Subregional pedoclimatic conditions with contrasted UV-radiation shape host-microbiome and metabolome phenotypes in the grape berry

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ARTICLE INFO

ABSTRACT

This study used integrative omics to address the response of key elements of the grapevine holobiont to contrasted pedoclimatic conditions found in distinct subregions of Douro Valley (Portugal). A metabolic OPLS-DA model predicted with 100 % accuracy the geographic origin of berries; higher UV radiation, higher temperature and lower precipitation stimulated the accumulation of phenolic acids, flavonols and malvidin conjugates, in detriment of amino acids, organic acids, flavan-3-ols, proanthocyanidins and non-malvidin anthocyanins. Metabarcoding showed a trade-off between bacteria and fungal diversity among subregions, with *Pseudomonas*, *Lactobacillus*, *Aspergillus* and *Penicillium* acting as intraregional microbial markers. The high phenotypic plasticity of berries and the role of microbes in this process are relevant upon current projections for increased UV radiation and temperature in Southern European viticulture, in a climate change scenario, with predicted impacts on regional wine quality and on the development of adaptation strategies for resilient viticulture.

1. Introduction

The characteristics of a wine region shape grape berry and wine sensory properties, and the terroir is envisioned as an interactive ecosystem that gathers physical factors such as climate (temperature, humidity, pluviometry, sunshine, wind) and topography (parcel orientation, altitude, slope), soil (composition, granulometry), biological elements (grape cultivar and microbiota), and human intervention through viticultural and oenological practices (Anesi et al., 2015; Belda et al., 2017; Bokulich et al., 2014). The supra-organism constituted by the grapevine and its microbial communities is known as a holobiont, considered as a "functional whole" composed of plant-microorganism interactions that define plant fitness (Bettenfeld et al., 2022). The existence of biogeographical assemblages of grape-surface microbiota is broadly acknowledged, although the impact of the climate, cultivar and vine management practices on the structure of microbial communities is far from being fully understood (Bokulich et al., 2014; Martins et al., 2023). Geographical delineations shown for vineyard yeasts suggest that grape-associated microbial biogeography is nonrandomly associated with regional, varietal, and climatic factors (Cheng et al., 2020; Gao

et al., 2019; Knight et al., 2020). Farmers currently take advantage of these factors, for instance, by employing practices of canopy management, such as reducing canopy density, to alter the microclimate of the fruiting zone in an attempt to positively shape the microbial consortium and reduce mold growth (Pugliese et al., 2011). In cultivars managed under the same regime within a wine farm, a cultivar signature was also shown to be strongly imprinted in the berry microbiota, with only 56 % of fungi Operational Taxonomic Units (OTUs) and 34 % of bacteria OTUs being ubiquitously detected (Martins et al., 2023). This effect was partly attributed to the distinct metabolic composition of the fruits, with over 7000 metabolite-microbiota correlations suggesting a host genotype mediated selection of specific taxa in the carposphere (Griggs et al., 2021; Martins et al., 2023). Many of these metabolites are microbial growth promotors or present antimicrobial activity, modulating their vegetative and sexual cycles, besides the microbe-microbe interactions (Griggs et al., 2021). Other factors including phytochemical treatments like fungicides, fertilizers and biostimulants add another level of complexity to this system, by shaping not only the microbiota, but also the grape berry metabolome (Martins et al., 2021).

In current viticulture, cultivars are distinguished by their phenotypic
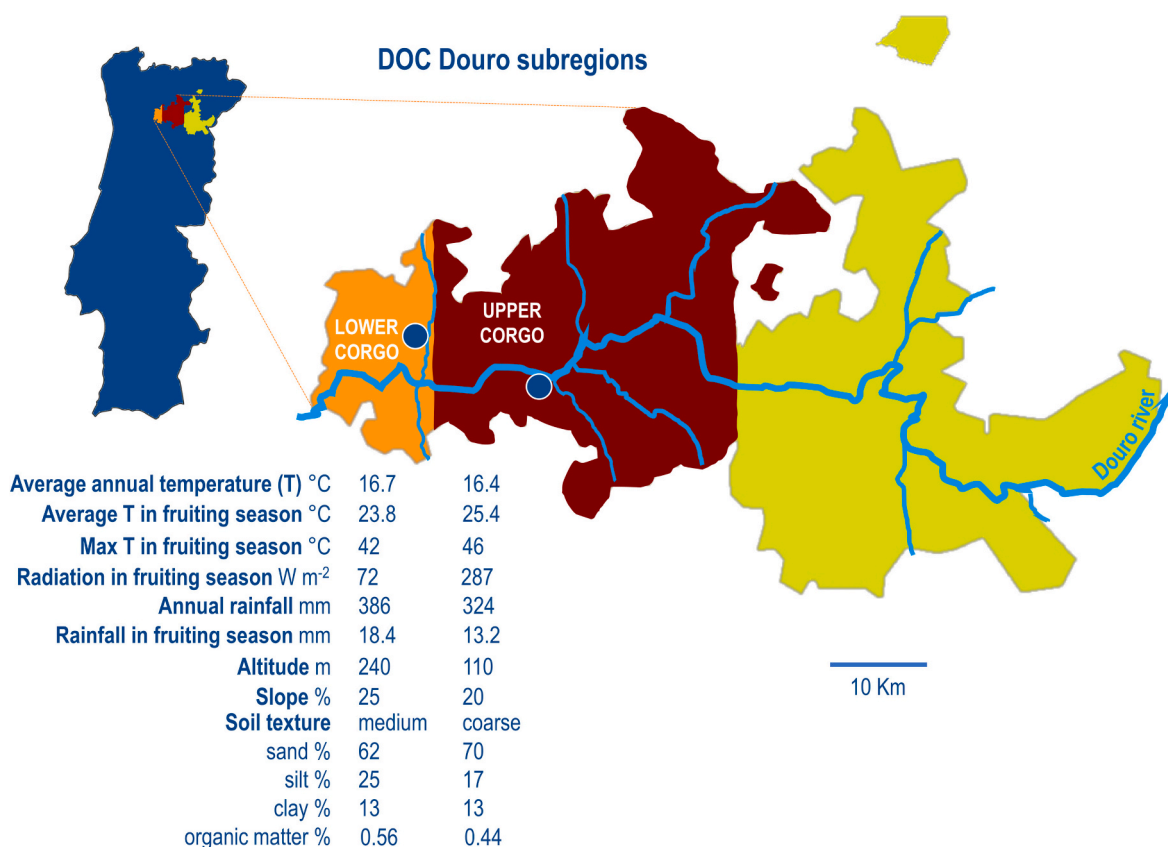
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Fig. 1. Characterization of the vineyard sites in DOC Douro subregions, separated by Corgo River, one of the tributaries of the Douro River: Lower Corgo (orange) and Upper Corgo (maroon). Annual values refer to the year of 2022 and values relative to the fruiting season are indicative of data collected from June to August 2022, at each farm locality. Mapping, climate data, topography and soil features of each farm were retrieved from information provided by Sogevinus S.A. (sogevinus .com), Douro Museum (museudodouro.pt) and Portuguese Institute for Sea and Atmosphere (ipma.pt). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

traits and adaptability to certain environments, associated to a genetic fingerprint that allows clonal variation (Griggs et al., 2021). Although advantageous upon a climate change scenario, the plasticity of the epigenome under different climates introduces bias in the systems used for cultivar identification (Anesi et al., 2015, Fabres et al., 2017, Antolín et al., 2020). One example of this effect was reported for Sousão, a highly reputed autochthonous Portuguese cultivar that is amply cultivated under the alias Vinhão in the humid wine region of Vinhos Verdes, but known as Sousão in Douro wine region, where it was first introduced for intensifying the color of Port wines (Soares et al., 2023). Despite showing substantial lower vigour in the dry climate of Douro region, it is the most used Portuguese cultivar for improving wine color quality and phenolic content, attributing pleasant raspberry and blackberry aromas (Soares et al., 2023).

Studies at subregional scale have been very useful to ascertain phenotypic variations in specific cultivars, particularly on grape amino acids, varietal thiols, phenolics and organic acids (Baleiras-Couto et al., 2023; Rienth et al., 2021), anthocyanins and flavonoid contents (Lemos et al., 2020), and non-volatile or volatile compounds in the berry, coupled to transcriptomics (Anesi et al., 2015; Wang et al., 2024), besides on berry surface fungi and bacteria (Chen et al., 2023; Gao et al., 2019; Portillo et al., 2016) and on soil bacteria (Zhou et al., 2021). However, integrative studies addressing the impact of subregional cues on a combination of berry quality traits are still very scarce, and focused, to the best of our knowledge, on *S. cerevisiae* populations coupled to grape phenolics (Cheng et al., 2020). The present study thus employed an integrative approach to unravel the combined effects of subregional environmental factors and topography on the plasticity of grape berry biochemical parameters, metabolic profile and surface microbial

communities, hypothesizing substantial intraregional variations on fruit quality traits. These studies are particularly relevant in the mountainous Douro region, among the oldest regulated wine regions in the world, characterized by hot and dry summers over a dryland regime, where climate change impacts are predicted to be site-specific and very heterogeneous (Prata-Sena et al., 2018; Santos et al., 2020). The region is subdivided into three subregions, characterized by intense seasonal variations, of which Lower Corgo is the westernmost subregion characterized by higher humidity and lower temperatures, while Upper Corgo is the central subregion, drier and with higher temperatures than Lower Corgo (Prata-Sena et al., 2018, Santos et al., 2020). Douro Superior is the subregion with most extreme weather conditions, where intense heat and water scarcity greatly challenge plant fitness and berry development (Prata-Sena et al., 2018, Santos et al., 2020). The present study focused on comparing the yet unexplored subtleties of the neighboring subregions, Lower and Upper Corgo, to uncover nuanced terroir-related phenotypic responses of the grape carposphere. Vines cv. Sousão of identical clones, rootstocks and age were selected within the same vintage and within a 14 km radius, to rule out, as much as possible, the influence of these factors, and focus on the plasticity of the vine holobiont at each subregional terroir.

## 2. Material and methods

2.1. Grape berry sampling

Forty bunches of grape berries at harvest-ripe stage (20°Brix, *E*-L 38; Coombe, 1987, Tornielli et al., 2023) were randomly collected from grapevines cv. Sousão in August 2022 from farms owned by the
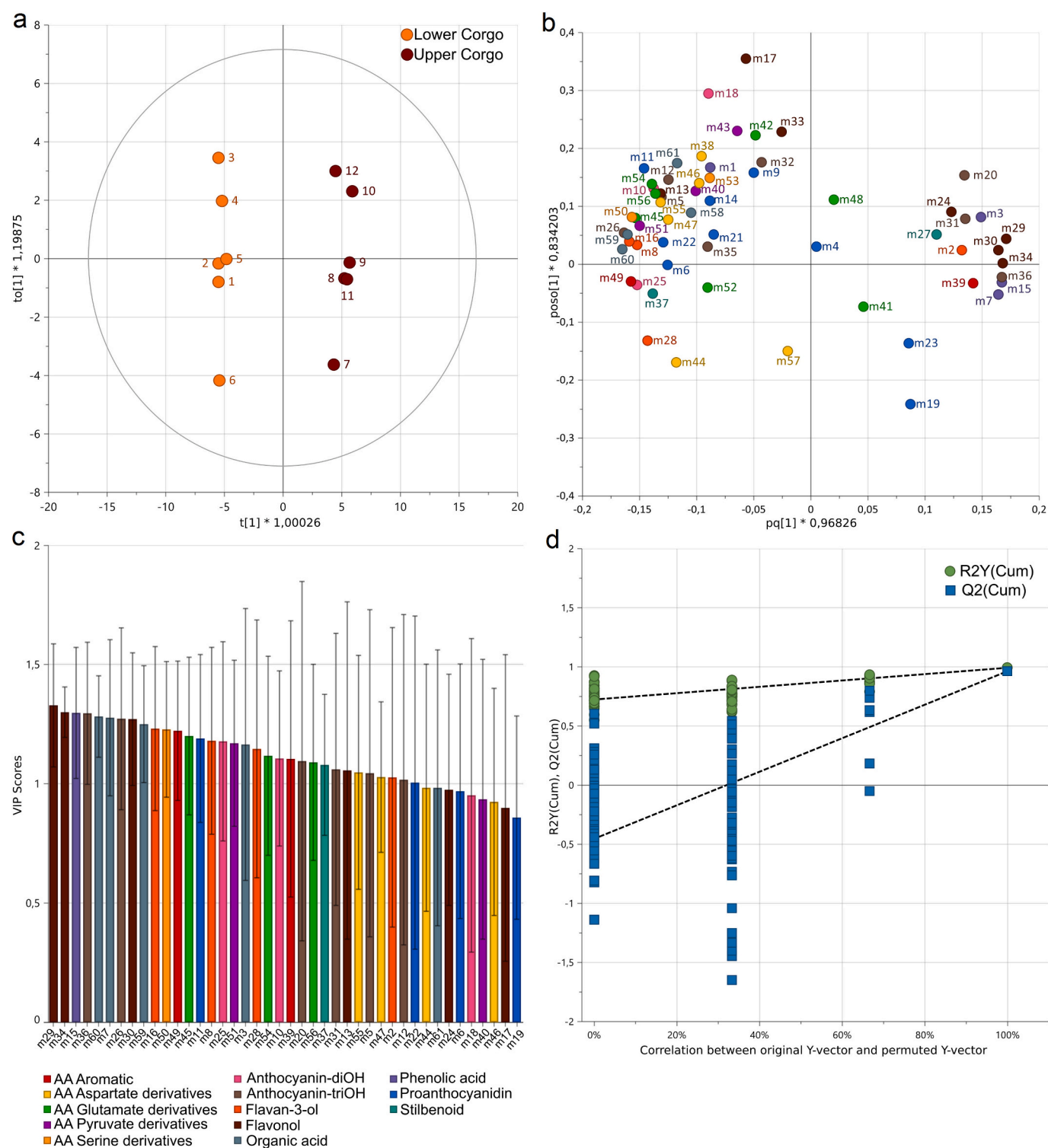
Fig. 2. Supervised classification using OPLS-DA with "DOC Douro subregions, Lower Corgo and Upper Corgo" as discriminant variable on metabolomic data of grape berries cv. Sousão (**a**). Loading plot (**b**). VIP-scores (**c**). Validation plot of 100 permutation tests for OPLS-DA model built for grape berry extracts (**d**). Numbers indicate the ID of the compounds as given in **Table S2** and colors indicate the metabolic class.

company Sogevinus, located in the North of Portugal, in two distinct subregions of Douro wine region: Upper Corgo (Quinta de S. Luiz, GPS coordinates: 41° 09′25.7″ N. 7° 36′ 57.5″ W) and Lower Corgo (Quinta do Bairro, GPS coordinates: 41° 11′ 55.4″ N. 7° 46′ 17.1″ W). In both farms, separated by 14 km, vines with identical rootstock, clone and age (R110//10103P; 7/8 years old) grew in a single Guyot training system with a northeast parcel orientation, on schist and greywacke complex

soil, and under a dryland regime. The two subregions are characterized by specific microclimates (Fig. 1). Briefly, Lower Corgo has higher rainfall and lower summer temperatures and solar radiation than Upper Corgo. The altitude and slope where the vines grew were also considerably higher in Lower Corgo, as well as soil silt content and organic matter, while soil sand content was lower. Identical phytosanitary treatments were applied in both vineyards respecting the suppliers'
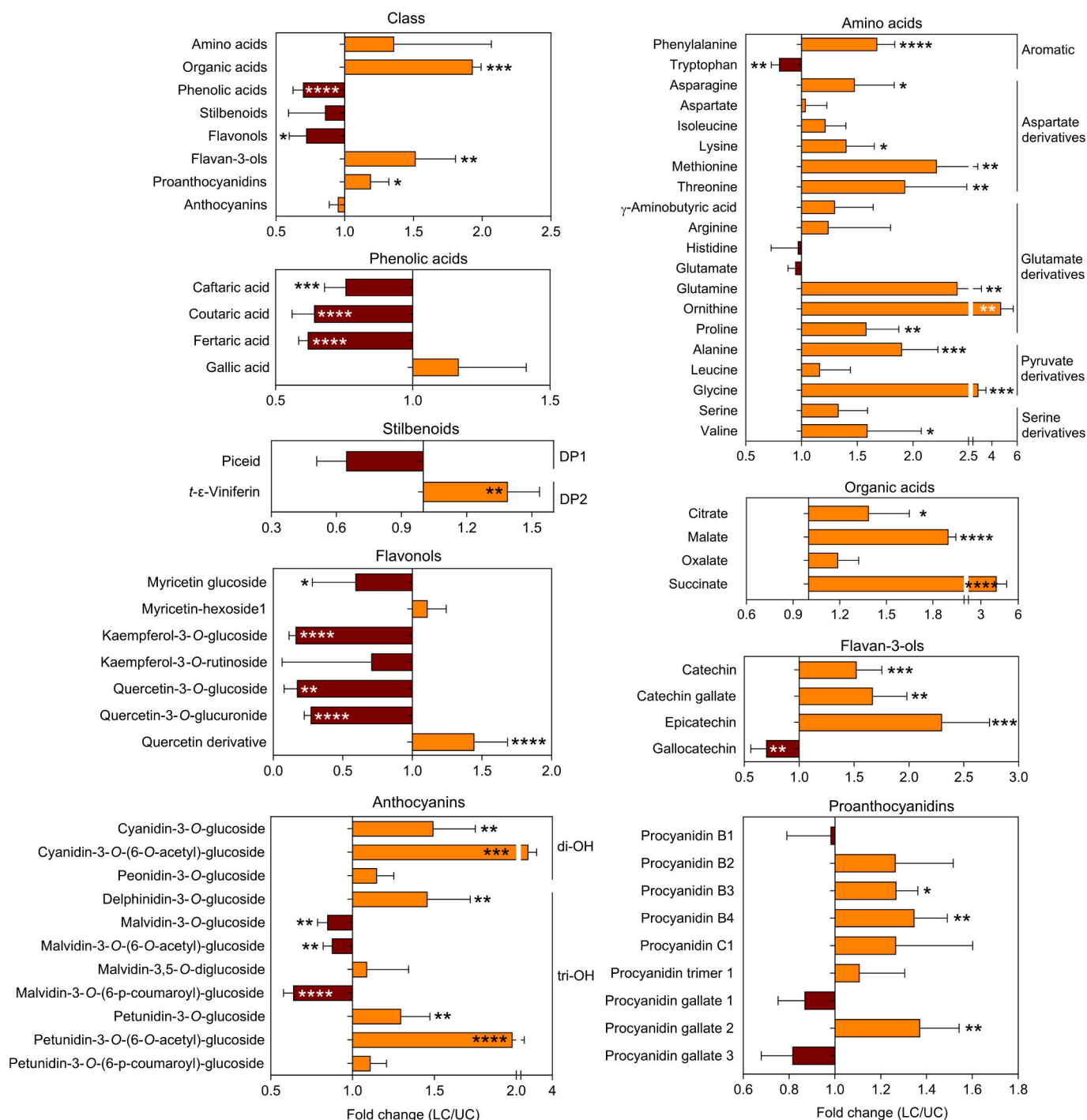
Fig. 3. Subregional metabolite signature of grape berries cv. Sousão. Results are expressed as fold change of the mean ± SD values obtained for fruits of Lower Corgo (LC) relative to Upper Corgo (UC), and asterisks indicate statistical differences: *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$; ****$P \leq 0.0001$; $n = 6$.

instructions, but with higher intensity in Lower Corgo, where disease pressure is generally higher. These comprised a combination of fungicides targeted at mildew, oidium and black rot, including a penetrating fungicide (Cupertine Super, IQV Agro Portugal S.A.) and systemic fungicides Luna Experience (Bayer), Ksar (Ascenza) and Sercadis (BASF Agricultural Solutions Portugal). The vineyards in Lower Corgo also received a single application of an insecticide targeted at cicadelids. All treatments were performed 1–3 months before harvest, respecting the safety intervals stipulated by the Portuguese legislation, and ensuring a minimal impact of the treatments on the fruit microbiota at harvest stage. To minimize the orchard effect as much as possible, berries were

collected from different rows and from different positions along each row, within a week prior to the winery harvesting date, and transported to the laboratory in individual sterile plastic bags, in refrigerated conditions. Independent pools of berries were used as biological replicates.

2.2. Fruit technical & biochemical traits

Berry dry weight and water content were determined on an analytical scale Mettler Toledo AG245, after dehydration at 60 °C for 3 d. The concentrations of reducing sugars, total phenolics, total anthocyanins and NH_4^+ were determined using classical spectrophotometric methods
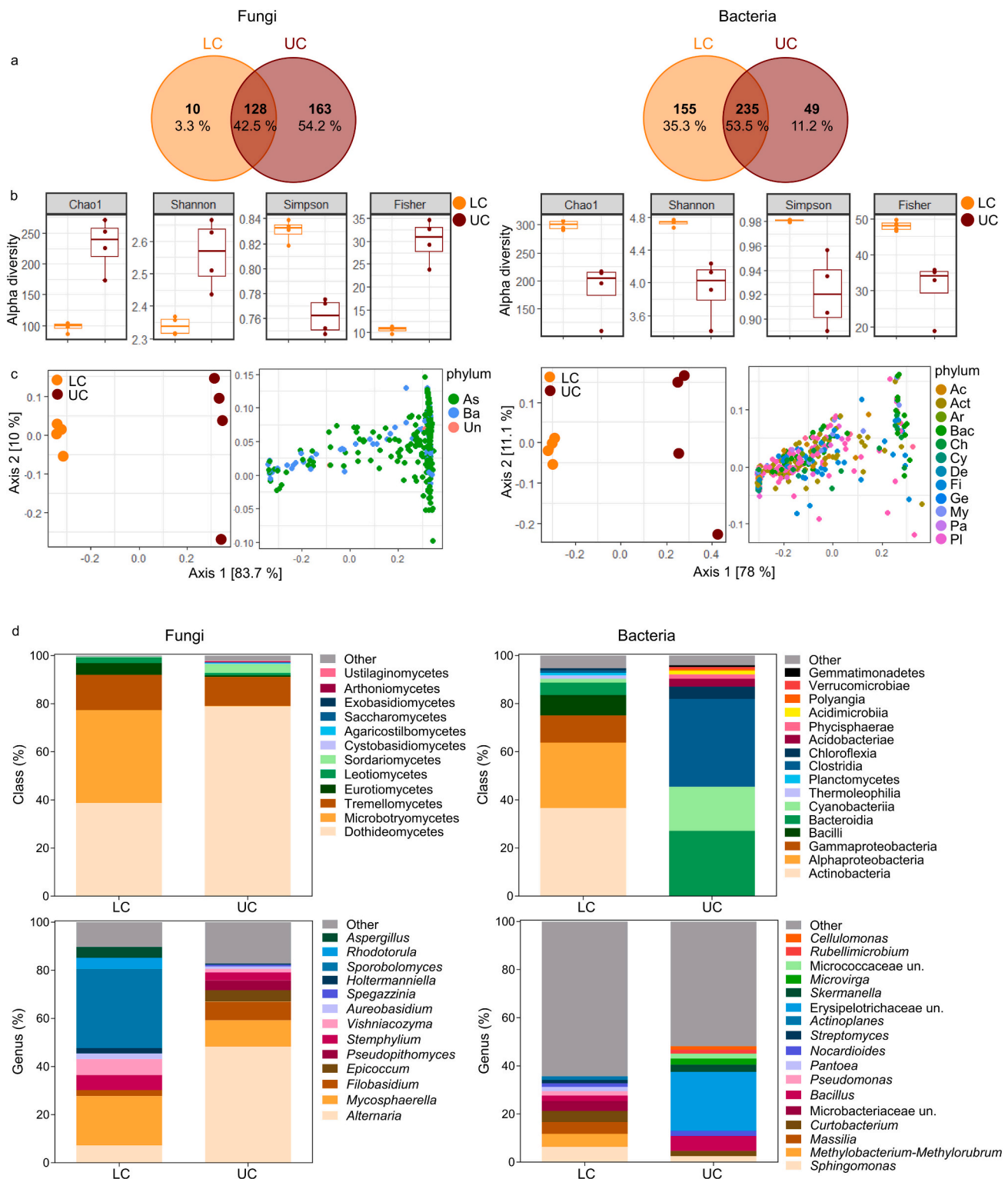
Fig. 4. Fungi and bacteria communities of grape berries cv. Sousão from the DOC Douro subregions Lower Corgo (LC) and Upper Corgo (UC). Venn diagrams (**a**) showing unique and shared OTUs. Alpha diversity (**b**) calculated with the Chao1, Shannon, Simpson and Fisher indexes; results are expressed as median ± SD of 4 biological replicates. Beta diversity (**c**) shown in PCoA plots using Bray Curtis distance matrix coefficients, presented by sample (left panel) and by phylum (right panel). Phyla names were abbreviated as follows: Ascomycota (As), Basidiomycota (Ba), Acidobacteriota (Ac), Actinobacteriota (Act), Armatimonadota (Ar), Bacteroidota (Bac), Chloroflexi (Ch), Cyanobacteria (Cy), Deinococcota (De), Firmicutes (Fi), Gemmatimonadota (Ge), Myxococcota (My), Patescibacteria (Pa), Planctomycetota (Pl), Unidentified (Un). Distribution of fungi and bacteria OTUs by class and genera (**d**), in grape berries cv. Sousão from the DOC Douro subregions Lower Corgo (LC) and Upper Corgo (UC). Only the 10 most abundant taxonomic groups are shown for each community, and rare population taxa were placed in artificial groups designated as "Other". For taxa without identification at genus level (un.), the assigned family is indicated. The statistical significance of the results was assessed by Kruskal-Wallis and Dunn's tests (B) or by PerMANOVA (C), as presented in **Table S5**.

detailed in Martins et al. (2023). Titratable acidity and pH were determined using a multi-parameter analyzer Consort C-860, as described before (Martins et al., 2023). The Students' *t*-test was used to identify statistically significant differences between parameters of berries located in Upper and Lower Corgo.

2.3. Metabolomic analysis

Berry metabolic profile (including polyphenols) was analyzed by UPLC-MS, as detailed previously (Billet et al., 2018; Li et al., 2024; Martins et al., 2020, 2023). Metabolite extraction was performed with 1 mL of 80 % (*v/v*) methanol from 25 mg of freeze-dried samples (*n* = 6). Following sonication for 30 min and maceration overnight, samples were centrifuged at 18,000*g* for 10 min and the supernatants were analyzed in an Acquity Ultra Performance Liquid Chromatography system coupled to a photo diode array detector (PDA) and a Xevo TQD mass spectrometer (Waters, Milford, MA) equipped with an electrospray ionization (ESI) source (Waters, Milford, MA). Analyte separation, chromatographic separation and identification of analytes were achieved as optimized previously Martins et al. (2020, 2023), using selected ion monitoring mode of the targeted molecular ions. Chromatogram and peak integration were achieved with the subroutine QuanLynx 4.1 for data mining and the ApexTrack algorithm, configured as described before (Martins et al., 2023). Multivariate Statistical Data Analysis (MVA) was performed using SIMCA P + version 17.0 (Umetrics AB, Umeå, Sweden). All variables were mean-centered and unit-variance (UV) scaled prior to MVA. Orthogonal Partial Least Squares Discriminate Analysis (OPLS-DA) according to the subregion was used as supervised clustering to identify the Variable Important in Projection (VIP > 1). Significant differences between the concentration of metabolites found in berries of each subregion were assessed with the Student's *t*-test in Prism®6 (GraphPad Software, Inc.).

2.4. Microbiota profile

The microbiota was isolated from the berry surface, as described previously (Martins et al., 2021, 2023), and microbial DNA was extracted with the DNeasy PowerSoil Pro Kit (Qiagen). Two 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 by the specialized services provider StabVida (Caparica, Portugal), using an in-house protocol based on Illumina 16S Metagenomic Sequencing Library Preparation protocol (15,044,223 Rev.B). lllumina MiSeq was performed with 300 bp paired-end sequencing reads, generating 64,362 to 389,326 raw sequence reads, analyzed with QIIME2 v2022.2. Data quality was assessed with Phred quality score, and reads denoising was performed with DADA2 plugin, as described before (Martins et al., 2021). Sufficient sequencing depth was visualized through alpha rarefaction curves (**Fig. S1**). Reads classification into Organized Taxonomic Units (OTUs) was achieved using UNITE database (release 8.3) and SILVA (release 138 QIIME) for ITS and 16S data, respectively. OTU information was imported into the R version 4.2.2 (https://cran.r-project.org/) and analyzed with phyloseq and vegan packages. The alpha and beta diversity were assessed as described previously (Martins et al., 2021, 2023). Non-parametric Kruskal-Wallis and Dunn's tests, and permutation-based ANOVA (PerMANOVA), were used to assess statistically significant differences between samples. Differential analysis was performed using edgeR in phyloseq_to_edgeR (PathoStat, v. 1.6.1), using an output of log2-fold-changes (logFC) of read counts with FDR correction (*P* < 0.05), as described previously (Martins et al., 2021, 2023). Fungi-bacteria dissimilarity networks were assembled with make_network function (Jaccard distance = 0.25). Barplots were designed in Prism®6 (GraphPad Software, Inc.) after collapsing OTUs at class and genus level. Illumina data generated in this work was deposited in Sequence Read Archive (SRA) from NCBI with the BioProject accession number PRJNA1191795.

2.5. Microbiota-metabolite correlations

Pearson correlations were calculated using the biological replicates of metagenomics and metabolomics data. The operational taxonomic units (OTUs) were grouped at the class level for fungi and at the phylum level for bacteria. Similarly, metabolites were grouped by metabolic class. Correlation analysis was performed using the corrplot R package (version 0.84).

3. Results

Grape berries cv. Sousão collected at harvest stage in Lower Corgo had significantly higher values of dry weight than those collected in Upper Corgo. The former also had 12 % more reducing sugars, but 32 % less total phenolics (**Table S1**). Other parameters including water content, titratable acidity, pH, total anthocyanins and NH4$^+$ were not markedly affected by subregional cues. Sixty-one metabolites were quantified by UPLC-MS in grape berries from Lower and Upper Corgo (**Table S2**). OPLS-DA analysis showed a clear separation in the metabolic profiles of berries of the two subregions (model diagnostic: R^{2}Xcum = 61.2 %, R^{2}Ycum = 99.3 % and Q^{2}cum = 96.4 %), with most metabolites being more concentrated in fruits from Lower Corgo (Fig. 2a, b). Metabolites with VIP > 1 included flavonols (m29, m34, m30, m13), phenolic acids (m15, m7, m3), anthocyanins diOH (m25, m10) and triOH (m36, m26, m20), organic acids (m60, m59), flavan-3-ols (m16, m8, m28), amino acids (m39, m45, m46, m47, m49, m50, m51, m54, m55, m56), and proanthocyanidins (m11, m22) (Fig. 2c). The permutation test (100 times) allowed the prediction at 100 % of the subregional origin of the berries based on their metabolic profile (Fig. 2d). Generally, berries from Upper Corgo were the richest in flavonols and phenolic acids, while berries from Lower Corgo were richer in organic acids, flavan-3-ols and proanthocyanidins (Fig. 3). Berries from Upper Corgo were also especially rich in tryptophan, while most other amino acids were more abundant in fruits from Lower Corgo. Additionally, berries from Upper Corgo were the richest in malvidin derivatives, while cyanidin, delphinidin and petunidin derivatives were more abundant in fruits from Lower Corgo. Only two stilbenoids were detected in the present study, of which *t*-ε-viniferin was more concentrated in berries from Lower Corgo.

Metabarcoding analysis allowed identification of 301 fungi OTUs and 439 bacteria OTUs in the surface microbiota of grape berries (**Table S3, S4**). Among these, 128 fungi OTUs and 235 bacteria OTUs were found in fruits from both subregions. Additionally, 163 fungi OTUs were exclusive of berries from Upper Corgo, while only 10 fungi OTUs were exclusively found in Lower Corgo (Fig. 4a). In contrast, 155 bacteria OTUs were exclusive of berries from Lower Corgo, while 49 bacteria OTUs were only detected in fruits from Upper Corgo. Alpha diversity analyses through the Chao1, Shannon, Simpson and Fisher indexes showed that the fungal diversity was higher in berries from Upper Corgo, while the bacteria diversity was higher in fruits from Lower Corgo (Fig. 4b, **Table S5**). The only exception was found with the Simpson index for the fungi community. Beta diversity analyses corroborated the marked compositional differences observed in the microbial profile of berries from the two subregions (Fig. 4c, **Table S5**), with Ascomycota being especially abundant in Upper Corgo. Particularly, Dothideomycetes dominated the fungi community of Upper Corgo (79 %), followed by Tremellomycetes (12 %) (Fig. 4d). The most represented genus in berries of this subregion was *Alternaria* (48 %). In contrast, Dothideomycetes shared its rank with Mycrobotryomycetes in berries from Lower Corgo (39 %), with *Sporobolomyces* being the most abundant genus (33 %) followed by *Mycosphaerella* (20 %). In parallel, Actinobacteria (37 %) and Alphaproteobacteria (27 %) dominated the bacteria community in berries from Lower Corgo, while Clostridia and Bacteroidia were dominant in fruits from Upper Corgo. A balanced distribution of the most abundant bacteria genera was found in berries from Lower Corgo, while an unidentified genus from *Erysipelotrichaceae*
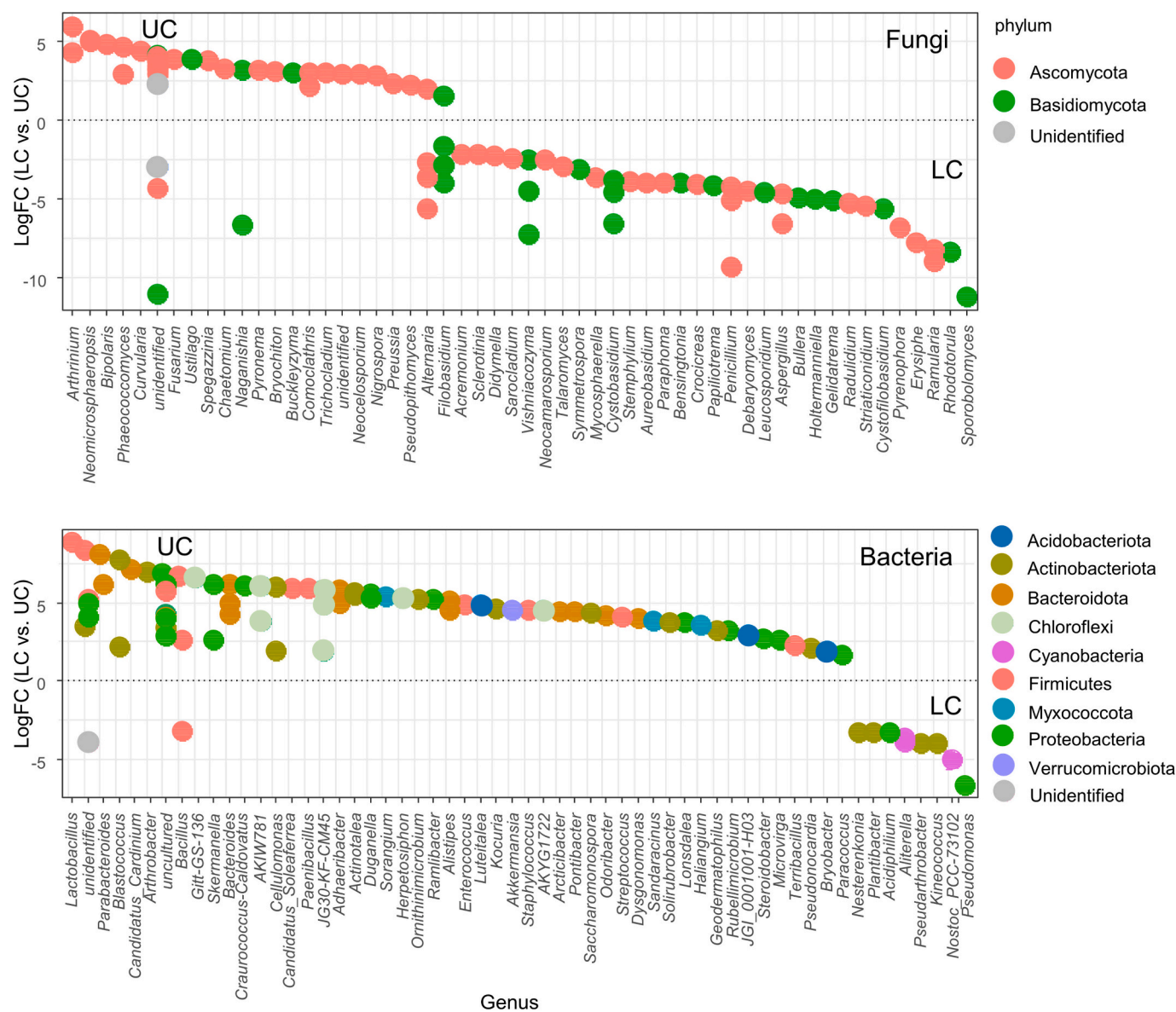
Fig. 5. Differential analyses of fungi and bacteria OTUs underlying significant differences between grape berries cv. Sousão from different DOC Douro subregions. The Y axis shows log2-fold-changes (logFC) for microbiota data of Lower Corgo (LC) versus Upper Corgo (UC) ($P \leq 0.05$). Each node represents a different OTU, identified at genus level and colored according to its corresponding phylum.

dominated the bacteria community of fruits from Upper Corgo.

Differential analyses highlighted the OTUs responsible for the significant differences ($P \leq 0.05$) observed in the microbial communities of berries from Lower and Upper Corgo (Fig. 5). The yeasts *Sporobolomyces*, *Rhodotorula*, *Holtermanniella* and *Bullera* were markers of the Lower Corgo microbiota, together with *Aureobasidium* and several pathogenic fungi including *Erysiphe necator*, *Ramularia*, *Aspergillus parasiticus*, *Penicillium thomii* and *P. citrinum*. In contrast, *Arthrinium*, *Neomicrosphaeropsis*, *Bipolaris*, *Phaeococcomyces*, *Curvularia* and *Fusarium* were characteristic of berries from Upper Corgo. In parallel, nine bacteria genera were top markers of fruits from Lower Corgo, including *Pseudomonas*, *Kineococcus*, *Pseudarthrobacter*, *Aliterella* and *Acidiphilium*. In turn, 77 other bacteria OTUs were more abundant in Upper Corgo, with *Lactobacillus* and *Parabacteroides* being exclusive of this subregion (Fig. 5). Dissimilarity analysis (Jaccard distance = 0.25) showed that the complexity of fungi-bacteria networks was similar in both subregions, denoting equivalent cluster complexity and class predominance, with Alphaproteobacteria and Actinobacteria acting as major intervenients (Fig. S2).

Pearson correlation matrixes (Fig. S3) allowed further visualization of positive correlations between the majority of fungi classes and flavonols, phenolic acids and anthocyanins, as previously denoted in berries from Upper Corgo (Figs. 3 and 4). In contrast, flavan-3-ols generally correlated negatively with most fungi classes, including Dothideomycetes, in berries from both Upper and Lower Corgo (Fig. S3). Consistent negative correlations between the bacteria community and fruit metabolites were observed for amino acids, Firmicutes and Patescibacteria. In parallel, the relationships between phenolic compounds and bacteria phyla while highly diverse, although negative correlations between stilbenoids and microorganisms were very significant in berries from Upper Corgo.

4. Discussion

The present study explored the influence of subregional terroir cues on the berry holobiont, considering the combined influence of environmental and topography features including the vineyard orientation, soil texture, altitude, slope, temperature and rainfall. Results

unequivocally demonstrated that there are marked differences in technical quality parameters and in the metabolic and microbiota profiles of grape berries cv. Sousão of the two locations of Douro subregions, separated only by 14 km. Although the soil has long been envisaged as a major reservoir of microbial communities that colonize aerial plant parts, recent studies in Douro region showed that only 10–30 % of microbial species found in the soil are also detected in berries (Teixeira et al., 2024). As the vineyards from both regions of Douro region addressed in the present study shared identical rootstocks, clone, age and training system, other factors inherent to the local terroir likely underlain the observed differences. Environmental heterogeneity associated to mesoclimate could originate more microbial dissimilarity than geographic distance, as suggested for cv. Chardonnay across California - USA (Bokulich et al., 2014), cv. Cannonau in Sardinia - Italy (Mezzasalma et al., 2017), cv. Grenache and Carignan in Catalonia - Spain (Portillo et al. 2016) and cv. Alvarinho in Monção and Melgaço - Portugal (Fernandes et al., 2023). At parcel scale, the terroir influence could be highlighted in a continuous space, by the combined use of spatial metabolomics and geographical information system mapping (Billet et al., 2023).

Contributing environmental factors for the marked variations in microbial communities observed in the present study could be the lower temperatures and higher humidity of Lower Corgo when compared to Upper Corgo, associated to finer soil texture with lower sand/silt ratio that could improve water retention capacity. The higher bacteria alpha diversity observed in berries from the former subregion adds novelty to previous reports showing lower diversity in the soil bacteria of cooler and wetter regions than warmer and drier sites (Zhou et al., 2021). In turn, the higher diversity of fungi in Upper Corgo could be associated to the positive effect of the higher temperature and dryness, as suggested in previous studies that predict increased microbial-rate metabolism (Gao et al., 2019). The higher fungi diversity in berries from Upper Corgo with lower precipitation further supports that rainfall could be a non-preponderant factor in determining fungal diversity in these Douro subregions, unlike previous reports in other wine regions (Gobbi et al., 2022). The higher altitude and slope found in vineyards of Lower Corgo could also be a contributing factor for the higher bacteria diversity found in this region, as previous studies showed relationships between the bacterial community and the altitude, latitude and longitude (Gao et al., 2019). Fungal diversity has also been positively associated with these factors (Gao et al., 2019), although results in the present study showed higher alpha diversity in Upper Corgo with markedly lower altitude. The lack of direct relationships between fungi diversity and altitude or rainfall jointly suggest that host-related factors likely exert stronger influence upon microbial communities than environmental conditions or topography per se.

As suggested by results in the present study, environmental factors and topography strongly shaped the berry metabolome, with a demarcated subregional signature being translated by the 100 % accuracy of the metabolic OPLS-DA model predicting the geographic origin of the berries. Such model was also able to predict the geographical origin of grape seed extracts according to the polyphenol composition (Munsch et al., 2024). Although reports on the influence of the altitude in total phenolics are contradictory (Jin et al., 2017), the higher total phenolic content observed in berries from Upper Corgo could be attributed to the warmer temperature and/or higher light exposure, as these factors were shown to stimulate the activity of PAL enzyme, the first of the phenylpropanoid pathway and secondary metabolism (Teixeira et al., 2013). The higher content of malate and succinate in fruits from Lower Corgo could be associated to the higher altitude of the vineyard, as observed in cv. Syrah (de Oliveira et al., 2019), and to the lower temperatures of this region, as excessive heat was shown to inhibit the accumulation of these organic acids (Rienth et al., 2021). This effect has been attributed to several cellular mechanisms including the stimulation of the activity of the NAD dependent malic enzyme, and the enhanced anaplerotic capacity of the TCA cycle. The markedly higher accumulation of amino

acids in berries from Lower Corgo could also be a consequence of the cooler and more humid microclimate, consistent with reports in three other Portuguese cultivars from two other wine regions (Teixeira et al., 2014). In turn, the increased accumulation of phenolic acids in berries from Upper Corgo could be indicative of an adaptive response to heat stress or drought that are more frequent in this subregion (Laddomada et al., 2021; Santos et al., 2020). Also, the increased abundance of flavonols in berries from this subregion could partially be associated to their accumulation in response to sunlight exposure, consistent with their UV radiation-screening activity and capacity to reduce light-induced oxidative damage (Cataldo et al., 2023; Cheng et al., 2020; Goufo et al., 2020; Teixeira et al., 2013), although the relationship between temperature and flavonol accumulation is still unclear (Rienth et al., 2021). The higher concentration of flavan-3-ols and proanthocyanidins in berries from Lower Corgo could be related to the positive influence of the altitude on the levels of catechin, epicatechin and tannins (Arias et al., 2022; de Oliveira et al., 2019; Jin et al., 2017; Lemos et al., 2020), but contrasts previous studies showing their over-accumulation in warmer areas, although the influence of environmental factors on their abundance is still regarded as elusive (Cheng et al., 2020). Equally, the content of t-Ɛ-viniferin was previously associated to water deficit and UV-C exposure, and shown to decline in dry seasons (Anesi et al., 2015), but its higher abundance in berries from Lower Corgo could be associated to the role of stilbenoids as phytoalexins, usually more abundant in wet climate where disease pressure is higher (Rocchetti et al., 2021). The regulation of the accumulation of these bioactive compounds in grape berries by factors such as UV exposure is highly relevant from a nutraceutical point of view, as many of these metabolites, including resveratrol and quercetin, have acknowledged antioxidant, antimicrobial, anti-inflammatory, and anti-carcinogenic activities (El Rayess et al., 2024; Sabra et al., 2021; Zhang et al., 2023; Zhou et al., 2022).

Several factors including altitude, temperature, light exposure and vine water status were also shown to modulate total anthocyanin content, with a stimulation in anthocyanin levels being observed at higher altitude, low to moderate temperature, and strong light, given their protective role from excessive sunlight (Anesi et al., 2015; Jin et al., 2017; Teixeira et al., 2013). In turn, high temperature was shown to inhibit anthocyanin accumulation and enhance its degradation, through the repression of biosynthesis regulators such as VviMYBA1 and VviUFGT, and the stimulation of the activity of peroxidases (Rienth et al., 2021). These global effects did not seem apparent in cv. Sousão berries in the present study, as total anthocyanin content was identical in fruits from Lower to Upper Corgo, however, with a marked trade-off between malvidin conjugates and all other anthocyanin types, which could be cultivar-specific. This assumption is supported by reports in cv. Merlot where sun exposure promoted the specific accumulation of delphinidin, petunidin and peonidin-based anthocyanins, while malvidin derivatives remained unaffected (Tarara et al., 2008). Results in the present study also complement previous reports showing that high temperature increases the proportion of tri-hydroxylated anthocyanins in various other cultivars, concomitantly with the overexpression of the acyltransferase gene Vvi3AT (Rienth et al., 2021). Additionally, water deficit was shown to stimulate the accumulation of tri-hydroxylated anthocyanins, and specifically, post-veraison water deficit was shown to enhance malvidin content (Teixeira et al., 2013), a factor that cannot be ruled-out on the outcome observed in berries from Upper Corgo in the present study.

The influence of isolated environmental factors on the fruit metabolome and microbial communities is further complicated by the intricate relationships between metabolites and microbiota. These relationships likely play a significant role in shaping the grape ecosystem, thereby mediating the interaction with the local terroir. The existence of associations between wine volatile compounds and specific bacteria and/or fungi subpopulations of the musts has been demonstrated in several cultivars including Cabernet Sauvignon and Chardonnay (Bokulich
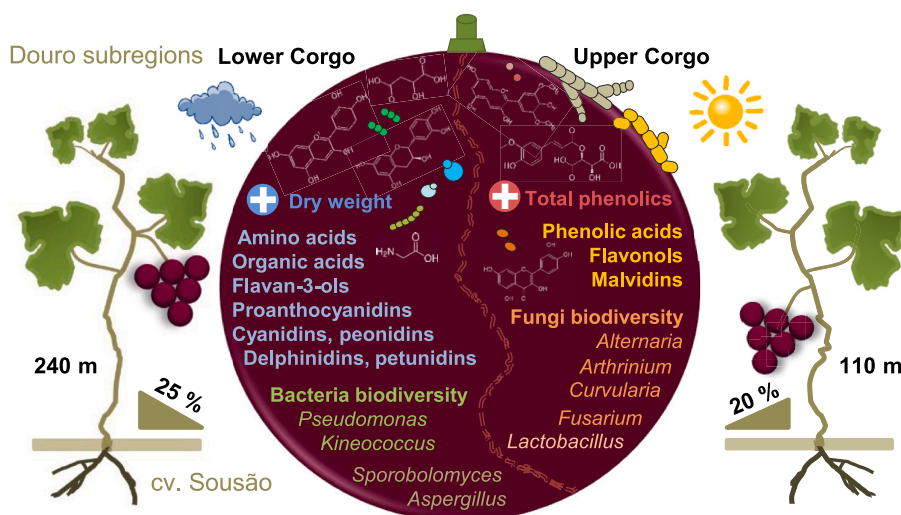
Fig. 6. Subregional-associated phenotype plasticity of cv. Sousão berries in Douro region.

et al., 2014; Chen et al., 2020; Chen et al., 2022; Liu et al., 2021), besides local Portuguese varieties from Douro region, among which, Sousão (Martins et al., 2024). In this recent study, the complexity of the wine volatile profile was shown to be largely dictated by the timing of the *Hanseniaspora-Saccharomyces* succession during spontaneous fermentations, which was cultivar-dependent, and species like *Lachancea quebecensis* correlated with several wine varietal metabolites (Martins et al., 2024). Berry parameters such as total phenolics were also found to associate with soil bacteria communities (Zhou et al., 2021), but the existence of specific metabolite-microbiota correlations in the grape berry itself has only recently been exposed, highlighting a connection between the microbial community, the host, and the macroscopic phenotype (Martins et al., 2023). In the three Portuguese cultivars Sousão, Touriga Nacional and Viosinho, over 7000 metabolite-microbiota correlations exposed a core of 15 metabolites linked to 11 microbial taxa, among which were several analytes markedly more abundant in berries from Lower Corgo explored in the present study. These included phenylalanine, asparagine, alanine, cyanidin-3-*O*-glucoside and petunidin-3-*O*-glucoside, besides several taxa found to be microbial markers of each Douro subregion. In this view, the dominance of *Aspergillus*, *Penicillium* and *Aureobasidium* in berries from Lower Corgo could be associated to the high content in phenylalanine, asparagine, alanine, methionine, glycine, threonine, ornithine, citrate, procyanidin B3 and the quercetin derivative, as specific positive correlations were found between these metabolites and these taxa (Martins et al., 2023). Concomitantly, the incidence of *Aspergillus* in these berries could further be linked to the low content in fertaric acid and malvidin conjugates, that were shown to correlate negatively with this filamentous fungus. Similar relationships could exist between the abundance of *Mycosphaerella*, *Pseudomonas Kineococcus* and *Acidiphilium* and the content in malate, *t*-ℰ-viniferin and catechin gallate in berries from Lower Corgo. In turn, the prevalence of *Alternaria*, *Arthrinium*, *Phaeococcomyces*, *Curvularia* and *Fusarium* in berries from Upper Corgo could be associated to the low abundance of malate, lysine, epicatechin, cyanidin and petunidin conjugates in these berries, as specific negative correlations were reported between these metabolites and these taxa (Martins et al., 2023). These complex associations likely command downstream microbiota-microbiota correlations, through antagonism and commensalism relationships that constitute the berry holobiont (Liu et al., 2019). For example, the growth of pathogenic fungi like *Botrytis cinerea*, *Alternaria alternata*, *Aspergillus niger* and *Penicillium expansum* was shown to be inhibited by *Pseudomonas* spp. (Andreolli et al., 2019; Niem et al., 2020). In turn, the sporulation of the causal agent of downy mildew may be hampered in the presence of *Alternaria alternata* (Musetti et al., 2006).

The wide biocontrol potential of *Aureobasidium pullulans* was also demonstrated, supporting its beneficial role in grapevine health (Ayogu et al., 2023). Thus, despite many fungi that act as microbial markers of each Douro subregion being generally classified as general plant pathogens, they could not trigger disease development in the berry, likely due to the presence of many other beneficial taxa such as *Aureobasidium*. Also, the low fungal diversity found in berries from Lower Corgo could have favored the dominance of bacterial species, yeasts and pathogenic fungi such as *Aspergillus* and *Penicillium*, that were otherwise overruled by beneficial filamentous fungi in fruits from Upper Corgo, a hypothesis that deserves further investigation. It is also relevant to note that the dominance of Alphaproteobacteria and Actinobacteria in fungi-bacteria relationships in both subregions is consistent with results from previous studies in cv. Sousão (Martins et al., 2021, 2023), despite not being the most abundant classes within the bacteria community of Upper Corgo, supporting their major influence on the structure of the grape berry microbial community of this cultivar. Lastly, it should be considered that the higher intensity of fungicide treatments in vineyards from Lower Corgo, as demanded by environmental conditions underlined by higher humidity, could also have contributed to the outcomes observed, as phytochemical treatments were shown to be capable of affecting not only the microbiota of the berry but also its metabolic profile, even though their reported effects on the microbial diversity are still inconsistent (Agarbati et al., 2019; Barata et al., 2012; Martins et al., 2020).

Taken together, results in the present study demonstrated that berries cv. Sousão display a high intraregional phenotypic plasticity, that likely translates not only the influence of environmental factors and topographical conditions, but also complex metabolite-microbiota and microbiota-microbiota relationships that are shaped by climatic subregional cues. This data may be useful for understanding adaptive responses to climate change in the next decades and for predicting the potential impacts in wine quality.

5. Conclusion

The present study exposed the marked impact of the subregional terroir on the metabolic and microbial profile of cv. Sousão berries, with evident trade-offs between fungi and bacteria diversity, associated to opposing trends in the accumulation of key metabolites for berry quality, denoting a high degree of intraregional-associated phenotypic plasticity (Fig. 6). Warmer and drier sites (Upper Corgo) are associated to a decrease in the abundance of amino acids, organic acids, flavan-3-ols, proanthocyanidins and non-malvidin-based anthocyanins, in favor of phenolic acids, flavonols and malvidin conjugates. Besides the

bioactive functions of these metabolites in the berry, further impacts are expected on wine production, given their contribution as yeast assimilable nitrogen during fermentation, as stabilizers of wine acidity, as antioxidants, as preservatives against spoilage and as aroma precursors, modulating wine astringency, flavour and color. The associated modifications in the microbial consortium, especially in the incidence of native yeasts, are also likely to influence the fermentation process and wine regionality. With downward trends in precipitation and higher temperatures projected as the main threats to Southern European viticulture during the growing season (Santos et al., 2019, 2020), these results can be valuable for interpreting how grape phenotypes might evolve in a changing climate.

Funding

CRediT authorship contribution statement

Viviana Martins: Writing – review & editing, Writing – original draft, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Cécile Abdallah:** Validation, Methodology, Investigation. **António Teixeira:** Writing – review & editing, Methodology, Formal analysis. **Carolina Moreira:** Writing – review & editing, Investigation. **Márcio Nóbrega:** Writing – review & editing, Resources. **Arnaud Lanoue:** Writing – review & editing, Resources, Methodology, Funding acquisition, Formal analysis. **Hernâni Gerós:** Writing – review & editing, Resources, Funding acquisition, Conceptualization.

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.

Acknowledgements

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2024.102139.

Data availability

Data generated in the present study was deposited in NCBI SRA (accession PRJNA1191795) and is available as supplementary files.

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