LWT - Food Science and Technology 193 (2024) 115769

Contents lists available at ScienceDirect

LWT

journal homepage: www.elsevier.com/locate/lwt

Combined omics expose microbial niches of fungi and bacteria correlating with wine volatile profiles in Douro wine region

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ARTICLE INFO

Keywords:
Hanseniaspora-Saccharomyces succession
Metabarcoding
Metabolite-microbiota correlations
Spontaneous fermentation
Wine aroma

ABSTRACT

Wine microbial communities establish complex ecological ecosystems that modulate the formation of aroma compounds, but only a few studies sought for correlations between specific microorganisms and wine volatiles. The present study combined metabarcoding and metabolomics for identifying microbial niches of fungi and bacteria correlating with the volatile profiles of wines of 3 renowned cultivars of the emblematic Douro region. Three major microbial niches were identified throughout the spontaneous fermentation processes, and the *Hanseniaspora-Saccharomyces* succession timing was cultivar-dependent. The largest niche included *Hanseniaspora*, *Aureobasidium*, *Alternaria*, *Rhodotorula*, *Sporobolomyces*, *Massilia*, *Bacillus*, *Staphylococcus* and *Cutibacterium*, that positively correlated with 7 metabolites, namely, acetoin, isoamyl acetate, ethyl propanoate, c-3-hexenol, phenylethyl acetate and 4-ethylphenol. The fermentative yeasts *S. cerevisiae*, *Torulaspora delbrueckii* and *Meyerozyma caribbica* strongly correlated with γ-butyrolactone, t-whiskylactone, isoamyl alcohol, ethyl decanoate, ethyl isobutyrate, diethyl succinate, isovaleric acid, 4-ethylguaiacol and 4-propylguaiacol. *Lachancea quebecensis* clustered with several pathogenic fungi (*Penicillium citrinum*, *Erysiphe necator*, *Sclerotinia sclerotiorum, Aspergillus, Mycosphaerella tassiana*) and bacteria (*Pseudomonas* spp., *Bacteroides acidifaciens*, *Pantoea*, *Stenotrophomonas* and *Enhydrobacter*), correlating positively with various monoterpenols and norisoprenoids including linalool and β-ionone, besides with benzyl alcohol, diacetyl, isobutyl acetate, ethyl-vanillate and methyl vanillinate. Metabolite-microbiota correlations denoted cultivar specificities likely underlying regional aromatic signatures.

1. Introduction

Wine production is amongst the world's oldest biotechnologies, entailing an intricate succession of microbial conversions of berry components into metabolites that stabilize the final product (Bisson & Walker, 2015). The must microbial consortium arises primarily from berries, thus, the community of oxidative and fermentative species that composes the wine microbiota is constantly evolving because of the metabolization of nutrients like sugars and the production of alcohol and other metabolites, including organic compounds and antimicrobial compounds (Bisson & Walker, 2015; Ding et al., 2021; Englezos et al., 2022; Renouf et al., 2005).

Because the complexity of wine is believed to increase with different microbial interactions, research directed to understand and manipulate the microbial biodiversity inherent to winemaking is highly relevant from an industrial point of view (Sirén et al., 2019). Studies based on metagenomic approaches contributed to the depiction of the microbial terroir, by investigating microbial populations associated with wine production (Belda et al., 2017; Bokulich et al., 2014; Marzano et al., 2016; Mezzasalma et al., 2017; Perpetuini et al., 2023; Pinto et al., 2015; Stefanini & Cavalieri, 2018; Suzzi et al., 2012; Tofalo et al., 2014). Metabolomics studies have also been very useful to differentiate wine aromas according to cultivar, region and starter inoculum (Deed et al., 2017; Martins et al., 2020; Roullier-Gall et al., 2014; Vararu et al.,

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https://doi.org/10.1016/j.lwt.2024.115769
Received 18 October 2023; Received in revised form 30 November 2023; Accepted 15 January 2024
Available online 16 January 2024
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2016). The combination of different omics approaches is very relevant to wine production and other food industries, to reveal biological pathways underlying the formation of specific aroma compounds (Sirén et al., 2019), but only few studies addressed the relationships between microbial populations and wine organoleptic properties (Perpetuini et al., 2022; Rossetti et al., 2023). Research available to date is focused on few cultivars including Vidal, Carbenet Sauvignon, Chardonnay and Petit Verdot, correlating specific bacteria and/or fungi subpopulations with volatile compounds or non-volatile metabolites including phenolics and organic acids, although many could not be identified from available databases (Bokulich et al., 2016; Chen et al., 2020; Chen et al., 2022; Liu et al., 2021). Other studies characterized the evolution of microbial populations during spontaneous fermentation and the chemical and sensory traits of wines, without seeking for microbiota-metabolite correlations (Bagheri et al., 2020; Böhmer et al., 2020; De Filippis et al., 2019; Patrignani et al., 2017).

The Douro region is an internationally renowned Portuguese viticulture region, characterized by the steep slopes of the Douro Valley, and is amongst the oldest delimited and regulated wine regions of the world. It is classified Word Heritage by the UNESCO since 2001, however, studies on the relationships between the metabolites and the microbiota of prominent cultivars in this region are very scarce. Only one recent study described the correlations between the grape berry metabolites and its microbial residents, showing that microbial diversity is generally associated to the high abundance of specific amino acids in the berry, concomitant with low level of some phenolics and wax components that may present antimicrobial activity (Martins et al., 2023). Indeed, the prevalence of plant pathogens such as *Erysiphe necator* and *Aspergillus carbonarius* in the berry was suggested to be linked to the levels of various triterpenoids (Martins et al., 2023). In the sequence of this work, the novelty of the present study relied on the identification of specific microbial niches of native fungi and bacteria associating with the formation of aroma compounds in wine. Through the innovative approach of combined omics, metabolite-microbiota correlations were disclosed during spontaneous fermentations of 3 renowned cultivars of the Portuguese Douro region. Results further unraveled the influence of the cultivar to the *Hanseniaspora-Saccharomyces* succession timing during fermentation. The metabolite-microbiota correlations with ρ values $>|0.95|$ are focal hypotheses generators for the search of causal relationships between fungi and bacteria communities and the wine matrix, particularly relevant in the wine industry.

2. Material and methods

2.1. Berry sampling and microvinifications

Mature grape berries (E-L 38) were randomly collected from grapevines of 3 of the most prominent cultivars in DOC Douro region (Sousão, Touriga Nacional and Viosinho), located in Quinta de S. Luiz (GPS coordinates: 41.158, −7.617). During the fruiting season, the average daily temperature was 12–33 °C, with a maximum temperature peak of 47 °C, and an average rainfall of 77 mm. Vineyards were growing on the same soil type of schist and greywacke complex, in dryland regime (non-irrigated) on a 15% slope and mostly oriented in west/southwest to east/northeast. To minimize the orchard effect, fifty bunches were collected from 30 grapevines per cultivar, and the bunches were randomly collected from different grapevine rows and from different positions along each row. All cultivars were subject to the same viticulture practices and conventional phytosanitary treatments following to the suppliers' instructions. These included a combination of fungicides targeted at mildew and oidium, namely a penetrating fungicide (Cupertine Super, IQV Agro Portugal S.A.) and systemic fungicides Luna Experience (Bayer), Ksar (Ascenza) and Talendo (Corteva). Nutricomplex fertilizer treatments enriched in macro and micronutrients including nitrogen, potassium and calcium were also applied in the months preceding harvest. Berries were transported to the laboratory in individual sterile plastic bags, in refrigerated conditions, and stored at 4 °C for 12 h prior to must preparation.

Bunches of each cultivar were separated into 4 groups and musts were prepared in quadruplicate ($n = 4$), according to the traditional methods for red and white wines, at laboratory scale (Martins et al., 2015, 2020). Musts of red cultivars (Sousão, Touriga Nacional) were prepared in 1 L glass flasks with 0.5 L of grape juice and 280 g of berry skins and seeds. Musts of the white cultivar (Viosinho) were prepared in a similar way, but the grape skins and seeds were removed after 24 h of maceration at 4 °C, allowing transfer of the skin aromas and microbiota to the musts, while preventing the start of fermentation (Lukić et al., 2015). No additives were added to the musts. Microvinifications were then allowed to occur spontaneously, by incubating the musts in the dark at 24 °C (for red cultivars) or at 16 °C (for the white cultivar), to mimic the conditions found in the cellar. Musts were stirred once a day, and the progress of fermentation was monitored through the estimation of sugar and alcohol levels using conventional methods detailed previously (Martins et al., 2015, 2020). Aliquots of 60 mL of musts, semi-fermented musts (7% alcohol) and wines (12% alcohol) were collected for analysis of the evolution of the microbiota detailed in the next section. The choice to collect semi-fermented musts with 7% alcohol was to ensure that the selective pressure of ethanol was already exerted on environmental fungi, while allowing to highlight possible differences in fermentative species that could be masked in musts with lower alcohol content.

2.2. Microbiota analysis

The microbiota was recovered through an adaptation of previously optimized methods (Martins et al., 2021; Mezzasalma et al., 2017). Freshly collected aliquots of 60 mL were centrifuged at 4500×*g* for 15 min and the pellets were washed in sterile 0.9% w/v NaCl. Genomic DNA was extracted from pellets with the DNeasy PowerSoil Kit (Qiagen). Library preparation, sequencing and initial bioinformatic analyses were performed by StabVida (Caparica, Portugal), in 4 biological replicates per cultivar/fermentation stage, totalizing 36 samples. Independent DNA libraries for fungi (ITS1 region between the 18S −5.8S rRNA gene) and bacteria (V3 – V4 variable regions of 16S rRNA gene) were prepared with an in-house protocol based on Illumina 16S Metagenomic Sequencing Library Preparation protocol (15044223 Rev.B). The Illumina MiSeq platform was used to sequence the DNA fragments, with MiSeq Reagent Kit v3, using 300 bp paired-end sequencing reads. Following analysis of the generated raw sequence data with QIIME2 v2022.2 (Caporaso et al., 2010) and assessment of data quality through the Phred quality score, the DADA2 plugin was used to denoise the reads (Callahan et al., 2016), which were organized in features and classified by taxon using UNITE database (release 8.3) for ITS and SILVA (release 138 QIIME) for 16S data, trained using scikit-learn. OTUs containing less than 10 sequence reads were filtered from the OTU list, together with those annotated as host mitochondria or chloroplast. OTUs detected in only one sample replicate were not considered for further bioinformatic analysis.

Data were analysed with phyloseq (McMurdie & Holmes, 2013) and vegan (Oksanen et al., 2013) packages in R version 4.0.4 (https://cran.r-project.org/). Alpha and beta diversity were calculated as described by Martins et al. (2021), using Bray–Curtis as measure of dissimilarity and the adonis2 function (package vegan, v. 2.5–7). The statistical significance of the results was assessed by Kruskal-Wallis and Dunn's tests, and by PerMANOVA, respectively. Barplots were assembled in Prism®6 (GraphPad Software, Inc.) by collapsing OTUs at class level using Microsoft Office Excel.

2.3. Wine volatile composition

The quantitative analysis of volatile compounds was carried out using previously optimized methods (López et al., 2002; Martins et al., 2020; Ortega et al., 2001). Major volatile compounds were quantified by GC with FID detection using 3 mL of wine containing internal standards [4-methyl-2-pentanol, ethyl heptanoate, heptanoic acid and 2-octanol (Sigma-Aldrich, St. Louis, Mo., USA)] and salted with 4.1 g of ammonium sulfate (Panreac, Barcelona, Spain) and extracted with dichloromethane (Fisher Scientific, Loughborough, UK). Minor volatile compounds were analysed by SPE and GC-Ion Trap-MS analysis using 50 mL aliquots of wine containing BHA solution [0.01 g of 3-*tert*-butyl-4-hydroxyanisole per g of ethanol (Sigma-Aldrich)] and a surrogate standards solution [3-octanone, β-damascone, heptanoic acid, and isopropyl propanoate (Sigma-Aldrich)]. Data were visualized through Principal Component Analysis (PCA) and heatmaps in R software version 4.0.4, using the packages FactoMineR 1.4.1 (Lê et al., 2008), Factoextra 1.0.5 (Kassambara, 2017) and ComplexHeatmap 1.18.1 (Gu et al., 2016) on Bioconductor 3.9. Significant differences between cultivars were marked by different letters, following Dunn's test, after assessment of normality and homogeneity with Shapiro and Bartlett's tests.

2.4. Correlation networks

Correlations between wine volatiles and fungi/bacteria OTUs were assembled from a matrix of OTU and metabolite values in Cytoscape version 3.8.1 using MetScape plugin (Cline et al., 2007), as described by Martins et al. (2021). Three different networks were generated using a perfuse force direct layout algorithm, each correlating wine volatiles to the microbiota of musts, semi-fermented musts or wines. For simplification purposes of the network comprising the microbiota of musts, only the top 50 most abundant fungi OTUs were considered. In each network, the node size is proportional to the correlation node strength ($ns = |\rho|$). Lines joining the nodes represent correlations with $\rho > |0.95|$; positive correlations are shown in red, while negative correlations are shown in blue.

3. Results

3.1. Evolution of microbiota composition from musts to wines

The metabarcoding approach used for the characterization of the microbiota of musts, semi-fermented musts and wines of the 3 cultivars rendered a total of 122,690 to 344,596 and 72,298 to 289,632 raw sequence reads for ITS1 and 16S regions, respectively. Alpha rarefaction curves denoted sufficient sequencing depth (Figure S1). A total of 257 OTUs were identified (Table S1). Musts of Viosinho presented the highest biodiversity, followed by musts of Touriga Nacional and Sousão (Fig. 1a, Table S2). While the fungi diversity decreased in all samples as fermentation progressed, an increase in the bacteria diversity was observed, more evidently for Viosinho. In semi-fermented musts, the highest fungi diversity was observed either for Touriga Nacional/Viosinho or for Sousão, denoted through the Chao1 and Shannon indexes, respectively. Nonetheless, at the end of fermentation, the Chao1 index translated a higher fungi diversity in wines of Viosinho, while no significant differences between cultivars were observed with the Shannon index.

Beta diversity expressed the differences in the composition of fungi and bacteria populations among samples (Fig. 1b and c), which were shown to be significant following PerMANOVA analyses (Table S3). The PCoA plots denote that the majority of fungi OTUs were present in musts, with only a few Ascomycota being present in semi-fermented musts and wines (Fig. 1b, **c**). Firmicutes were exclusively detected in musts and semi-fermented musts of Touriga Nacional, while Bacteroidota dominated the musts of Sousão. Actinobacteriota OTUs were more pronounced in semi-fermented musts and wines of the red cultivars and evenly present in all samples of Viosinho.

A clear separation between the musts of the 3 cultivars was observed upon closer inspection of the compositional differences of fungi communities at each fermentation stage (Fig. 2). In semi-fermented musts, the separation was mostly evident between Sousão and the other two cultivars, while in wines, samples of Sousão and Touriga Nacional clustered closely together (Fig. 2a). The number of fungi OTUs decreased sharply from musts to wines (Fig. 2b). Dothideomycetes was the most abundant class in musts of Viosinho (63%), followed by Saccharomycetes (22%) and Leotiomycetes (7%) (Fig. 2c). While Dothideomycetes was also the most represented class in musts of Touriga Nacional (50%), followed by Eurotiomycetes (25%), Saccharomycetes was the most abundant class in musts of Sousão (76%). In semi-fermented musts, the majority of OTUs were detected in samples of Viosinho and Touriga Nacional (Fig. 2b), with Saccharomycetes surpassing all other taxonomic classes (98%), and even becoming completely dominant in Sousão (Fig. 2c). In wines, the dominance of Saccharomycetes became even more pronounced for Touriga Nacional and Viosinho (Fig. 2c), although a few OTUs of Dothideomycetes and Microbotryomycetes were still detected in the latter (<0.5%) (Fig. 2b, **c**).

At genus level, the top 15 most abundant fungi OTUs in musts included *Hanseniaspora*, *Alternaria*, *Penicillium*, *Mycosphaerella*, *Aureobasidium*, *Sporobolomyces, Rhodotorula, Botryosphaeria* and *Saccharomyces* (*cerevisiae*), the latter found in similar amounts in the 3 cultivars (1–2%) (Fig. 3). In semi-fermented musts, *Hanseniaspora* spp., *Saccharomyces* (*cerevisiae*), *Lachancea* and *Aureobasidium* comprised the top 5 most abundant OTUs, while the counts of the remaining genera decreased dramatically. Noteworthy, at this stage, *Saccharomyces cerevisiae* dominated the samples of Sousão (70%), while *Hanseniaspora* dominated the samples of the other two cultivars. Indeed, *S. cerevisiae* was marginally detected in semi-fermented musts of Touriga Nacional (0.2%) and Viosinho (0.002%). In wines, *S. cerevisiae* largely dominated the fungi consortia of Sousão and Touriga Nacional (>98%), followed by *Hanseniaspora*, and by *Lachancea* in the latter cultivar. In contrast, *Hanseniaspora* dominated the wines of Viosinho (99%), marginally followed by *S. cerevisiae* (0.2%), *Rhodotorula* and *Aureobasidium*. In the bacteria community, the most abundant OTUs in musts and semi-fermented musts included the genera *Pseudomonas*, *Tumebacillus*, *Massilia*, *Pantoea*, *Curtobacterium*, *Bacillus* and *Cutibacterium*. In turn, *Bacillus* and *Staphylococcus* predominated in the wines of Viosinho, *Cutibacterium* predominated in the wines of Sousão, and *Stenotrophomonas* was the most abundant in the wines of Touriga Nacional (Fig. 3).

3.2. Wine volatile profile

Sixty-four volatile compounds were identified in wines of Sousão, Touriga Nacional and Viosinho, 40 of them at supra- or peri-threshold concentration levels (Table S4). Principal component analysis exposed a separation between the volatile profiles of the 3 cultivars (Fig. 4); the first dimension (Dim1, 47.2%) denoted the separation between wines of Viosinho and of the red cultivars, and the second dimension (Dim2, 40.2%) showed a separation between wines of Sousão and of Touriga Nacional. Most volatile compounds were more abundant in wines of the red cultivars. A heatmap analysis showed that, in general, 22 metabolites were more abundant in wines of Sousão, 23 metabolites were more abundant in wines of Touriga Nacional, 10 metabolites were evenly abundant in wines of the red cultivars, and only 9 metabolites were abundantly detected in wines of Viosinho (Fig. 5, Table S5). In the latter, these included the acetates isoamyl acetate and phenylethyl acetate, the ester ethyl propanoate and the carbonyl compound acetoin. Four alcohols were tendentially more abundant in wines of Sousão than in wines of the other two cultivars, including isoamyl alcohol and methionol (Fig. 5). The highest concentration of acetic acid was detected in wines of Touriga Nacional (440 mg L^{-1}), followed by wines of Viosinho (353 mg L^{-1}) (Table S4, S5). In turn, butyric, hexanoic and octanoic acids were lowest in wines of Viosinho, while wines of Touriga Nacional contained the lowest levels of isobutyric acid. The majority of branched

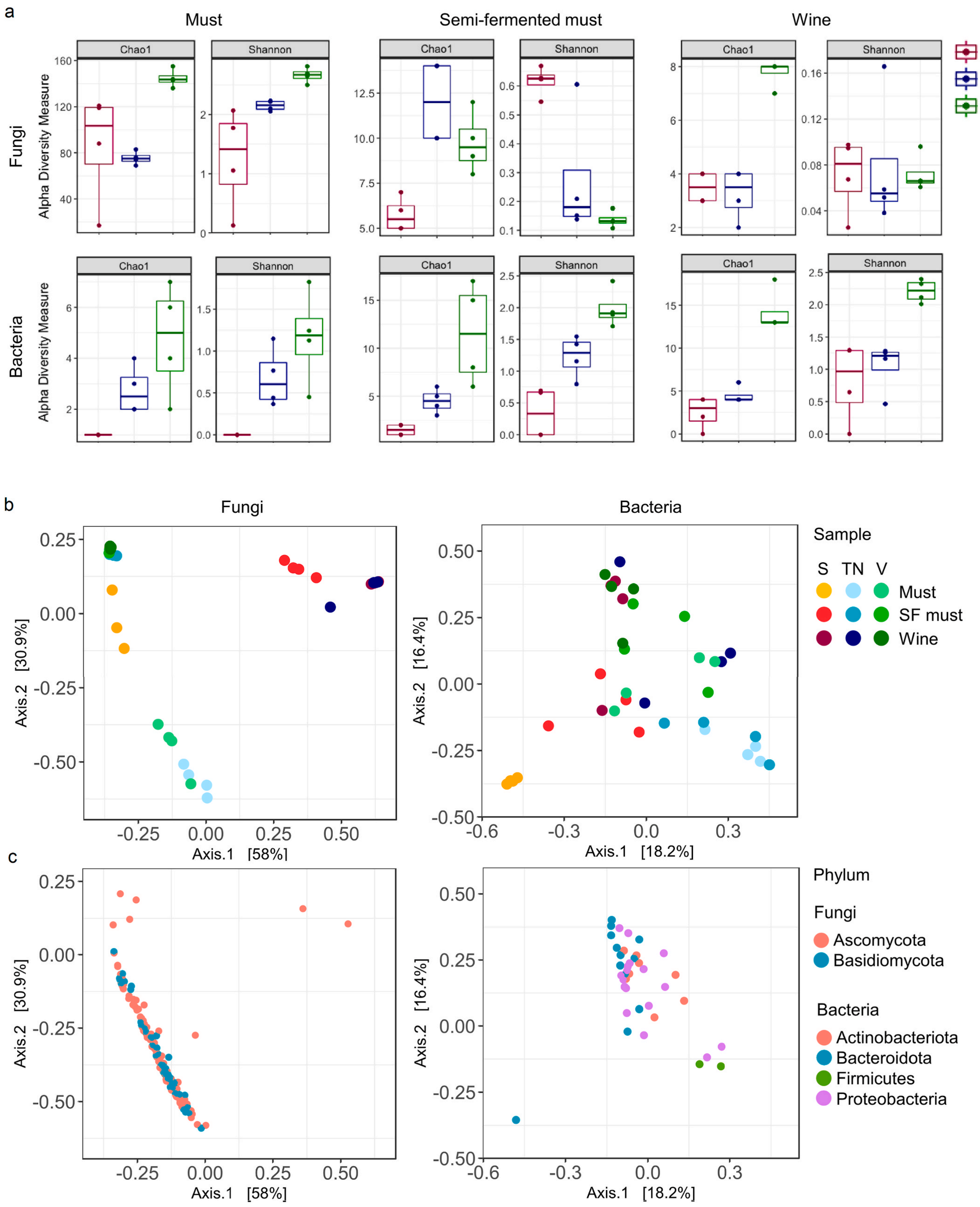

Fig. 1. Diversity of fungi and bacteria communities of musts, semi-fermented musts (SF) and wines from grape berries Sousão (S), Touriga Nacional (TN) and Viosinho (V). Alpha diversity calculated with the Chao1 and Shannon indexes (**a**). Results are expressed as median ± SD of 4 biological replicates. The statistical significance of the results was assessed by Kruskal-Wallis and Dunn's tests, as presented in Table S2. Beta diversity PCoA plots using Bray Curtis distance matrix coefficients, presented by sample (**b**) or by phylum (**c**). The statistical significance of the results was assessed by PerMANOVA, as presented in Table S3.

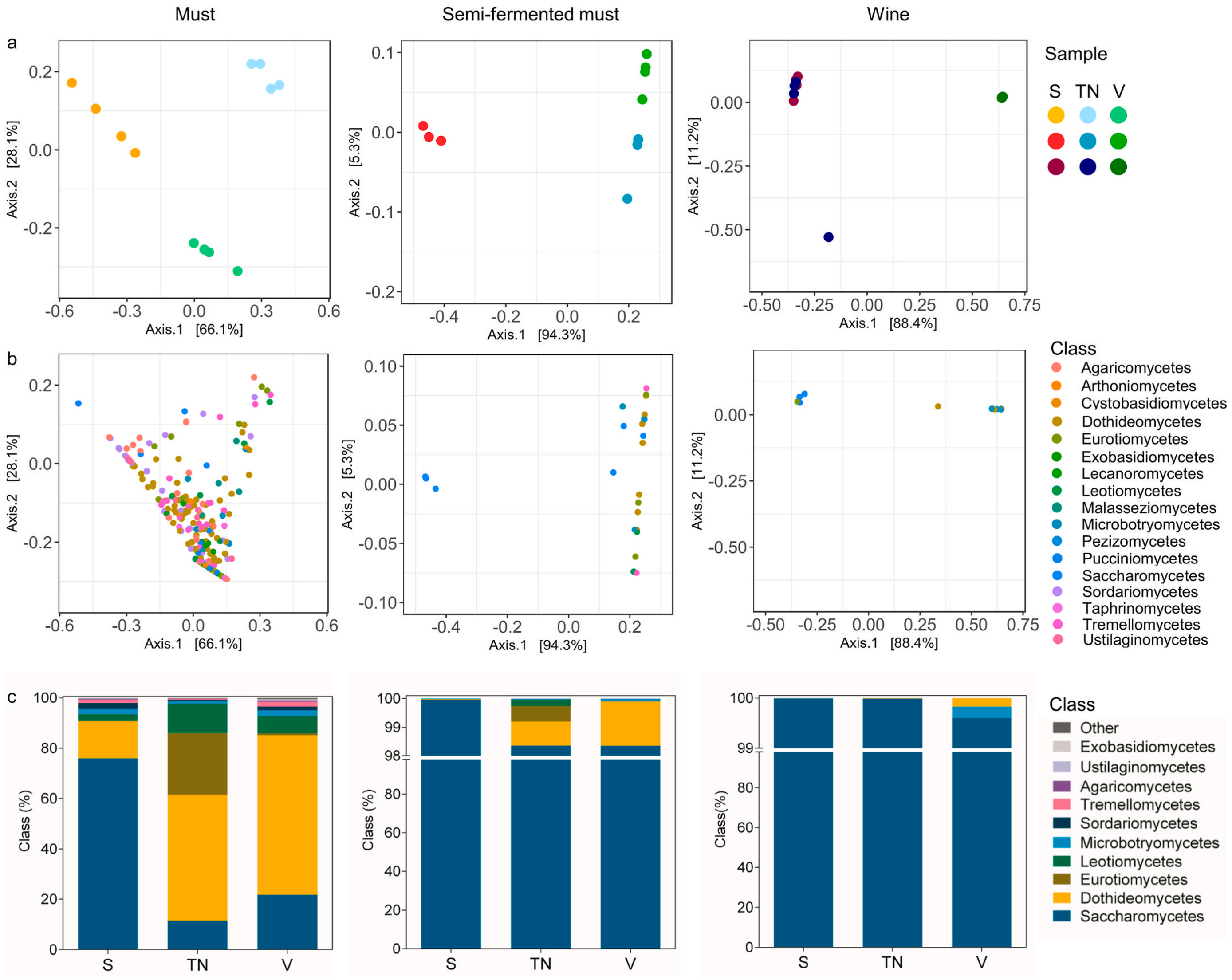

Fig. 2. Detailed distribution of fungi OTUs by class in musts, semi-fermented musts and wines from grape berries Sousão (S), Touriga Nacional (TN) and Viosinho (V). PCoA plots using Bray Curtis distance matrix coefficients are presented by sample (**a**) or by class (**b**). Barplots show the relative abundance of each class (**c**); particularly for musts, only the 10 most abundant classes are shown, and the remaining classes were placed in an artificial group designated as "Other".

ethyl esters and other miscellaneous esters were generally more abundant in wines of Sousão, unlike most linear ethyl esters, although these wines contained very high levels of ethyl decanoate (0.9 mg L^{-1}). In turn, wines of Sousão were generally the least rich in acetates, while lactones were abundant, especially t-Whiskylactone (Fig. 5, Table S4).

Wines of Touriga Nacional contained high levels of the monoterpenols linalool, geraniol and α-terpineol, while linalool acetate content was higher in wines of Viosinho (Fig. 5). Higher levels of the norisoprenoids α-ionone and β-ionone were detected in wines of Touriga Nacional than in wines of Sousão and Viosinho, respectively. In turn, β-damascenone was more abundant in wines of Sousão than of Touriga Nacional. The carbonyl compound diacetyl reached its highest levels in wines of Touriga Nacional (2.6 mg L^{-1}), while acetaldehyde was equally abundant in wines of the red cultivars (Table S4, S5). The total abundance of vanillin derivatives was higher in wines of Touriga Nacional, with ethyl vanillate and methyl vanillinate levels being significantly higher than in wines Viosinho. Additionally, syringaldehyde and acetovanillone were more abundant in wines of Sousão than in wines of the white cultivar (Fig. 5). The total abundance of phenols was also higher in wines of Touriga Nacional (Table S4), although many metabolites in this class were detected in similar levels in wines of Sousão, including 4-ethylguaiacol.

The determination of odor active values (OAV; Table S4) exposed the compounds that, in theory, contribute to the global aroma (OAV >1) and the compounds that could contribute to the global aroma through synergistic effects (OAV between 0.1 and 0.99). Noteworthy, caution was exerted with the interpretation of OAVs differences, because although they provide a first glance of the relative importance of the compounds for the aroma, having a high OAV is not necessary for, nor does it guarantee, an effect on the aroma of wine (Escudero et al., 2004). The general analysis of OAV suggested that wines of Viosinho could have weaker oxidative and lactic odors than red wines (Table S4). In turn, wines of Sousão may denote stronger fusel and green notes and cheesy odors, while citric, animal and leather odors may be more marked in wines of Touriga Nacional. Flowery, fruit and sweet odors are expected to be less prominent in these wines, but more demarcated in Viosinho and Sousão.

3.3. *Correlations between the microbiota and the wine volatiles – identification of microbial niches*

Correlation networks built from matrixes of Pearson correlation

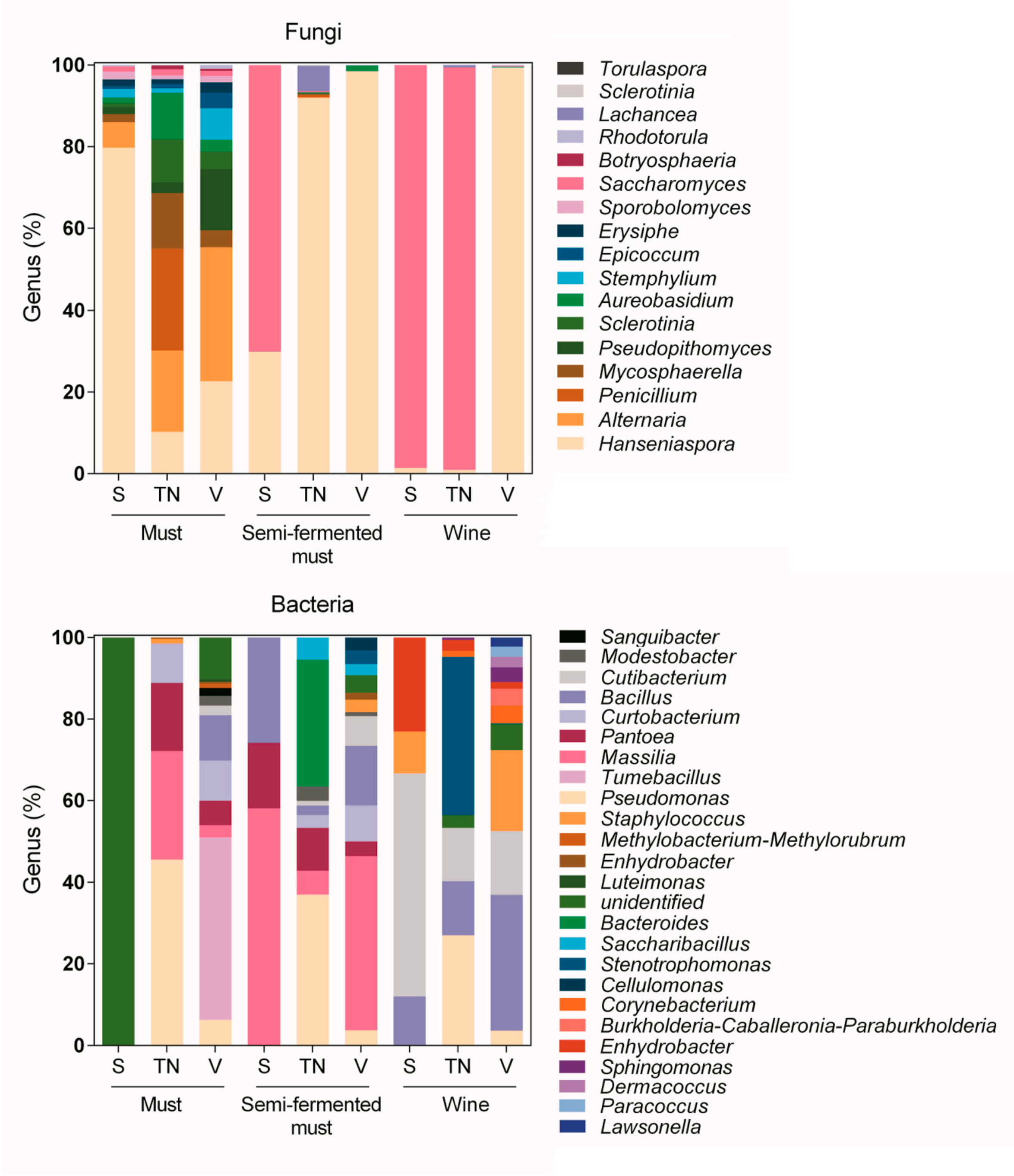

Fig. 3. Genera identity and percentage of the top 15 most abundant OTUs in musts, semi-fermented musts and wines of Sousão (S), Touriga Nacional (TN) and Viosinho (V).

coefficient values (ρ) exposed the associations between the wine volatile compounds and the fungi/bacteria OTUs detected in musts, semi-fermented musts and wines (Fig. 6, Table S6; Table S7; Table S8). A distinctively large and dense cluster characterized all networks, composed of the highest number of metabolites and OTUs. Although the composition of the OTUs could vary throughout fermentation, the metabolites in this cluster were generally maintained, and comprised 3 acids, 2 acetates, 4 phenols, 3 ethyl esters, 1 alcohol, 1 monoterpenol, 1 vanillin derivative, 1 lactone and 2 carbonyl compounds. The nature of the correlations established by these metabolites was also identical throughout fermentation; 11 correlated negatively with OTUs, namely the acids butyric, hexanoic and octanoic, acetaldehyde, vanillin, γ-nonalactone, 2 ethyl esters and 3 phenols, and 7 correlated positively, namely acetoin, c-3-hexenol, isoamyl and phenylethyl acetates, linalool acetate, ethyl propanoate and 4-ethylphenol. The metabolites in this largest cluster strongly correlated to 35 OTUs in the must, among which 27 fungi and 8 bacteria, mostly representing Dothideomycetes, Sordariomycetes and Tremellomycetes, and including the genera *Alternaria*, *Penicillium*, *Rhodotorula*, *Acremonium*, *Filobasidium*, *Vishniacozyma*, *Holtermanniella*, *Cutibacterium* and *Bacillus*. The remaining metabolites were distributed in other clusters, most of them weakly correlating to a small number of OTUs in the must (small nodes). These included 7 Dothideomycetes, 4 Gammaproteobacteria, 5 Sordariomycetes and 3 Saccharomycetes. *Hanseniaspora* integrated a cluster together with *Lachancea quebecensis*, *Alternaria angustiovoidea*, *Penicillium citrinum*, *Curtobacterium*, *Enhydrobacter* and an unidentified Erysipelotrichaceae,

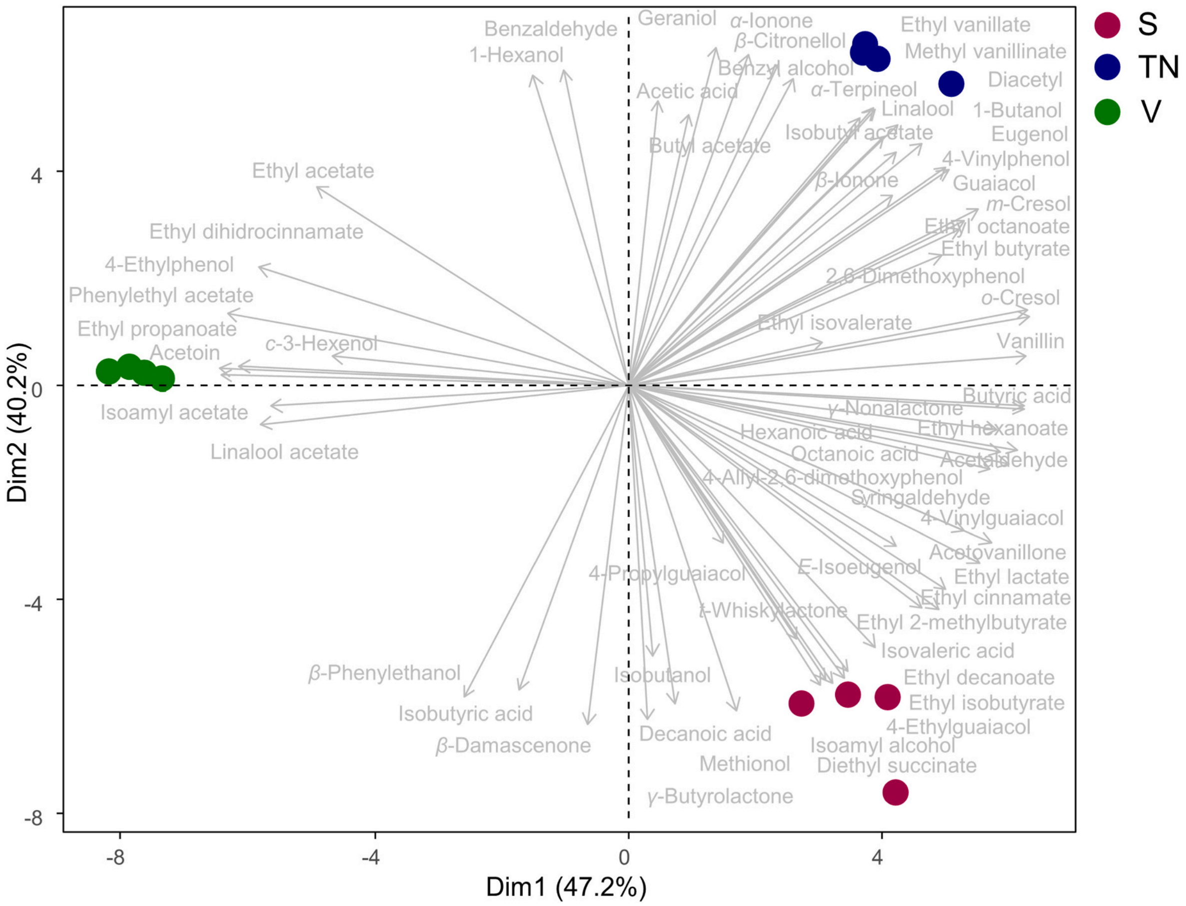

Fig. 4. Principal Component Analysis BiPlot of volatile profiles of wines Sousão (S), Touriga Nacional (TN) and Viosinho (V). For each cultivar, the 4 biological replicates in the score plot are shown with the same color, and the length of the arrows associated to each volatile compound is proportional to its contribution to the overall sample distribution. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

correlating with 11 metabolites including benzaldehyde and 1-hexanol (negative correlations), and t-whiskylactone, isoamyl alcohol, γ-butyrolactone, 2 phenols and 4 ethyl/miscellaneous esters (positive correlations). *S. cerevisiae* integrated another cluster together with *Aureobasidium*, *Sporobolomyces roseus*, *Rhodotorula babjevae*, *Filobasidium chernovii*, *Mycosphaerella tassiana, Pseudomonas* and *Massilia*, correlating positively with benzyl alcohol, acetic acid, butyl acetate, β-citronellol, geraniol and α-ionone, and negatively with β-phenylethanol, isobutyric acid and β-damascenone (Fig. 6, Table S6).

In semi-fermented musts and wines, the correlations in the largest cluster involved substantially less fungi OTUs than in musts, and more bacteria OTUs. These included *Aureobasidium*, *Alternaria* spp., *R. babjevae*, *S. roseus, Hanseniaspora clermontiae*, *H. uvarum*, *Massilia, Bacillus* and *Staphylococcus*. Several metabolites that were previously placed in secondary smaller clusters appeared now in the periphery of the largest cluster, including acetovanillone, ethyl cinnamate, ethyl acetate, ethyl lactate and ethyl-2-methylbutyrate, together with *Hanseniaspora* spp. In semi-fermented musts, secondary clusters comprised strong correlations (large nodes) with 15 fungi and 4 bacteria OTUs, including the yeasts *S. cerevisiae*, *L. quebecensis*, *Torulaspora delbrueckii*, *Meyerozyma caribbica*, and *Filobasidium wieringae*, the pathogenic fungi *M. tassiana*, *P. citrinum*, *Erysiphe necator*, *Sclerotinia sclerotiorum* and *Aspergillus* sp., and the bacteria *Pantoea*, *Pseudomonas,* and *Bacteroides acidifaciens* (Fig. 6, Table S7). Together with *Pseudomonas* and *Bacteroides*, the filamentous fungi and *Lachancea* integrated a cluster with negative correlations with isobutyric acid and β-phenylethanol, and with positive correlations with 3 monoterpenols including linalool, 2 alcohols including benzyl alcohol, α- and β-ionone, ethyl-vanillate, methyl-vanillinate, diacetyl and isobutyl acetate. In turn, *S. cerevisiae, T. delbrueckii* and *M. caribbica* integrated another cluster, negatively correlating with 1-hexanol and benzaldehyde, and positively correlating with γ-butyrolactone, t-whiskylactone, isoamyl alcohol, ethyl decanoate and isobutyrate, diethyl succinate, isovaleric acid and 2 phenols. Many OTUs that figured as protagonists in the network of semi-fermented musts maintained their relevance in wines. These included *T. delbrueckii, L. quebecensis, P. citrinum, Pseudomonas and B. acidifaciens* which still established most of their correlations with the same metabolites as in semi-fermented musts. Additionally, *Pantoea*, *Stenotrophomonas* and *Enhydrobacter* integrated the cluster populated by *L. quebecensis, P. citrinum and B. acidifaciens, while S. cerevisiae* integrated the largest cluster (Fig. 6, Table S8).

4. Discussion

4.1. The Hanseniaspora-Saccharomyces succession timing is cultivar-dependent

Although skin contact time with the musts and maceration are known to influence the microbial consortium of the musts (Guzzon, Malacarne, Larcher, Franciosi, & Toffanin, 2020; Englezos et al., 2022), these factors did not seem to be preponderant in the present study for the selected cultivars, as musts of the white cultivar contained the highest microbial diversity. The influence of grape berry metabolites over its microbial residents likely explains this result, as direct correlations were found between the levels of amino acids, phenolics and waxes and the microorganisms present at the fruit surface (Martins et al., 2023). For

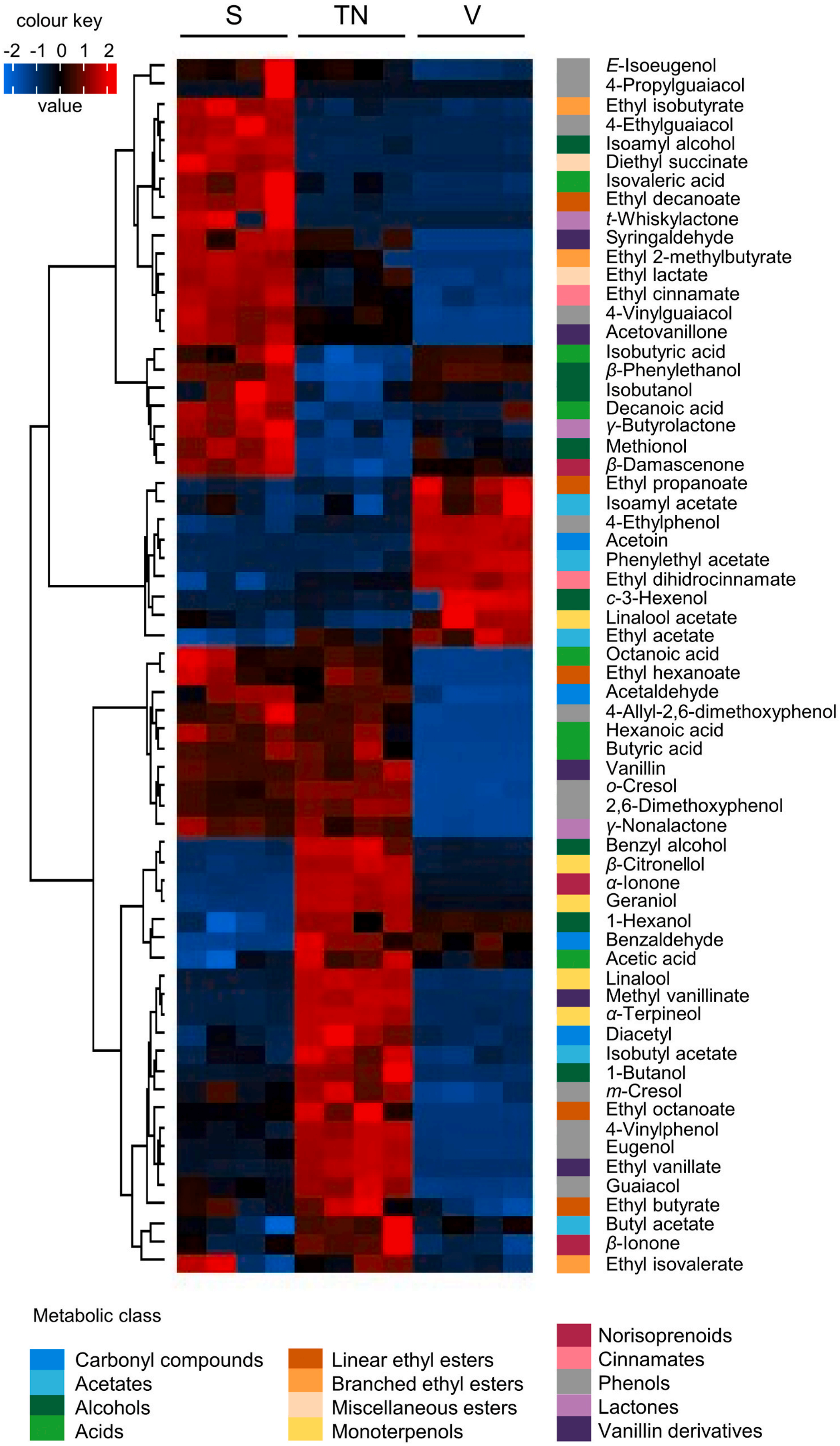

Fig. 5. Heatmap of the volatile compounds quantified in wines Sousão (S), Touriga Nacional (TN) and Viosinho (V). Values were centred and scaled in the row direction to form virtual colors as presented in the color key. Each row represents a metabolite, colored according to its metabolic class. For each cultivar, 4 columns corresponding to the 4 biological replicates are presented. Hierarchical clustering was performed in the row direction to group metabolites showing similar profiles. Statistically significant differences between cultivars following Dunn's test are detailed in Table S5. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

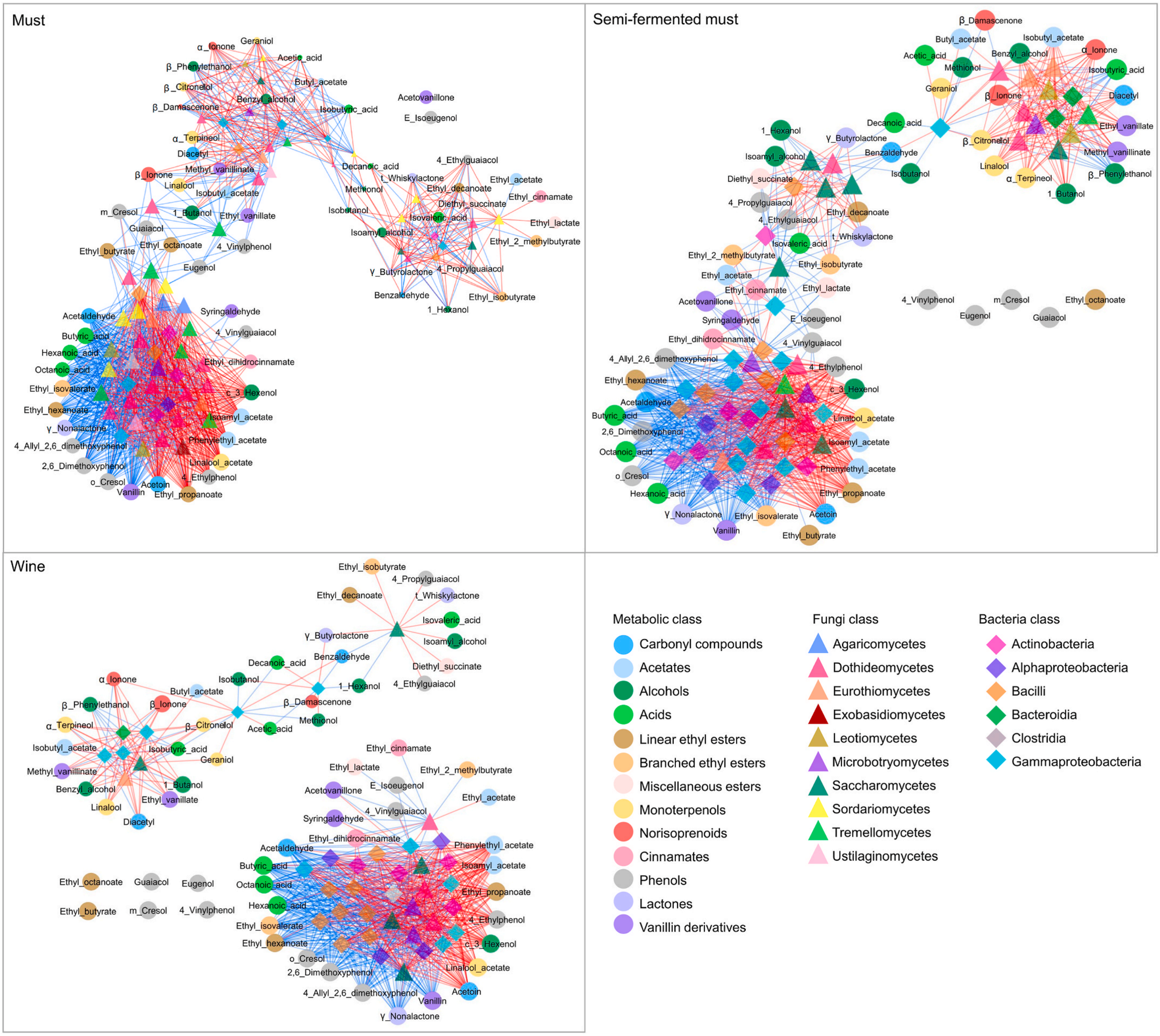

Fig. 6. Correlation networks of wine metabolites and OTUs of musts, semi-fermented musts or wines of Sousão, Touriga Nacional and Viosinho. Each node represents a metabolite (circle), a fungi OTU (triangle) or a bacteria OTU (diamond), and is colored according to the corresponding metabolic or taxonomic class. Lines joining the nodes represent correlations with $\rho>|0.95|$; positive correlations are shown in red, while negative correlations are shown in blue. Node size is proportional to the correlation node strength ($ns = |\rho|$). For simplification purposes, only the top 50 most abundant fungi OTUs were considered in the assembly of the network of the must. For more details, see Tables S6, S7 and S8. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

example, berries of Viosinho contained the highest levels of yeast-assimilate amino acids like phenylalanine, asparagine and alanine, that correlated positively with the majority of microbial OTUs. In turn, fruits of the red cultivars contained higher levels of specific phenolics and wax-related compounds with antimicrobial properties, in line with their lower microbial alpha diversity (Martins et al., 2023).

In the present study, the initial abundance of *S. cerevisiae* and *Hanseniaspora* populations in the musts did not influence how they evolved during fermentation, i.e. their ability to dominate the microbial consortium throughout the fermentation process. Rather, this was dictated by the fermentation conditions and, likely, by strain types that best adapted to them (Blanco et al., 2006). Indeed, *Hanseniaspora* dominated the semi-fermented musts of Touriga Nacional, suggesting that the strains present in this cultivar are more resistant to alcohol than the ones present in Sousão, while the *S. cerevisiae* strains thrived later in the fermentation process, becoming only rapidly dominant towards the end of fermentation when alcohol levels surpassed 7%. Accordingly, strain-associated ecological interactions besides physico-chemical conditions were shown to be dominant factors in the contribution of each species to the outcome of the fermentation (Bagheri et al., 2020). These effects thus likely translate the differences in the timing of *Hanseniaspora-Saccharomyces* succession in fermentations of the red cultivars addressed in the present study. In addition to the contribution of the different strains of *Hanseniaspora* and *Saccharomyces* present in the must, the fermentation temperature also seems to have a marked impact on the *Hanseniaspora-Saccharomyces* succession. Indeed, at lower fermentation temperatures used for the white cultivar, the presence of non-*Saccharomyces* yeasts in the beginning of alcoholic fermentation hampered the growth of *S. cerevisiae* yeasts, possibly by consuming a significant portion of the nitrogen and oxygen from the must (Liu et al., 2017). Accordingly, the increased thermotolerance of *Hanseniaspora* spp. was demonstrated, as well as the ability of these non-*Saccharomyces* yeasts produce significant alcohol levels of up to 11% (Jolly et al., 2003; Mančić et al., 2022). Research focused on cultivar-specific strain succession during fermentation will further elucidate the contribution of different genotypes to wine aroma.

4.2. The diversity of fungi but not of bacteria decreases throughout fermentation

Results in this study showed that cultivars could be distinguished by the relative abundance of key microbial taxa present in musts. Dothideomycetes, Saccharomycetes and Eurotiomycetes took the leading role, mostly represented by *Alternaria*, *Hanseniaspora* and *Penicillium*, while *Cladosporium*, *Aureobasidium*, *Vishniacozyma* and *Botryotinia* were predominant, besides *Hanseniaspora*, in other cultivars (Böhmer et al., 2020; Bokulich et al., 2016; Chen et al., 2020; Chen et al., 2022; Liu et al., 2021). Besides the cultivar, the vineyard management regime also influences the berry fungi diversity, and previous studies showed an exclusive detection of *S. cerevisiae* in conventional grapes, and of *Penicillium* in organic grapes (Perpetuini et al., 2022; Rossetti et al., 2023).

The observed decrease in fungi population diversity throughout fermentation is well documented, as most environmental fungi cannot survive the severe fermentation conditions characterized by high osmotic pressure, increased temperature due to yeast activity and high production of ethanol (Chen et al., 2022; Englezos et al., 2022; Liu et al., 2017; Liu et al., 2021; Mezzasalma et al., 2017). Nonetheless, the overall abundance of the microbial population increases, as resistant species prosper in this environment. The dominance of Saccharomycetes, especially *S. cerevisiae,* in wines of the red cultivars is in agreement with previous studies, and probably associated to its well reported resistance to ethanol (Böhmer et al., 2020; Bučková et al., 2018; Garofalo et al., 2016; Li et al., 2018). However, despite *S. cerevisiae* being envisaged as the lead fermentative species, results in the present study showed that non-*Saccharomyces* yeasts like *Lachancea* and *Torulaspora* are also detected at the end of fermentation, complementing previous studies (Bagheri et al., 2020; Böhmer et al., 2020; Bučková et al., 2018; Li et al., 2018; Portillo & Mas, 2016; Stefanini et al., 2016).

In the present study, the fermentation process had higher impact on the diversity of fungi populations than of bacterial communities, although the dominant bacteria taxa varied from musts to wines, in line with previous reports (Böhmer et al., 2020; Mezzasalma et al., 2017). *Pseudomonas*, *Tumebacillus*, *Massilia* and *Pantoea* prevailed in the musts in the present study, the former also dominating musts of Vidal, followed by *Sphingobium*, *Sphingomonas* and *Massilia* (Chen et al., 2020), while studies in other cultivars like Pinot blanc and Cabernet Sauvignon reported the high abundance of *Lactococcus*, *Bacillus* and *Acetobacter*, besides *Pseudomonas* (Böhmer et al., 2020; Bokulich et al., 2016; Bučková et al., 2018; Portillo & Mas, 2016). The abundance of *Pseudomonas* decreased throughout fermentation in the present study, while *Bacilli* and *Staphylococcus* became generally more represented, in analogy to studies in other cultivars (Chen et al., 2020; Mezzasalma et al., 2017).

4.3. Three major niches of yeasts, environmental fungi and bacteria co-correlate with the richness of wine metabolites

Although varied microbial interactions are believed to improve wine complexity (Sirén et al., 2019), results in the present study showed that increased microbial biodiversity in the must does not necessarily associate with a more varied assortment of aroma compounds in wine. The cultivar effect was largely evident both in metabolites linked to varietal and fermentative aromas. The high abundance of acetates in Viosinho is in line with the expected outcome of white wine fermentations, while the high content of terpenols in Touriga Nacional is likely a varietal trait (Petronilho et al., 2020). The higher occurrence of terpenols in wines is related to the β-glucosidase activity of yeasts associated to the grapes, which can release glycosidically bound grape-derived terpenes, thereby contributing to varietal aroma in wines (Rossetti et al., 2023). The concentration of other compounds in wine such as β-damascenone is also linked to the prevalence of fungi genera with an enzymatic reservoir capable of converting grape precursors (such as C13-norisoprenoid glycosides derived from carotenoid metabolism) to odor active compounds during fermentation, by acid- or yeast-mediated hydrolysis (Parker et al., 2017; Rossetti et al., 2023).

The 18 metabolites that mostly distinguished the profile of wines of Viosinho from wines of the red cultivars composed the densest cluster of metabolite-OTU correlations. These reflected connections with many environmental fungi including *Aureobasidium* and *Alternaria* spp., respiratory yeasts including *Rhodotorula babjevae*, *Sporobolomyces roseus*, *Holtermanniella* and *Filobasidium* spp., a diversity of bacteria taxa including *Massilia*, *Bacillus*, *Staphylococcus* and *Cutibacterium*, and the weakly fermentative yeast *Hanseniaspora* spp. Positive correlations between these taxa and 7 metabolites (acetoine, isoamyl acetate, ethyl propanoate, c-3-hexenol, phenylethyl acetate, 4-ethylphenol) may reflect several types of contribution, including direct production by the microorganism, synergistic/antagonistic relationship with the microorganism that produces the metabolite, or ability of the microorganism to render substrates more accessible to other microorganisms that will convert them, for instance, through the production of enzymes that modify grape integrity and enhance the release of aroma-active compounds (Bozoudi & Tsaltas, 2018; Di Francesco et al., 2015; Oluwa, 2020; Zhu et al., 2017). *Hanseniaspora* spp. are considered as high producers of esters (Mančić et al., 2022) and acetoin (Ciani & Maccarelli, 1997), correlating positively with ethyl propanoate, ethyl acetate and acetoin in the present study, but not with several others esters, among which ethyl butyrate, in line with reports in wines of Petit Verdot (Chen et al., 2022). The positive correlation of *Hanseniaspora* with c-3-hexenol complements previous studies in these wines, while the negative correlation with 1-hexanol is in line with reports in Vidal (Chen et al., 2020).

Besides *Hanseniaspora*, some species of *Aureobasidium*, *Rhodotorula* and *Sporobolomyces* are capable of producing glucosidases, pectinases, proteases and lipases (Bozoudi & Tsaltas, 2018; Kot et al., 2016; Kot et al., 2021; Mančić et al., 2022), further denoting their contribution to aroma development in wine. Accordingly, results in the present study showed positive correlations of *Aureobasidium, R. babjevae* and *S. roseus* to an array of volatile compounds likely associated to regional wine aroma, complementing previous studies linking them to the production of ethyl octanoate, isobutyl acetate, diethyl succinate and various alcohols not detected in the present study (Bozoudi & Tsaltas, 2018; Liu et al., 2021). In turn, species of *Alternaria* were shown to antagonize the growth of other fungi in grapes, while producing toxins which could compromise the performance of other microorganisms during fermentation (Musetti et al., 2006; Prendes et al., 2015). This taxon correlated with over 25 wine metabolites in the present study, including esters, phenols, vanillin derivatives, alcohols, acetates, carbonyl compounds, monoterpenols and acids, complementing previous reports predicting associations with citronellol, ethyl acetate, decanoic acid, isoamyl acetate, phenylethyl acetate and β-damascenone (Chen et al., 2020; Liu et al., 2021). The yeast-bacteria compatibility for wine making is a topic of increasing interest, and the presence of *Massilia*, *Bacillus*, *Staphylococcus* and *Cutibacterium* in the densest metabolite-OTU cluster suggests that their contribution to wine aroma deserves further investigation. Novel metabolite-OTU correlations comprising these genera complement studies in wines Petit Verdot, showing correlations of *Massilia* with isoamyl alcohol and 2-heptanol, and of *Bacillus* with a vast array of alcohols, acetates, ethyl esters and acids (Chen et al., 2022), most not detected in wines in this study.

In the present study, the fermentative yeasts *S. cerevisiae*, *L. quebecensis, T. delbrueckii* and *M. caribbica* populated secondary clusters in the metabolite-OTU networks, translating specificities of the fermentations of the red cultivars. In musts, the strength of these correlations was particularly weak, as these pitching yeasts were still marginally detected. However, as fermentation progressed, correlations became stronger, thus suggesting that the semi-fermented stage is the most feasible for predicting volatile profiles mediated by mildly and strongly fermentative yeasts. Furthermore, it is pivotal to stress that at each fermentation stage, *S. cerevisiae* correlated with different wine volatiles, highlighting the contribution of distinct strains in succession to the final volatile profile, as discussed previously. The positive correlations of *S. cerevisiae, T. delbrueckii* and *M. caribbica* with γ-butyrolactone, t-whiskylactone, isoamyl alcohol, ethyl decanoate, ethyl isobutyrate, diethyl succinate, isovaleric acid, 4-propylguaiacol and 4-ethylguaiacol complement previous reports in wines of Pinot Noir, Chardonnay, Vidal and Petit Verdot, showing associations of the first two yeasts with several esters, acetates and alcohols, among which isoamyl, ethyl, pentyl and hexyl acetates and ethyl lactate, hexanoate and octanoate (Chen et al., 2020; Chen et al., 2022; Liu et al., 2021). Additionally, the negative correlation of *S. cerevisiae* with 1-hexanol is in line with the study in Petit Verdot (Chen et al., 2022). The ability of *Meyerozyma* spp. to produce β-glucosidases further supports its beneficial effect in varietal aroma (Silva et al., 2005; Liu et al., 2021). In the present study, monoterpenols and norisoprenoids correlated with a few OTUs in secondary clusters, among which *L. quebecensis*, highlighting the role of specific taxa on their release from the fruit matrix. Correlations of this species with benzyl alcohol, diacetyl, isobutyl acetate, ethyl-vanillate and methyl vanillinate, isobutyric acid and β-phenylethanol add novelty to previous reports showing modulation of wine alcohols, acids and acetates by *Lachancea* spp. in mixed fermentations with *S. cerevisiae* (Benito et al., 2016; Fairbairn et al., 2021; Gobbi et al., 2013; Porter et al., 2019). In the present study, many metabolites correlating with *Lachancea* also shared associations with pathogenic fungi and bacteria, likely resulting from direct interactions amongst the microorganisms, and also from the enzymatic activity and ability to form hyphae or pseudohyphae that modify grape berry integrity and modulate nutrient availability (Barata et al., 2012). Accordingly, *Pseudomonas* spp. were shown to inhibit the mycelial growth of *Aspergillus niger* and *Penicillium expansum*, triggering grapevine immune responses against infection, together with *Pantoea* (Andreolli et al., 2019; Aziz et al., 2016; Gruau et al., 2015). Additionally, *Pseudomonas* has been shown to associate with the formation of aroma compounds including ethyl caprylate (Wang et al., 2022), while *Penicillium* correlated with 15 volatile compounds in wines of Vidal (Chen et al., 2020), *Pantoea* correlated with 5 volatiles in wine of Petit Verdot (Chen et al., 2022) and *Mycosphaerella tassiana* correlated with 8 metabolites in wines of Pinot Noir and Chardonnay (Liu et al., 2021).

4.4. Conclusion

The present study elucidated the contribution of native fungi and bacteria communities to the evolution of fermentation and volatile profiles of wines of the DOC Douro region. Results exposed major microbial niches that can be useful when seeking predictions of the relative abundance of wine metabolites, comprising environmental fungi highly abundant in musts (*Aureobasidium, Alternaria, Penicillium, Erysiphe, Sclerotinia, Mycosphaerella*), respiratory yeasts (*R. babjevae, S. roseus, Holtermanniella, Filobasidium*), weakly fermentative yeasts (*Hanseniaspora*), mildly and strongly fermentative yeasts (*S. cerevisiae, L. quebecensis, T. delbrueckii* and *M. caribbica*) and bacteria (*Pseudomonas, Massilia, Bacillus, Staphylococcus, Cutibacterium, Bacteroides, Pantoea, Stenotrophomonas, Enhydrobacter*). Novel metabolite-microbiota correlations denote cultivar specificities that likely underlie regional aromatic signatures that deserve further investigation. While OAV values provided an indication of the expected odor of the wines, further sensory evaluation will elucidate the specific contribution of the different species of the microbial consortium to the wine aroma.

Funding

This work was supported by the "Contrato-Programa" UIDB/04050/2020 (DOI 10.54499/UIDB/04050/2020) and by DOI 10.54499/DL57/2016/CP1377/CT0027 to VM funded by portuguese national funds through the FCT I.P. The work was also supported by FCT, CCDR-N (Norte Portugal Regional Coordination and Development Commission) and European Funds (FEDER/POCI/COMPETE2020) through the projects GrapeMicrobiota (PTDC/BAA-AGR/2691/2020) and AgriFood XXI (NORTE-01-0145-FEDER-000041). This work benefited from the networking activities within the CoLAB VINES & WINES and the COST Action CA 17111 INTEGRAPE. AT was supported by an Auxiliary researcher contract/position within the project "Fleurs locales" (SOE4/P5/F1011). LAAE acknowledges the continuous support of Diputación General de Aragón (T29) and European Social Fund.

CRediT authorship contribution statement

Viviana Martins: Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Writing – original draft, Writing – review & editing. **Ricardo López:** Formal analysis, Funding acquisition, Investigation, Methodology, Resources, Writing – review & editing. **António Teixeira:** Formal analysis, Methodology, Writing – review & editing. **Hernâni Gerós:** Conceptualization, Funding acquisition, Resources, Writing – review & editing.

Declaration of competing interest

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

Data availability

Data will be made available on request.

Acknowledgements

The authors would like to acknowledge CoLAB VINES&WINES: ADVID - Association for the Development of Viticulture in the Douro Region, and Sogevinus Fine Wines S.A. for networking activities and for providing the experimental vineyards used in this study. In particular, the authors also thank Catarina Barbosa (CoLAB VINES&WINES, ADVID) and Márcio Nóbrega (Sogevinus Fine Wines, S.A.) for their assistance in the field.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2024.115769.

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