu and Howell, 2021; Zhang *et al*., 2019). After fruit set, veraison marks a crucial turning point for mycobiota composition with the beginning of ripening and major modifications in berry composition. Indeed, fungal diversity increases during ripening, which is primarily attributed to changes in berry nutrient composition (Liu *et al*., 2021). Ripening creates favourable conditions for fungal development, such as increased water availability (a_w), decreased acidity and a simultaneous rise in sugars (Wu *et al*., 2011). At this stage, the diversity and number of fungal taxa increase. While *Cladosporium* and *Aureobasidium* persist as the dominant genera at veraison on Pinot noir grapes (Liu and Howell, 2021; Zhang *et al*., 2019), several yeast genera, including *Vishniacozyma, Cryptococcus* and *Filobasidium*, can also increase in relative abundance (Zhu *et al*., 2021; Liu and Howell, 2021). The harvest period is often characterised by an increase in the relative abundance of various mould genera, such as *Alternaria*, *Penicillium*, *Botrytis*, *Erysiphe* and *Aspergillus* (Liu and Howell, 2021; Zhang *et al*., 2019; Zhu *et al*., 2021). However, after harvest, when grapes are crushed to obtain the must for winemaking, a significant reduction in mould diversity is observed (Martiniuk *et al*., 2023). Filamentous fungal populations may decline due to stress, oxidative conditions, acidity, and the liquid state encountered in the must (Zhu *et al*., 2021). In contrast, non-*Saccharomyces* yeasts, which are better adapted to these conditions and are underrepresented on grape berries, tend to increase in relative abundance. Yeast genera such as *Pichia, Hanseniaspora, Starmerella, Metschnikowia* and *Torulaspora* tend to dominate at this stage (Barata *et al*., 2012; Martiniuk *et al*., 2023). After *Saccharomyces cerevisiae* inoculation, alcoholic fermentation starts and this yeast species ultimately dominates in the resulting wine (Goddard, 2008). The majority of these genera and species are not specific to grape vine bunches and are found on other fruits, plants and various food matrices. For example, *A. pullulans* is a ubiquitous yeast found in a variety of environments, including, foods and crops (Samson *et al*., 2004; Deshpande *et al*., 1992). *Alternaria alternata* is isolated from cereals, nuts, citrus fruits, pome fruits and stone fruits (Samson *et al*., 2004). *Botrytis cinerea* is found mainly in humid temperate climates, on flowers, leaves and stems, and causes fruit and leaf rot in grapes, strawberries and vegetables (Samson *et al*., 2004). *Cladosporium* species are ubiquitious saprophytic fungi often found on decaying substrates and a wide range of food products (Samson *et al*., 2004). *Penicillium* are also saprophytic fungi and are known to decompose fruit; they are found on crops and as food contaminants (Pitt and Hocking, 2009). For example, *Penicillium bialowiezense* is found in processed food products, vegetables, mushrooms and soil (Samson *et al*., 2004); *Penicillium brevicompactum* is found on cereals, coffee beans, meat products and in refrigerators; and *Penicillium citreonigrum* is found on rice and in soil (Pitt and Hocking, 2009).

Certain fungal species, such as *Botrytis, Penicillium, Aspergillus* and *Alternaria* spp., are known as common grape contaminants, which cause the spoilage of both grapes and wines (Steel *et al*., 2013). They can have a particularly negative impact on the sensory characteristics of wines. Off-flavours in wine result from molecules with potent olfactory and/or gustatory properties (Scott *et al*., 2022); different undesirable volatile fungal compounds associated with rotten grape bunches that cause organoleptic defects in wine have been documented in the literature. For example, grapes infected by grey rot (caused by *B. cinerea*) can lead to typical earthy or musty odours due to geosmin or 2-methylisoborneol (2-MIB) production in vinified wines (La Guerche *et al*., 2007; Rousseaux *et al*., 2014). Moreover, geosmin production by *B. cinerea* increases when specific strains of *Penicillium expansum* are present (Correia, 2012; Darriet *et al*., 2000). In addition to *B. cinerea*, other berry-associated fungal species that produce undesirable volatile compounds and spoiled wines have already been described. For example, 2-MIB was detected in grapes infected by and/or in wines produced from grapes contaminated by *Penicillium thomii*, *P. expansum*, *Rhizopus nigricans* and *Aspergillus niger* (Rousseaux *et al*., 2014). Another molecule, 2-isobutyl-3-methoxypyrazine (IPMP), linked to herbaceous and green notes in wines, has been shown to be produced by *P. thomii, P. expansum, Penicillium glabrum,*

Penicillium spinolusum and *A. niger* (Rousseaux *et al*., 2014). As well as these well-known off-odours, fresh mushroom aroma (FMA) has also been described. Typically, this off-odour does not seem to occur directly in grapes or musts (Meistermann *et al*., 2021; Delcros *et al*., 2022), but rather appears in wines after alcoholic fermentation (La Guerche *et al.*, 2006; Pons *et al.*, 2011). Preventing this off-odour from being produced is thus very challenging. To date, different volatile compounds have been linked to this off-odour: primarily 1-octen-3-ol, 1-nonen-3-one, 1-octen-3-one (Steel *et al.*, 2013; Rousseaux *et al.*, 2014), as well as several fungal species: *B. cinerea, Erysiphe necator, Alternaria spp., A. niger, Apergillus nigri, Crustomyces subabruptus, P. thomii, P. brevicompactum* and *P. glabrum* (Darriet *et al*., 2002; Lopez Pinar *et al*., 2016; Meistermann *et al*., 2021; Rousseaux *et al*., 2014; La Guerche *et al.*, 2006, Börjesson *et al.*, 1992; Kaminski *et al*., 1974), although other species could also be involved. Recently, Delcros *et al*. (2023) identified 1-hydroxyoctan-3-one as a molecule involved in FMA off-odours. This newly-described volatile compound was hypothesised to be a potential precursor or derivative for 1-octen-3-one in wines produced from musts that were contaminated with *C. subabruptus* in the laboratory. Among the molecules associated with FMA, 1-octen-3-one is known to be the most odoriferous, with a reported chemical spoilage index (i.e., the minimum concentration leading to spoilage perception) of 40 ng/L in neutral white wine (Pons *et al.*, 2011) or 15 ng/L in model solution similar to wine (La Guerche *et al*., 2006). Moreover, a fresh mushroom odour at 20 ng/L has been detected in Alsace white wine and Champagne still wine (Pons *et al.*, 2011), while that of 1-hydroxyoctan-3-one is ten thousand times higher (Delcros *et al*., 2023). The cited off-flavour compounds can be derived from secondary metabolism and produced by and/or result from functional complementarity, during which fungal metabolites can act as substrates for metabolisation by other organisms, leading to deviations; however, information related to metabolic pathways and putative complementarity between taxa is scarce (Calvo and Cary, 2015; Keller *et al*., 2005)

In this study, we compared the composition and dynamics of mycobiota from vine to wine, obtained from 31 parcels, to evaluate whether grape mycobiota dynamics and its interactions plays a role in FMA off-odour production. The wines resulting from micro-winemaking for the studied parcels were sensorially characterised and mycobiota composition was compared to pinpoint fungal species potentially associated with the FMA defects.

MATERIALS AND METHODS

1. Grape berry sampling and lab scale micro-winemaking

1.1. Sampling area

The selected vineyards comprised 31 parcels equally distributed throughout the study area. These vineyards were in the Northeast region of France, spanning the Côtes des Blancs to the Massif Saint Thierry. Vineyards growing Pinot noir (n = 16) and Meunier (n = 15) grape varieties were selected. Sampling was done at 5 different stages in 2021 and included 3 berry ripening stages (fruit set: BBCH 71, veraison: BBCH 81, harvest: BBCH 89, according to Lorenz *et al*. (1995)), as well as the corresponding grape musts and laboratory-scale white wines.

1.2. Laboratory-scale micro-winemaking

Forty kilograms of grapes from each parcel were harvested without sorting and crushed using a small hydraulic press (20 L, Speidel); each resulting must was transferred into a 2L-fermenter. Musts were inoculated with 0.1 g/L of *Saccharomyces cerevisiae* (in-house strain), and alcoholic fermentation was monitored by weight loss due to CO_2 release. Malolactic transformation was initiated by inoculating *Oenococcus oeni* (in-house strain) and monitored by malic acid quantification according to the 2021 International Organisation of Vine and Wine (OIV, 2021) procedure. Still wines were stored at 3-4 °C for 1 month before sensorial analysis.

1.3. Sensorial and chemical analyses of wines

After settling, musts were sampled to quantify acetic acid (g/L), gluconic acid (g/L), glycerol (g/L), malic acid (g/L), total acidity (g/L H_2SO_4), ammonium nitrogen (mg/L), assimilable nitrogen or alpha-amino nitrogen (mg/L), potential degree of alcohol or refractometry degree (% Vol), pH and total SO_2 (mg/L). Briefly, malic acid was quantified using the colorimetric method by NADH absorption at 340 nm after enzymatic transformations applying procedures described in OIV (2021). The remaining protocols employed were analogous to those described by OIV (2021), but with an internal and accredited referenced method. Briefly, acetic acid, gluconic acid, glycerol and nitrogen content were quantified using the colorimetric method after enzymatic transformations, whereas potential degree of alcohol was quantified by automated IR spectrophotometry and SO_2 by iodometric titration of free and combined SO_2. In addition, Intensity Signal Anti-*Botrytis* (Botrytis Alert, United Kingdom) was used to estimate *Botrytis* infection applying the manufacturer's procedures (Botrytis Alert, 2020). This technique is based on the affinity of the monoclonal antibody BC12.CA4 with the mycelial wall of *Botrytis cinerea* (Dewey *et al*., 2000). Briefly, 40 µL of grape must were added to 4 mL buffer solution in test bottles. The lower part of the *Botrytis* test strip was immersed in five drops of this solution for 10 min. The test line score card, with values ranging from 0 to 5 (0 = healthy to 5 = high level of *Botrytis*), was used.

Still wines were tasted by three different panels of tasters: an expert oenologist panel (n = 4), a Research and Development (R&D) sensory division panel (n = 10) and a laboratory panel (n = 5). The expert oenologist panel had been specifically trained to detect the fresh mushroom defect in wines. Then panellists were asked to determine their perception of FMA in wines, based on a score from 0 to 5, with a score of 0 corresponding to no FMA off-odour detected and a score of 5 to strong intensity. In addition, the level of 1-octen-3-one (ng/L) was determined by GC-MS analyses (GC: Thermo scientific, Colone DB5 Agilent).

1.4. Sampling procedure for microbiological analyses

At each sampling stage and in each of the 31 parcels, 30 grape bunches were randomly harvested from the top, middle and bottom of the vine stock, except for fruit set when 60 grape bunches were harvested to compensate for weight differences. Grapes were washed by agitation on a shaking table (130 Hz, 1h with turnover at half-time) using a wash solution (sterile distilled water supplemented with 9.2 g/L NaCl and 0.2 % (w/v) Tween 80). The volume of added washing solution was equal to half of the total weight of 30 harvested bunches. After filtrations, using autoclaved coffee filters, cell pellets were obtained by centrifugation (20 min, 10,000 *g*). Cell pellets were stored at -20 °C for metagenetic analyses. Cryotubes were also prepared by centrifuging another 50 mL of cell suspension at 10,000 *g* for 20 min. After removing the supernatant, the cell pellets were resuspended in 0.8 mL of sterile distilled water containing 9.2 g/L NaCl and transferred into cryotubes. A volume of 0.8 mL of 50 % glycerol (v/v) was added, and the cryotubes were stored at -80 °C for microbial enumerations and isolation procedures. For fruit set, musts and wines, 20 mL of cell suspensions were mixed with 10 mL of glycerol 50 % (w/w) before storage at -80 °C. Before plating samples on medium, they were centrifuged (20 min, 10,000 *g*) and resuspended in 20 mL of sterile distilled water with 9.2 g/L NaCl.

2. Culture-dependent approach

2.1. Fungal counts

Once prepared, the cell suspensions obtained directly after washing grapes or from resuspended fruit set, must and wine samples were plated. To do so, serial dilutions were performed and 100 µL or 50 µL (depending on stage) were inoculated using an easySpiral Dilute plater (Interscience) to enumerate total fungal populations, as well as each mould morphotypes on M2Lev (malt extract 20 g/L, yeast extract 3 g/L, agar 15 g/L, bioMérieux, supplemented with penicillin 10,000 U/L and streptomycin 100 mg/L, incubation at 25 °C for 5 to 7 days).

2.2 Conservation, dereplication and fungal identifications

A collection of cultivable filamentous fungi was created; it was based on a selection of a maximum of 10 isolates representing all observed morphotypes for moulds and a maximum of 5 isolates representative of yeasts for each sample on M2Lev medium. All isolates were cryopreserved in 1 mL of 15 % glycerol (v/v) at -80 °C. The sampling limit of 15 isolates per sample (n = 31) was established for the five stages to ensure the acquisition of a reasonable and treatable number of isolates. The final collection yielded a total of around 1,200 fungal isolates.

A dereplication step comprising the presumptive identification of isolates was performed by either high throughput FTIR (Fourier Transform Infra-Red Spectroscopy) or M13 PCR for yeasts (as described by Coton *et al*. (2017)), and MALDI-TOF for filamentous fungi. FTIR analyses were done with 3 technical replicates using a FTIR Bruker (Tensor 27, Bruker Optics, Champs sur Marne, France, coupled to a high-throughput module, HTS-XT, Bruker Optics). Briefly, the spectrum generated by FTIR for each yeast isolate was compared to a dataset reference library (2,500 spectrum) to obtain a presumptive identification and secondly, a dendrogram was built to group isolates belonging to the same species into clusters (as described by Coton *et al*. (2017)). For M13-PCR, the genetic profiles were clustered and dendrograms created based on their similarities using the Bionumerics 6.6 software (as described by Coton *et al*. (2017)). For moulds, MALDI-TOF (VITEK MS) was used as previously described by Penland *et al*. (2021), and the generated MALDI-ToF spectra were compared to the Biomerieux database (VITEK MS V3.2 Knowledge Base) for presumptive identifications. Based on pre-identification data and dendrogram clustering, selected representative isolates of each group were then identified at species-level by sequencing taxonomically relevant DNA targets. From the 1,200 dereplicated isolates, 350 representative yeasts and 140 representative moulds were selected. Yeast identifications were performed with amplification and sequencing of the D1-D2 domain using the NL1/NL4 primer pair (Kurtzman and Robnett, 1997). For moulds, DNA regions yielding the highest taxonomic resolution, namely the β-tubulin gene for *Penicillium* (using the Bt2a/Bt2b primers; Glass and Donaldson (1995)), the actin gene (using the Act512F/ Act783R primers; Carbone and Kohn (1999)) for *Cladosporium* and *Aspergillus,* and the internal transcribed spacer (ITS) region for other moulds (using the ITS 4/ ITS 5 primers; White *et al*. (1990)) were targeted depending on the considered genus.

The PCR conditions used in this study associated with the barcodes described above are mentioned in Table S1A. The sequences obtained from Eurofins genomics EU using the same primer pairs were blasted using BlastN (Altschul *et al*., 1990).

3. Metagenetic analyses

3.1. Selection of primers and taxonomic level resolution assessment

First, a large database containing 473 ITS sequences was created with the compilation of ITS region (5.8S ribosomal RNA and large subunit LSU) belonging to 416 fungal species, representing grape-berry-associated taxa either identified in this study or in the literature. These sequences were extracted from the version (2021-05-10) of "UNITE+INSD" dataset. In order to select the best primer pair, 15 forward primers were tested *in silico* using Geneious prime (V2022.1.1, 2022, Biomatters Ltd.) software to determine their affinity with our sequence database; these primers were: "g ITS7", "f ITS7", "ITS86F", "ITS3 mix", "ITS3", "58 A1F", "58 A2F", "ITS3 Kyo1", "ITS3 Kyo2", "ITS3 tagmix 1", "ITS3 tagmix 2", "ITS3 tagmix 3", "ITS3 tagmix 4", "ITS3 tagmix 5", "GTAA", available sequences: Tedersoo *et al*. (2015), Toju *et al*. (2012), Morales-Cruz *et al*. (2018), and 7 reverse primers: "ITS4 ngs", "ITS4", "ITS4 – B", "ITS8 mum", "ITS4 Kyo1", "ITS4 Kyo2", "ITS4 Kyo3", available sequences: Tedersoo *et al.* (2015), Toju *et al.* (2012), Morales-Cruz *et al.* (2018), White *et al.* (1990). After multiple combinations of primer pairs,

ITS3 (Fwd: GCATCGATGAAGAACGCAGC)/ITS4kyo1 (Rev: TCCTCCGCTTWTTGWTWTGC) were chosen for their higher number of targeted sequences present in the created database. Then, the taxonomic resolution of the ITS2 sequences was determined and the database updated when appropriate to include any new species ITS2 sequences from our samples. Overall, the final database contained 515 sequences and the most recent version (2023-07-18) of "UNITE+INSD" dataset was implemented. In order to extract all the sequences to create phylogenetic trees, the sequences were trimmed to keep only the amplification target with Geneious prime. Sequences belonging to the same species were removed from the database when more than 99 % similar in the distance matrix. Thus, the final database used to construct the phylogenetic trees included 473 sequences belonging to 401 fungal species. The procedure to build the phylogenetic tree was the same as that described by Belair *et al*. (2023). Briefly, the extracted ITS2 sequences were aligned using the *MAFFT* tool v7 (Katoh *et al*., 2019) and edited using *Gblocks v0.91b* (Dereeper *et al*., 2010). Then, a phylogenetic tree was constructed using *BEAST2 v2.6.1* (Bouckaert *et al*., 2014) and the Bayesian inference calculation method. The best models were estimated for each sequence database with *bModel test* and *BEAUti2* (package and extension of *BEAST2 v2.6.1* respectively). Bayesian inference analysis was conducted with Markov Chain Monte Carlo (MCM) method that performed 3 repetitions of 100 million generations, and each sampling at every 1,000 generations. At the output of the independent inference bayesian analysis, convergence was established by *Tracer software* (v1.7.1) (Rambaut *et al*., 2018) and the files were combined using the *LogCombiner* package in BEAST. Posterior probabilities of the tree were calculated by *Treeannotator* in BEAST and the phylogenetic tree was generated using *FigTree v1.4.4* software (http://tree.bio.ed.ac.uk/software/figtree). The phylogenetic tree is available in Figure S1.

3.2. Total DNA extraction, amplification and sequencing

Total DNA was extracted in triplicate from the cell suspensions collected from grapes, musts and wines using the Quick DNA Fecal/Soil Microbe Miniprep kit (Zymo Research, USA) according to the manufacturer's instructions. DNA samples were adjusted to a concentration of 10 ng/µL DNA after quantification using a nanodrop spectrophotometer. As described above, the ITS2 region was selected with the ITS3 /ITS4-Kyo1 primer pair to amplify fungal targets. Amplicon libraries and Illumina MiSeq PE 300 bp sequencing were performed with FLD_ill adapter (forward - ACACTCTTTCCCTACACGACGCTCTTCCGATCT / reverse GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT) using 2 runs of amplification: PCR indexation for adapter indexation and PCR amplification for barcode amplification. ITS2 amplicons of all 423 samples (from all parcels and 5 stages) were sequenced in 3 separate sequencing runs to reach the expected sequencing depth using Illumina sequencing (MiSeq PE 300 bp) at Genome Québec Innovation Center (Montréal, Canada). The PCR conditions used are mentioned in Table S1B.

3.3. ITS2 sequencing analysis workflow

Optimal parameters for trimming forward and reverse read sequences were determined by the *Figaro* R v.4.1.3 package (Weinstein *et al*., 2019; with estimated amplicon length set at 500 bp and minimum overlap length of 20 bases). Next, the *Dada2* R v.4.1.3 package (Callahan *et al*., 2016) was used for removing ambiguous bases, denoising and filtering according to the previously determined Figaro trimming parameters (299 and 261 for forward and reverse reads trimming parameters). ASVs were independently inferred from the forward and reverse reads of each sample using the run-specific error rates, and read pairs were merged with an overlap setting of 12 bases minimum (default parameter). The read counts of the 3 runs for each of the ASVs were summed. Finally, the taxonomic assignment of each ASV was performed using BLASTn (Altschul *et al*., 1990) against the UNITE fungal database (latest available version V9.0, 2023-07-18). Before statistical analyses, ASV tables were filtered to remove ASV which sum of reads was inferior to 0.00001 % of the total number of reads, using the "*filter_otus_from_otu_table.py*" QIIME script (Caporaso *et al*., 2010). Sequences of the top 200 ASVs were blasted on NCBI blast nucleotide website to control the taxonomic assignment obtained. In addition, taxonomic placement of the top 200 amplicon sequence variant (ASV) sequences, obtained by metabarcoding, was checked by adding the obtained ASV sequences to the initial database and building a new phylogenetic tree as described above.

3.4. Co-occurrence networks

Significant pairwise correlations between the top 200 ASVs were established for each stage using Sparse Correlations for Compositional data algorithm implemented in the SparCC python module (Friedman and Alm, 2012). Before analysis, the top 200 ASVs of each stage were extracted from the ASV table. The co-occurrence networks were plotted for each stage at the genus level using the *qgraph* R package (Epskamp *et al*., 2012). Only correlations with an absolute correlation strength value greater than 0.3 (|coefficient correlation (= corr) | > 0.3) and *p*-value inferior to 0.05 were plotted.

4. Statistical analysis

Statistical tests described below were performed with JMP (V15.1, 2020, SAS). The oenological characteristics of the 29 laboratory-scale musts were compared to the reference value (Prat-Bernachot *et al.*, 2022) based on the conformity test. Anova and Kruskal-Wallis tests were conducted to compare the sensory classes for all measured compounds. Depending on the normality of their residual distribution, Wilcoxon pairwise tests were conducted for 1-octen-3-one and Tukey HSD for SO_2 concentration. A Chi-2 test was performed to determine whether the occurrence of FMA was significantly higher according to the considered grape variety or sampling area. To assess the effect of FMA sensorial classes on fungal abundances, obtained by cultural analysis, Anova test was used followed by Tukey HSD test for multiple mean comparisons based on a level of significance set at $\alpha = 0.05$.

The non-parametric Kruskal-Wallis test followed by the Wilcoxon pairwise test was used in case of non-normal distribution of data residues evaluated by the Shapiro-Wilk test.

Alpha-diversity indices (number of observed genera, Simpson and Shannon indices) were calculated using the ASV table rarefied at the lowest sequencing depth (14,563 reads). Statistical analyses were performed with either Wilcoxon for Observed or Tukey HSD for Simpson and Shannon also with 'JMP' software.

The fungal genera and species level relative abundance barplots were generated with R software version 4.2.2 (R Consortium, 2024) under the RStudio environment (2022-10-31). For metagenetics, the medians of relative abundance were calculated for each stage and for each genus with phyloseq R package (version 1.46.0). Only the genus with a median relative abundance < 0.1 % in each of the 5 stages were assigned as '< 0.1 %' in the legend. For cultural data, the means of relative abundance were calculated independently of stage (ie., for the 5 stages) for each species using Excel (V2208 of Microsoft 365 Enterprise). Only the species with relative abundance < 0.3 % were assigned as '< 0.3 %' on the legend.

To determine the impact of various factors on mycobiota composition and structure between the three sensorial classes (Fresh mushroom aroma, other off-flavours, without off-flavours), R software version 4.2.2 (R Consortium, 2024) under the RStudio environment (2022-10-31) with phyloseq package (version 1.46.0) was used to perform Adonis test and Principal Coordinate Analysis Plot (PCoA), based on Bray-Curtis distances calculated at genus level with Hellinger-transformed ASV tables for metagenetic data.

For count data, R software version 4.1.3 (R Consortium, 2024) and RAM package (version 1.2.1.7) was used to perform PCoA and Adonis test, based on Bray-Curtis distances calculated at species level with log10-transformed enumeration count tables. The indicspecies R package (version 1.7.14) was used to find genus, among the top genera with medians of relative abundance > 0.1 %, that were significantly associated with a particular sensorial class at each stage (De Cáceres *et al.*, 2010). Indicator species take into account specificity (probability that a given site belongs to the sensorial class group given the fact that a taxon has been found) and fidelity (probability of finding a given taxon in sites belonging to a given sensorial class), the statistical significance (α = 5 %) of the relationship was tested by permutation (De Cáceres *et al.*, 2010). Differentially-abundant genera among sensorial classes (with differences in average relative abundance > 1 %) identified by the metagenetic approach was evaluated using the Wilcoxon pairwise test with JMP software (V15.1, 2020, SAS).

RESULTS

1. Chemical and sensorial characteristics of laboratory-scale wines

1.1. Classification of wines according to sensory characteristics

Twenty-nine lab-scale wines (2 litres) were obtained from the 31 parcels in the original experimental design. Two vineyards were severely damaged by hail and mildew during the growing season and were thus excluded. Both alcoholic and malolactic fermentations were completed over one month. Based on sensorial analysis and 1-octen-3-one quantification, three

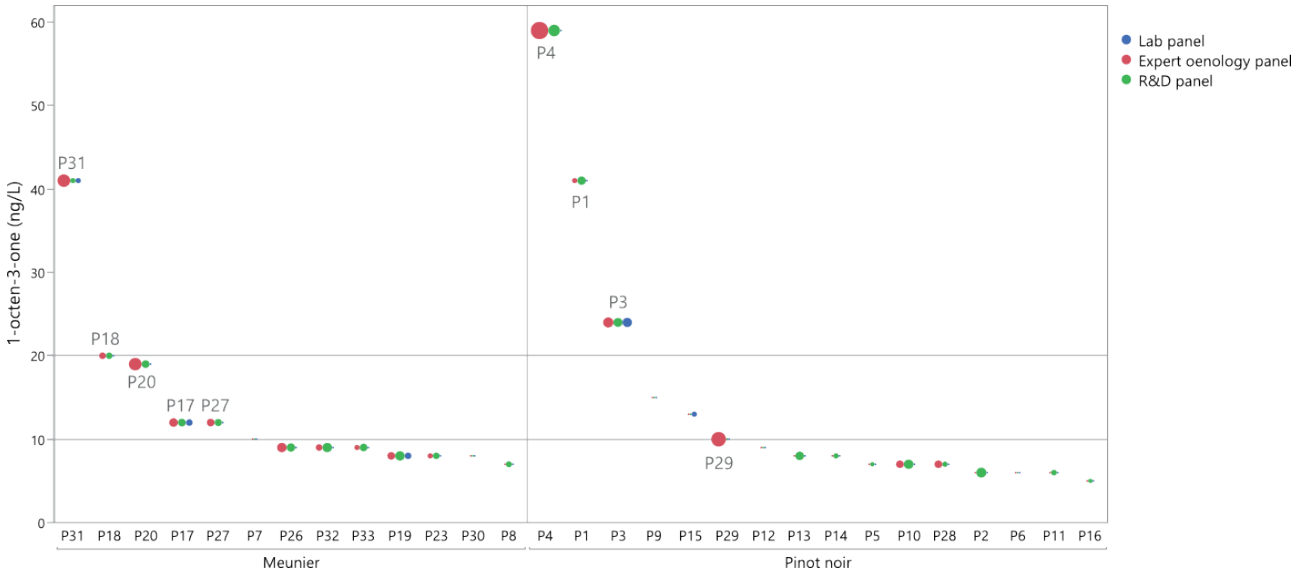


FIGURE 1. Levels of 1-octen-3-one (ng/L) in each laboratory-scale wine; results of the sensory evaluation for the "fresh mushroom aroma".

The dot size is proportional to the considered sensory panel average score (score ranging from 0 to 5). Blue = laboratory panel, red = Oenologist and green = R&D. The two grey lines represent the conventional perception threshold (20 ng/L) and the oenologist perception threshold (10 ng/L) respectively.

sensorial classes were defined, namely fresh mushroom aroma off-odours (FMA), other off-odours and without off-odours. Wines with levels of 1-octen-3-one above 20 ng/L (i.e., the perception threshold in white wine) and classified as "FMA" by at least two of the three sensory panels, were automatically classified as "FMA" (Figure 1). Wines with 10 to 20 ng/L 1-octen-3-one and classified as "FMA" by the oenologist expert panel, were also considered as belonging to the FMA class (Figure 1). Both criteria, based on quantitative and sensory data, were used because other molecules than 1-octen-3-one can cause FMA off-odours and the perception of the FMA defect varies depending on the panel. Overall, 9 wines were classified as "FMA". No off-odours were detected in 4 wines (NOF class; P9, P12, P16, P30); while for the remaining 16 wines, off-odours other than FMA were detected. The different organoleptic deviations perceived were herbaceous, earthy, musty or dusty in the 'other off-odours' class.

1.2. Chemical characteristics of musts for the different sensory classes of wines

No significant differences in oenological parameters were observed among the three sensorial classes, except for 1-octen-3-one in wine, which was used to define the FMA class (Table 1). Musts exhibited several oenological properties that closely aligned with those of the reference average for 2021 in the studied area (Prat-Bernachot *et al.*, 2022), such as gluconic acid, total acidity and ammonium nitrogen contents. This suggests that the obtained laboratory-scale musts were very similar to their full-scale counterparts. Despite this overall similarity, some deviations were observed in specific components in our experimental must. Notably, the levels of acetic acid and α-amino nitrogen significantly surpassed the average values for the 2021 vintage, as indicated by the conformity test (p-value = 7.7×10^{-16} and p-value = 2.6×10^{-6}, respectively). Conversely, the content of of glycerol, malic acid, refractometer degree (or 'Brix'), and pH exhibited values significantly below the reference average for 2021 (Table 1). The impact of vineyards and cultivars on FMA occurrence was evaluated. FMA wines were detected for both grape varieties, five from Meunier and four from Pinot noir; these wines originated from berries sampled from four different vineyards across the studied region. There was no significant impact of the sampling area (Chi-2 test: p-value = 0.16) or grape variety (Chi-2 test: p-value = 0.58) on the occurrence of any sensorial class.

2. Fungal diversity and dynamics from vine to wine

2.1 Fungal community diversity and dynamics from fruit set to wines

Two complementary cultural and molecular approaches were used to determine how fungal diversity and dynamics change during three different berry ripening stages, in musts and in wines. Cultural analysis revealed that total fungal counts were mainly driven by the sampling stage. Fungal abundance

List of compounds analysed in musts	FMA (n = 9) Mean ± standard deviation	Other (n = 16) Mean ± standard deviation	Without off-flavour (n = 4) Mean ± standard deviation	Comparison of sensorial classes: p-value	Reference average for vintage 2021**	Conformity test (n = 29) p-value
Acetic acid (g/L)	0.07 ± 0.01	0.07 ± 0.02	0.06 ± 0	0.61	0.02	**7.7E-16*** **
Gluconic acid (g/L)	0.06 ± 0.08	0.05 ± 0.06	0.02 ± 0.01	0.35	0.06	0.39
Glycerol (g/L)	0.21 ± 0.2	0.17 ± 0.12	0.13 ± 0.03	0.97	0.24	**0.03***
Malic acid (g/L)	7.57 ± 1.14	7.32 ± 1.25	7.63 ± 1.1	0.87	8.2	**0.004****
Total acidity (g/L H_2SO_4)	8.43 ± 1.02	8.28 ± 1.17	8.43 ± 0.92	0.94	8.3	0.81
Ammonium nitrogen (mg/L)	132.33 ± 23.38	120.25 ± 29.97	105.5 ± 19.74	0.26	112.00	0.06
Alpha-amino nitrogen (mg/L)	278.44 ± 62.16	243.25 ± 70.94	210.5 ± 49.47	0.22	176.00	**2.6E-6*** **
Refractometer degree (%Vol)	9.84 ± 0.56	9.43 ± 0.67	9.63 ± 0.33	0.28	9.9	**0.01***
pH	3 ± 0.07	2.98 ± 0.09	2.97 ± 0.05	0.83	3.03	**0.005***
SO_2T (mg/L)	14.11 ± 3.76 [b]	15.69 ± 4.33 [ab]	20.25 ± 1.5 [a]	**0.0486***	unavailable	unavailable
1-octen-3-one (ng/L)	26.44 ± 16.86 [a]	8 ± 1.79 [b]	9.25 ± 4.19 [b]	**0.0004*** **	unavailable	unavailable
Intensity Signal Anti-BOT	34.6 ± 26.72	31.52 ± 14.08	24.67 ± 3.68	0.73	unavailable	unavailable

TABLE 1. Oenological characteristics of laboratory-scale musts and their comparison with the 2021 vintage "average", which serves as the benchmark for Champagne wines.

The reference values of musts are based on yearly data (Prat-Bernachot *et al.*, 2022). All values mentioned were measured in must, except for 1-octen-3-one, which was measured in wine. Parametric (Anova) and non-parametric tests (Kruskal-Wallis) were performed between sensorial classes for all measured compounds (letters represent the groups of significant difference evaluated by the Tukey HSD or the Wilcoxon pairwise test). Conformity test between the average values of the 29 musts and the reference values of the vintage 2021 average for Champagne must was also performed for each compound.

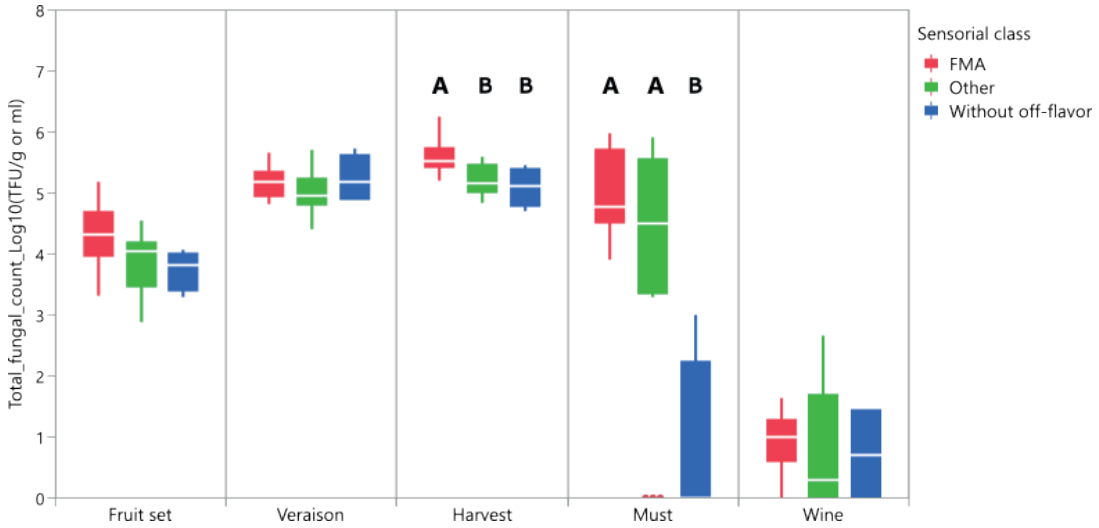


FIGURE 2. Comparison of total fungal abundance (Log10(TFU/g or /ml)) at each stage of sampling between the different sensorial classes, letters represent differences between groups (performed with either Wilcoxon at must stage or Tukey HSD at harvest).

increased slightly from fruit set to the harvest stage (average: 4 to 5.3 log CFU/g) before drastically decreasing in both musts and wines (average: 3.8 to 0.82 log CFU/mL), with counts being below the detection threshold for 6 and 8 out of 29 musts and wines respectively (Figure 2).

A total of 685 isolates of moulds and 552 isolates of yeasts were obtained. These isolates underwent dereplication, clustering (as described in the material and methods section for IRTF and Maldi-TOF dereplication) and species-level identification. Among the representative mould isolates, species level (n = 26) or genus level (n = 11) identifications were carried out; the isolates that could not be identified using the taxonomic targets were labelled "Unknown". For yeast isolates, genus or species level (n = 14 and 13 respectively) identifications were also obtained and some unidentified yeasts observed. Early stages of grape berry development (fruit set and veraison) were dominated by *A. pullulans* (56 % mean relative abundance at fruit set, and 58 % at veraison) and species belonging to the *Cladosporium cladosporioides* species complex (19 % at fruit set, and 14 % at veraison), while the other genera (25 % and 28 % at fruit set and veraison respectively) varied greatly depending on the considered parcels, in particular *Vishniacozyma* spp. and *Filobasidium* spp. representing up to 53 % and 62 % of total fungal counts respectively (Figure 3A). At harvest, the number of mould species increased with the emergence of *B. cinerea* in 9 parcels (relative abundance reaching up to 29 %), *P. brevicompactum* in 8 parcels and *P. bialowiezense* in 6 parcels. When present, *Penicillium* spp. representing up to 29 % of total counts. For yeasts, *A. pullulans* globally remained abundant (31 %) while *Vishniacozyma carnescens* and *Hanseniaspora uvarum* were more frequently isolated compared to previous stages (in 17 and 9 parcels with relative abundances up to 69 and 96 % respectively; Figure 3A).

In musts, the abundance of most mould species declined (total fungal count 3.8 log CFU/mL). This decrease was less drastic for the *Penicillium* genus, with only a 1 log decrease compared to harvest (Figure 4A). Among *Penicillium*, *Penicillium* spp. (considered as unknown species; in 2 parcels with relative abundance up to 100 %), *Penicillium corylophilum* (in 3 parcels with relative abundance up to 100 %), *Penicillium crocicola* (in 2 parcels with up to 45 % relative abundance), were the most abundant followed by *P. citreonigrum* (in 1 parcel with up to 100 % relative abundance) *and P. bialowiezense* (in 3 parcels with up to 30 % relative abundance). For yeasts, the dominant species in musts included *H. uvarum* (in 15 parcels with up to 100 % relative abundance), *Pichia kluyveri* (in 6 parcels with up to 91 % relative abundance) and *Starmerella bacillaris* (in 5 parcels with up to 52 % relative abundance). Moreover, *P. kluyveri* and *S. bacillaris* were exclusively detected at must stage by the cultural approach (Figure 3A). At the wine stage, almost only *S. cerevisiae* was detected, as expected, as it was inoculated to drive the fermentation.

To be able to take into account the non-cultivable or less competitive mycobiota, a metagenetic analysis targeting the ITS2 region was also performed. The predominant fungal genera and their dynamics during grape berry ripening and micro-vinifications were confirmed by metabarcoding. Indeed, grape berries from fruit set to harvest were dominated by *Aureobasidium* (mean relative abundance from fruit set to harvest across all samples: 63% at fruit set, 58 % at veraison and 36 % at harvest), *Cladosporium* (29 % at fruit set, 24 % at veraison and 17 % at harvest) and *Vishniacozyma* (6 % at fruit set, 2 % at veraison and 5 % at harvest), while relative abundance of *Botrytis* and *Penicillium* increased from veraison to harvest (16 and 0.2 % at veraison and harvest versus 39 and 1.8 % respectively). After grape crushing, yeast genera dominated in musts, especially *Starmerella*, *Metschnikowia*, *Pichia* and *Hanseniaspora* (representing respectively 30, 10, 5 and 4 % relative abundance in musts), before being outcompeted by *Saccharomyces* after inoculation (Figure 3B).

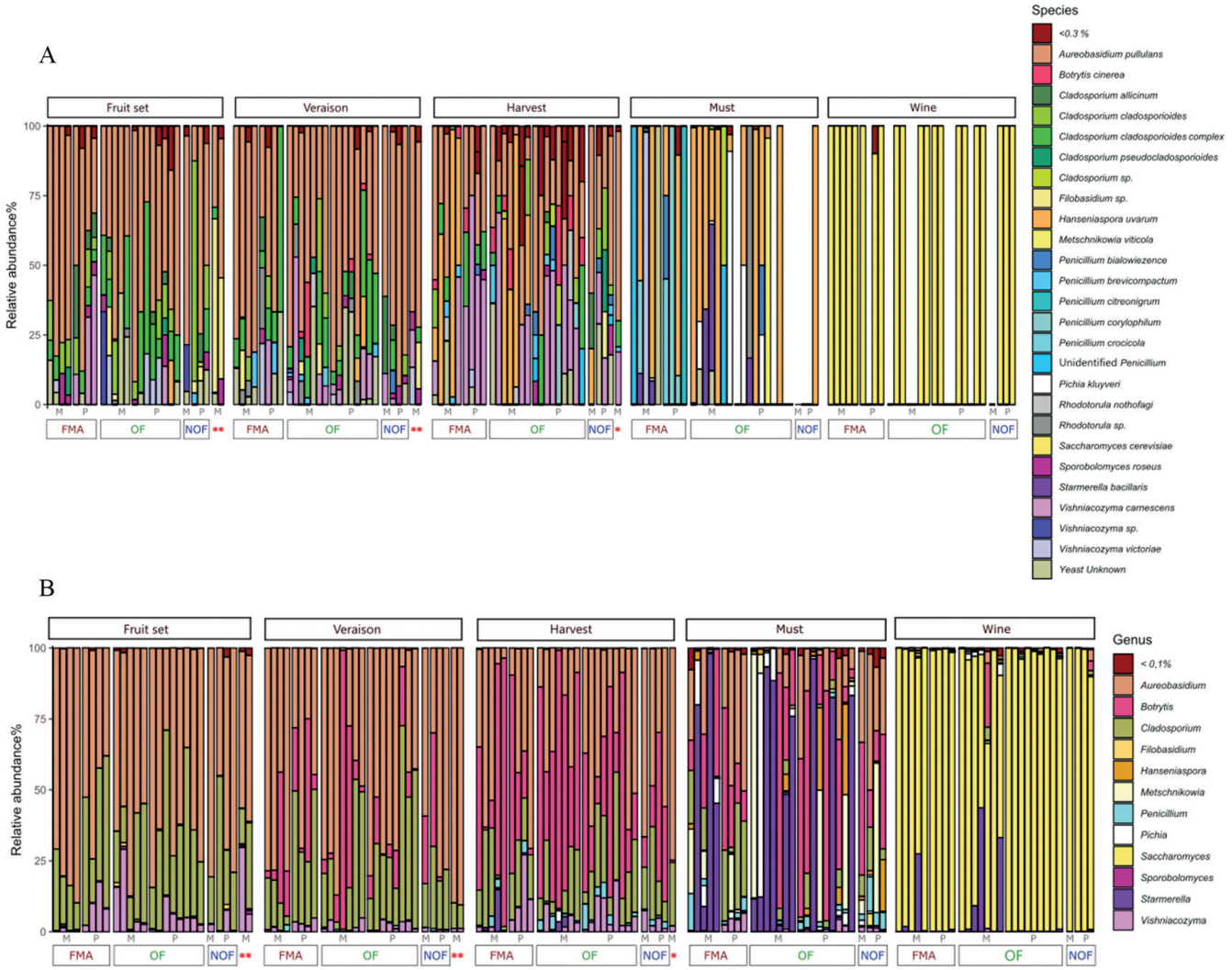


FIGURE 3. A) Relative abundance of each identified species in grapes, musts and wines (with mean abundance > 0.3 % for all stages) using the culture-dependent approach. B) Relative abundance of each identified genus in grapes, musts and wines (with > 0.1 % median abundance for each stage) using the culture-independent approach (MiSeq Illumina sequencing targeting the ITS2 region).

For both methods, relative abundance was plotted for each parcel and for the 5 sampling stages. The parcels were classified according to their sensorial wine class assignment: Fresh Mushroom Aroma (FMA), Other Off-Flavours (OF) and No Off-Flavour (NOF); and according to the grape variety: either Meunier (M) or Pinot Noir (P) respectively. *Parcels not included in the micro-winemaking process.

Although metagenetic data detected *Penicillium* at a relatively low abundance, the DNA of this genus persisted in musts (mean relative abundance from harvest set to must: 1.8 to 2.1 %), similarly to cultural data (mean relative abundance from harvest to must: 5.1 to 17.1 %). Unlike results obtained with the cultural method, higher relative abundances of *Aureobasidium* and *Botrytis* were detected in musts with metagenetic approach, reaching up to 94 % and 86 % respectively, when present (Figure 3B), versus 4 % and 0.2 % respectively based on cultural data (Figure 3A).

2.2 Impact of variety on fungal dynamics

Based on the cultural approach, total fungal abundances were not significantly different between Meunier and Pinot noir at veraison, harvest and wine stages. However, at fruit set and must stages, abundances were significantly higher for Meunier than Pinot noir (at fruit set: p-value = 0.013, t test and at must stage: p-value = 0.001 Kuskal-Wallis test). The disparity between the mean values did not exceed 1 log at fruit set (3.84 log CFU/g for Pinot noir and 4.31 log CFU/g for Meunier), while a 2-log difference was observed in musts (2.92 log CFU/mL for Pinot noir and 4.88 log CFU/mL for Meunier). Among the different fungal species present at these stages, *A. pullulans* counts at fruit set were significantly higher on Meunier than on Pinot noir (t test: p-value = 0.0039). Additionally, *H. uvarum* abundances were higher in Meunier musts than in Pinot noir musts (Kruskal-Wallis: p-value = 0.041, respectively). When applying the metagenetic approach, regarding the significant differences in relative abundance above 1 % at the fruit set and veraison stages, *Aureobasidium* was more abundant on Meunier, while *Cladosporium* was more prevalent on Pinot noir.

At harvest, *Starmerella* was observed to be more prevalent on Meunier, while *Vishniacozyma* was more abundant on Pinot noir. In Meunier musts, *Pichia* and *Starmerella* were more abundant, while *Aureobasidium, Botrytis, Cladosporium, Penicillium* and *Vishniacozyma* were more abundant in Pinot noir musts. *Starmerella* was also more abundant in wines made from Meunier grapes than those from Pinot noir grapes (p-value < 0.05, Kruskal-Wallis test).

3. Relationship between mycobiota and wine sensory classes

3.1. α and β-diversity analysis of fungal communities from grape to must as potential indicators of sensory wine classes

Alpha-diversity analyses were conducted using the ASV tables at the genus level (Figure S2). The number of observed genera (Observed index in figure S2) was significantly higher in the "Without off-flavour" musts than in the "FMA" and "Other off-flavors" musts. In contrast, Shannon and Simpson indices (Figure S2) were significantly lower only in "Other off-flavours" musts.

The comparison of mycobiota composition for the three wine sensorial classes was performed with a β-diversity analysis based on both cultural and metagenetic approaches, using PCoA (Bray-Curtis distance with Hellinger transformation) (Figure S3). No significant difference in mycobiota composition was observed between the three sensorial classes, except at the must stage using the metagenetic approach (p-value = 0.002, R^2 = 0.108, Figure S3A, S3B). Interestingly, at harvest, total fungal abundances were significantly higher (Figure 2) in vineyards where grape micro-winemaking led to FMA wines than in those classified as "other off-flavours" and "without off-flavour", although mean differences over the group average were minor (< 1 log). In the musts generating "FMA" and "other off-flavours" classes of wines, total fungal abundances were also significantly higher than in those "without off-flavour", with mean differences over 4 and 3 log CFU/mL respectively.

3.2. Fungal species potentially associated with FMA wines

Based on the cultural approach, *Penicillium* was the only differentially-abundant genus in musts resulting in FMA wines compared to the other classes with a difference of more than or equal to 3 log CFU/mL (Figure 4A; Kuskal-Wallis test, p-value = 0.0014). Furthermore, *Penicillium* spp. were significantly more prevalent in musts generating FMA wines compared to the other sensory classes (Chi-2 test: p-value = 0.004; Figure 1A). We were, however, unable to pinpoint any differentially abundant *Penicillium* species between sensorial classes. The *Penicillium* genus represented 47 % of total fungal composition in "FMA" musts, including *P. corylophilum* (1.55 log CFU/mL), *Penicillium* spp. (0.56 log CFU/mL), *P. citreonigrum* (0.52 CFU/mL), *P. crocicola* (0.75 log CFU/mL) followed by *P. bialowiezense, P. brevicompactum* and *Penicillium scabrosum* (0.72, 0.38 and 0.35 log CFU/mL respectively). *Penicillium* spp. represented only 5 % of total genera detected at the must stage for the "Other off-flavours" sensorial class, which included *P. expansum* (0.22 log CFU/mL), *P. bialowiezense* (0.21 log CFU/mL), *Penicillium* spp. and *P. brevicompactum* (0.19 log CFU/mL for both), while no *Penicillium* species were detected in the musts leading to "Without off-flavour" wines.

Based on metagenetic data, the indicator species analysis showed that *Cladosporium* (p-value = 0.0056, stat = 0.402) and *Vishniacozyma* (p-value = 0.0283, stat = 0.327) in musts were associated "FMA" wines; while *Penicillium* (p-value = 0.0007, stat = 0.485) at that stage was associated with the "Without off-flavour" class (data not shown). In addition, *Starmerella* (p-value = 0.0067, stat = 0.425) was associated with "FMA" and "Other" musts, and *Aureobasidium* (p-value = 4e-04, stat = 0.527) with "FMA" and "Without off-flavour" musts (data not shown).

In addition to the indicator species analysis, variation of relative abundances of the main genera was evaluated (with a median relative abundance > 0.1 %). Compared to both "Without off-flavour" and "FMA" musts, the *Cladosporium* and *Aureobasidium* genera showed significantly lower relative abundances than in musts from the "Other" category. Conversely, the relative abundance of *Vishniacozyma* members was significantly lower in the "Other" category compared to "Without off-flavour" musts (Figure 4B). Concerning *Starmerella*, its relative abundance was significantly higher in "Other" than in "Without off-flavour" musts. Less dominant genera, which were characterised by a mean abundance of less than 0.1 %, were more abundant in "FMA" musts compared to both "FMA" and "Other" musts (Figure 4B). Additionally, members of the *Penicillium* and *Hanseniaspora* genus were significantly more abundant in "Without off-flavour" musts compared to "FMA" musts (Figure 4B).

4. Mycobiota interactions and networks

In order to study the interactions between genera potentially associated with FMA wines, and other either positively or negatively correlated genera, co-occurrence networks (based on the top 200 ASV detected with metagenetic data) were created from fruit set to must stage. Overall, a few correlations were observed and conserved between veraison and harvest, including a negative correlation between *Botrytis* and *Aureobasidium,* and positive correlations between *Vishniacozyma* and *Cladosporium,* and between *Vishniacozyma* and *Aureobasidium* (Figure 5). In musts, the number of significant correlations increased and *Penicillium* was positively correlated with *Botrytis* (coefficient of correlation r = 0.28), *Cladosporium* (r = 0.61), *Vishniacozyma* (r = 0.57) and *Aureobasidium* (r = 0.57). *Botrytis* was also positively correlated with *Cladosporium* (r = 0.5), *Aureobasidium* (r = 0.44) and *Vishniacozyma* (r = 0.34), while it was negatively correlated with *Metschnikowia* (r = -0.33). *Penicillium* and *Botrytis* were also negatively associated with *Pichia* (r = -0.72 and -0.42, respectively) and *Starmerella* (r = -0.75 and -0.32, respectively) (Figure 5).

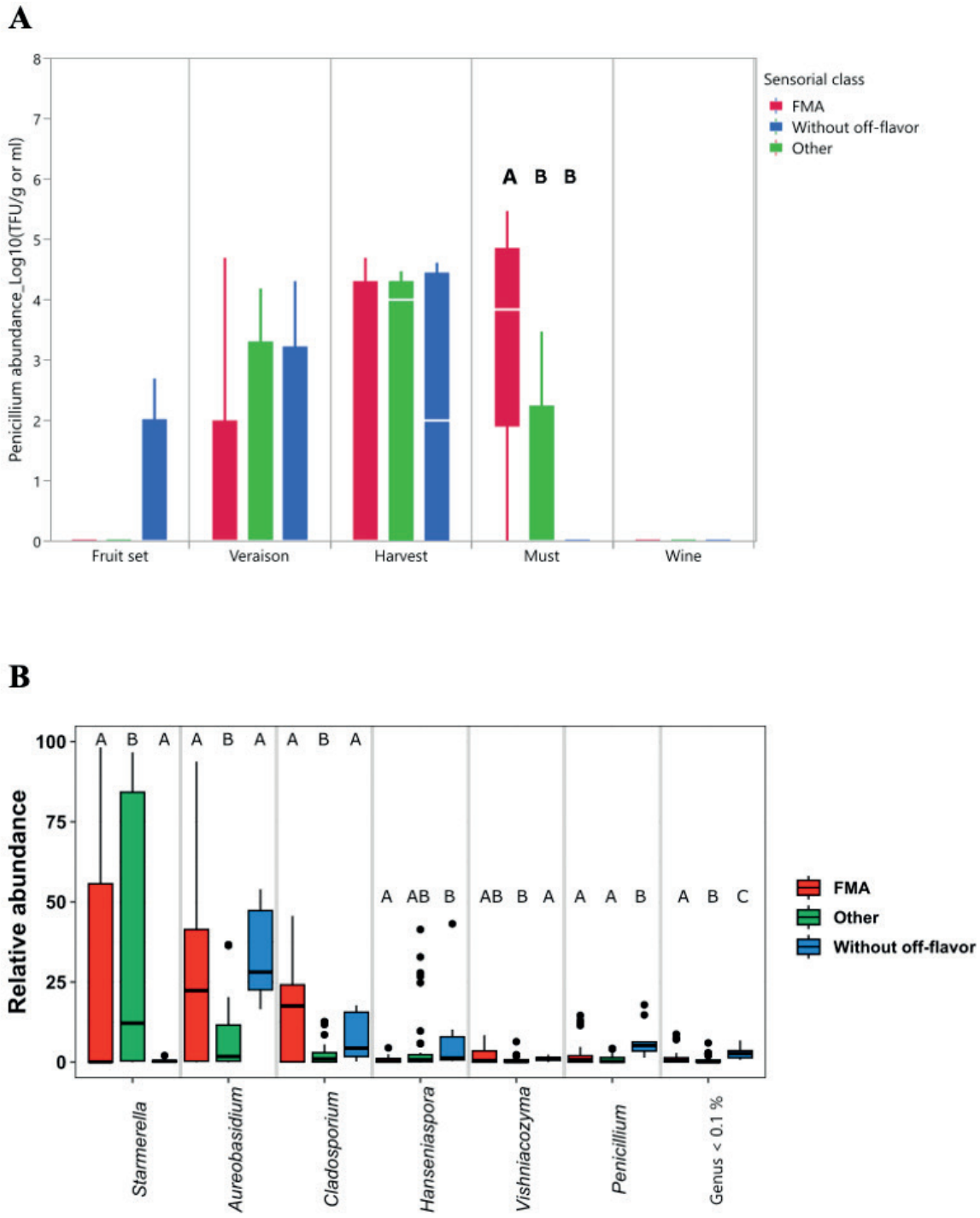


FIGURE 4. (A) *Penicillium* abundance in Log10 (TFU/g for fruit set, veraison and harvest or TFU/ml for must and wine) at each stage. The letters represent the significant difference in abundance between the sensorial classes (performed with Wilcoxon pairwise test). (B) Relative abundance of the top genera (> 0.1 %) for each sensorial class detected at must stage with metagenetic approach. Letters represent the significant difference of relative abundance between the sensorial classes (performed with Wilcoxon pairwise test).

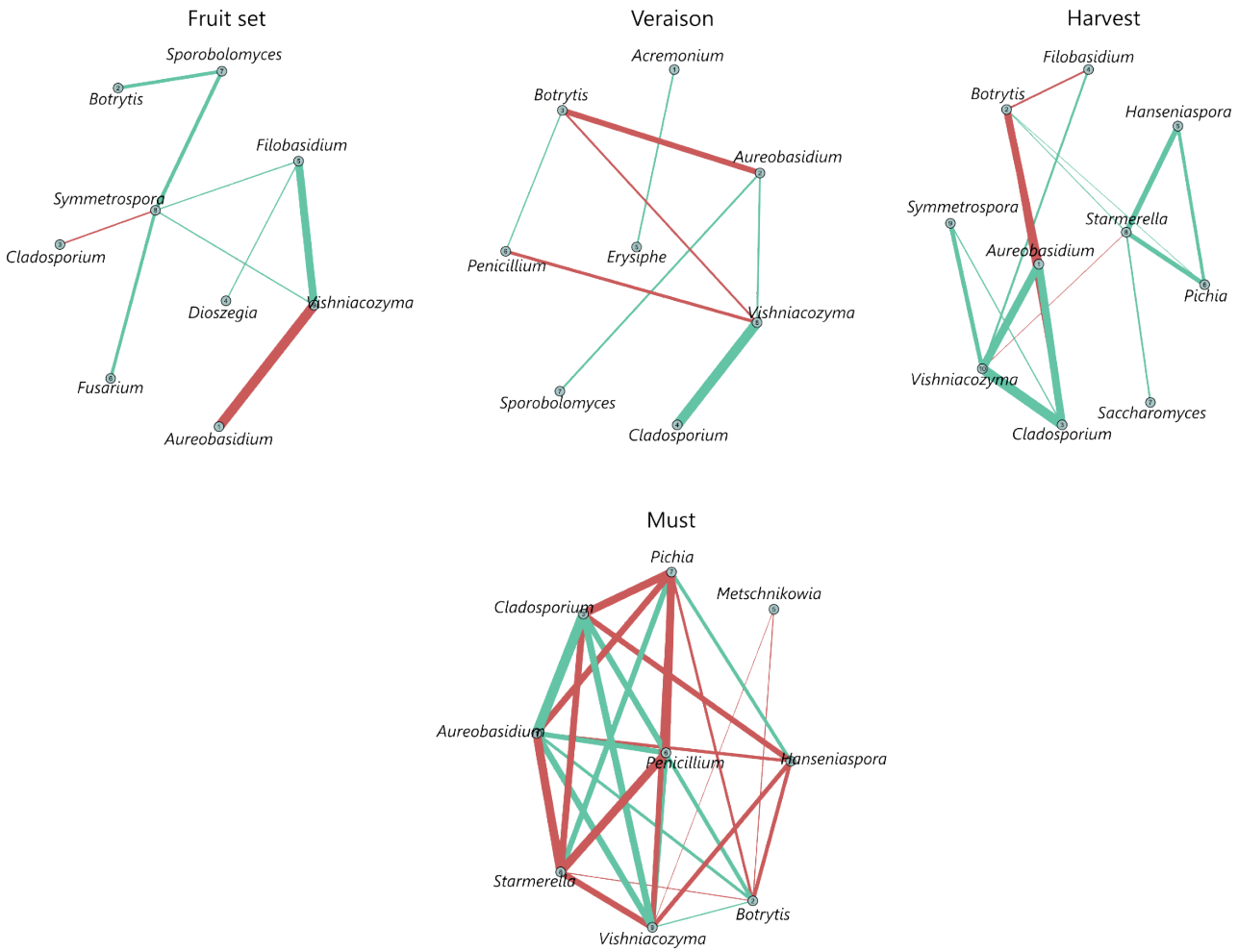


FIGURE 5. Co-occurrence networks between top 200 ASV from fruit set to must stages. Only the significant correlations ($p < 0.05$) with correlation coefficient higher than 0.3 or less than -0.3 were plotted. Green bars represent the positive interactions and red bars the negative interactions. The width of the bar is proportional to the intensity of correlation (the largest bars correspond to correlation coefficients closer to 1 or -1).

DISCUSSION

Only few studies have examined fungal dynamics from vine to wine (Martiniuk *et al*., 2023), most of them describing the microbial ecology of grapes (Liu and Howell, 2021), while other studies focused exclusively on the fermentative stages (Steenwerth *et al*., 2021). For this study, we microvinified 40 kg of grapes for each of the monitored plots to thoroughly describe mycobiota diversity and dynamics from vine to wine. Using complementary culture-dependent and -independent methods, similar trends on mycobiota composition and dynamics were observed. Fungal counts increased during berry ripening due to the physiological changes in berry composition during maturation, in association with the decrease in acidity and increase in total sugar (Zhu *et al*., 2021), before decreasing in musts. Such a decrease in filamentous fungal abundance in must during the pre-fermentation stages has already been well documented; it is mainly caused by the transition from solid state to liquid, acidity level and possible addition of sulfites in musts, which all cause both oxidative and toxic stresses for moulds (Grangeteau *et al*., 2017). However, the genus *Penicillium* also decreased in abundance but noticeably remained at a relatively high abundance in musts (1.5 log TFU/mL) compared to other moulds (ranging from 0 to 0.2 log TFU/mL).

At fruit set, the dominant species on both berry varieties corresponded to *Cladosporium* spp. (mainly represented by *Cladosporium allicinum* and *C. cladosporoides* complex) and *A. pullulans*, as previously reported by Liu and Howell (2021) on Pinot noir grape berries in Australia. At veraison, higher diversity was observed with *Penicillium* spp. (mainly represented by *P. brevicompactum, P. bialowiezense), B. cinerea, V. carnescens,* and *H. uvarum,* whose abundances tended to increase up to harvest. *Penicillium* spp. have previously been shown to be among the main moulds on Pinot noir grapes at harvest in the Burgundy region in 2008, with 58.5 % of all filamentous fungal populations belonging to this genus (Diguta, 2010). The identified species included *Penicillium spinulosum, P. expansum, Penicillium minioluteum, P. glabrum, P. thomii, P. brevicompactum, Penicillium chrysogenum,* as well as *C. cladosporioides* (15.1 %) and *B. cinerea* (15.1 %).

After grape crushing, as already described by Grangeteau *et al*. (2016) for Chardonnay musts, *Penicillium* spp. were the only filamentous fungi to persist at high abundance in both Meunier and Pinot noir musts, although variations among the main identified species (i.e., *P. corylophilum, P. citreonigrum, P. crocicola, P. bialowiezense, P. scabrosum*, and *P. expansum)* were observed. Before post-fermentation decline, other moulds including *Cladosporium, Botrytis, Aureobasidium and Filobasidium* in Pinot noir musts and yeasts including *Vishniacozyma*, *Pichia* and *Starmerella,* were also detected; this correlates well with previous studies of Pinot noir grape berries (Martiniuk *et al*., 2023). In the pre-fermentation stage, non-*Saccharomyces* yeasts, such as *Hanseniaspora, Starmerella, Pichia or Metschnikowia,* as observed in our study, are also considered as dominant genera by Steenwerth *et al*. (2021). Consistent with the findings of Windholtz *et al*. (2021) and Zhu *et al*. (2021), *H. uvarum, P. kluyveri*, *S. bacillaris* and *Metschnikowia pulcherrima* were found to be the most abundant species in must, while subdominant yeast species included *Metschnikowia viticola, Rhodotorula* spp. and *Vishniacozyma victoriae,* which were only sporadically detected in musts. Noteworthy, the cultural approach yielded a species-level resolution, unlike most studies, thus providing a more in-depth $e^{-i\omega t}$ view of fungal diversity. However, these fungi were not detected in the same samples via our cultural approach, probably due to the fact that these moulds may not be in viable forms in musts as reported by Stefanini and Cavalieri (2018).

After determining mycobiota composition and dynamics in grape berries, we focused on identifying the microorganisms potentially responsible for the FMA defect in laboratory-scale wines produced from unsorted grape bunches. Nine of the wines were characterised by levels of 1-octen-3-one over the 10 ng/L threshold and/or perceived as having the FMA defect by sensory panellists. Compared to the other volatile compounds associated with FMA notes, the olfactory detection threshold of 1-octen-3-one is known to be very low, with a chemical spoilage index reported at 40 ng/L in neutral white wine, 15 ng/L in model solution similar to wine, and at 20 ng/L in white wine for Champagne elaboration (Pons *et al*., 2011; La Guerche *et al*., 2006). This molecule is the main contributor to the FMA off-odour; however, 1-octen-3-ol, 2-heptanol, 2-octen-1-ol and 1-nonen-3-one have also been reported to contribute to this off-odour (olfactorily detected at 40 µg/L in model wine, 50 µg/L in water, 40 µg/L in water, and 0.008 µg/L in water respectively; Steel *et al*. (2013); La Guerche *et al*., 2006), as has more recently 1-hydroxyoctan-3-one (Delcros *et al*., 2023), which was identified in Pinot noir musts contaminated with *C. subabrutptus*. The detection threshold of 1-hydroxyoctan-3-one - which according to a hypothesis formulated by Delcros *et al*. (2023) may also represent a potential precursor of 1-octen-3-one or, conversely, a product - was ten thousand times higher than 1-octen-3-one (0.2 mg/L).

To evidence markers of the FMA defect in wines, global analyses of fungal diversity were conducted. For β-diversity analyses, only a slight difference in the overall mycobiota composition at must stage was observed between the different sensory classes. However, the α-diversity analysis showed that must mycobiota associated with wines characterised as without off-flavour had a significantly higher number of genera (mean = 16) compared to that of musts with off-flavours (mean = 10 and 11, for FMA and Other off-flavours musts). This suggests that a richer fungal community could potentially reduce the risk of organoleptic deviations during fermentation, or that in the vineyards used to produce wines with off-odours, a few species dominate the ecosystem, thus making it difficult to detect the low frequencies of some groups. Cultural analyses highlighted that wines with organoleptic deviations (i.e., 'FMA' or "Other off-flavours", described as earthy, mouldy etc.) were associated with significantly higher fungal abundances at harvest (for FMA) and in musts (for FMA and Other off-flavours), suggesting that this could help predict the risk of FMA spoilage. In addition, the cultural analyses also revealed that the abundance of *Penicillium* spp. was higher in musts leading to FMA wines compared to the other classes, suggesting that the risk to produce FMA wines is increased when the *Penicillium* load reaches a certain threshold level. This genus has already been described as being associated with the FMA defect; for instance, *P. thomii* (more recently revised as a clade containing *P. crocicola;* Houbraken *et al.* (2014)) is considered as a grapevine spoiler, because it colonises the surface of grape bunches and produces undesirable volatile compounds (such as 1-octen-3-ol and 2-methylisoborneol, responsible for FMA and earthy/moldy off-odours respectively) that can be detected in wine (La Guerche *et al*., 2006). *P. brevicompactum* and *P. glabrum* have also been associated with FMA production (La Guerche *et al.*, 2006; Börjesson *et al.*, 1992). In the present study, *P. bialowiezence, P. brevicompactum, P. citreonigrum, P. corylophilum, P. crocicola, P. scabrosum* and some unidentified *Penicillium* spp. were detected in the musts. Overall, these results suggest a strong involvement of *Penicillium* spp. in the FMA off-flavour defect, although the exact mechanisms by which these taxa induce FMA off-flavours have yet to be determined. Concerning yeasts, the relative abundance of the *Starmerella* genus was significantly higher in musts leading to wines with "Other off-flavours" compared to the two other sensory classes. However, it is unlikely that this distribution is linked to off-flavours in wines, particularly as the species *S. bacillaris* - which is the most frequently isolated species from grapes and must within the *Starmerella* genus - is often used in oenology for must bioprotection (Nadai *et al*., 2021). A more plausible explanation for the higher relative abundance of *Starmerella* in musts with defects could be the confounding effect with grape variety. Indeed, of all the detected genera, *Starmerella* was significantly more abundant in musts and wines from Meunier vineyards compared to those from Pinot noir vineyards, while the 'without off-flavour' sensory class was composed of only one Meunier vineyard versus four Pinot Noir vineyards. The analyses using the complementary metagenetic approach evidenced *Cladosporium* and *Vishniacozyma* as indicator genera of the FMA class in must, but it failed to reveal any significant differences in terms of fungal counts, as was observed via the cultural method. In addition, these two genera were strongly positively correlated to *Penicillium* in

musts, based on the SparCC co-occurrence networks. Except for *Cladosporium herbarum,* which has been reported to be involved in corked wine (Daly *et al*., 1984), *Cladosporium* was rarely linked to organoleptic defects. Similarly, *Vishniacozyma* has frequently been isolated on grape berries and is one of the dominant yeast species on grape bunches (Martiniuk *et al*., 2023), but its potential influence on wine quality and off-flavour is unknown (Zhu *et al*., 2021) and should be further investigated. Overall, these results highlight the possibility that these two genera play an indirect role in *Penicillium* development, promoting its establishment and consequently the production of FMA in the resultant wines. These trends, observed in musts, represent the only identified markers which could help predict the risk of FMA occurrence before grape crushing.

In addition to the multiple taxa potentially involved in the production of FMA, some interactions might impact positively or negatively the production of molecules associated with FMA. Indeed, some species may promote the implantation of the spoilage species responsible for FMA, and thus would be indirectly involved in the occurrence of this spoilage, while, conversely, other taxa may repress these spoilage species, via, for example, competition or the secretion of antifungal molecules (Nadai *et al*., 2021). In the context of negative interactions revealed in this study, potential suppressors against *Penicillium* (considered as a potential FMA producer) and *Botrytis* (as major contributor of many wine defects) were identified through negative correlation interactions between taxa. They correspond to both the *Pichia* and *Starmerella* genera in musts, while the *Metschnikowia genus* was only negatively correlated with *Botrytis*. Notably, as mentioned previously, *S. bacillaris,* a non-*Saccharomyces* yeast commonly used in grape fermentation alongside *S. cerevisiae*, has been found to exhibit antifungal activity against moulds, particularly *B. cinerea* (Nadai *et al*., 2021). Similarly, *P. kluyveri,* known for its contribution to wine aroma, glycerol, ethanol yield, and killer toxin production, has been shown to have antimicrobial potential against various spoilage yeasts in wine (Vicente *et al*., 2021). Furthermore, the use of *M. pulcherrima* as a biocontrol agent in must has been shown to prevent wine spoilage caused by *H. uvarum*, producing acetic acid during fermentation (Gerbaux and Davanture, 2019). The introduction of *M. pulcherrima* and *Torulaspora delbrueckii* in Semillon before alcoholic fermentation not only reduced SO_2 levels, protecting against browning, but also decreased the relative abundance of *Botrytis, Aspergillus,* and *Aureobasidium* due to antifungal and antioxidative activities (Windholtz *et al*., 2021; Milan *et al.*, 2022). Thus, in an FMA context, it would be interesting to further evaluate the potential repression effect of *M. pulcherima, S. bacillaris*, and *P. kluyveri*, which were among the dominant yeast species in musts, against fungal spoilage species.

CONCLUSION

This study describes for the first time fungal diversity and dynamics from vine to wine in a study area located in north-east France and how mycobiota composition contributes to the FMA defect. The changes in grape mycobiota composition in relation to berry ripening stage, must, wine and grape variety were thoroughly investigated and described. Subsequently, analyses were carried out, which showed that musts resulting in wines with FMA off-flavors had the highest fungal abundances. In particular, *Penicillium* counts were higher in musts generating FMA wines than in other categories. *Vishniacozyma* and *Cladosporium* were identified as potential taxa indirectly involved in the FMA defect and positively correlated with *Penicillium* in musts. Moreover, based on the co-occurrence networks generated in this study, we hypothesise that three yeast species, *M. pulcherima, S. bacillaris and P. kluyveri,* are potential biocontrol agents against *Penicillium* and *B. cinerea*. The actual involvement in FMA of the identified fungal species, as well as the potential positive and negative interactions, should be further investigated, in particular via challenge-test and co-culture experiments. Overall, this study provides a better understanding of the fungal actors potentially involved in the development of the FMA off-flavour in wines, as well as potential solutions worth exploring in the future.

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