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Spontaneous fermentation of Maraština wines: The correlation between autochthonous mycobiota and phenolic compounds

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ABSTRACT

Understanding fungal community dynamics during fermentation is important for assessing their influence on wine's phenolic content. The present study represents the first effort to explore the correlation between the autochthonous mycobiota of Maraština grapes collected from Dalmatian winegrowing sub-regions in Croatia and the phenolic composition, as well as the physicochemical parameters of wines produced through spontaneous fermentation. The metataxonomic approach revealed *Metschnikowia pulcherrima*, *Metschnikowia fructicola* and *Hanseniaspora uvarum* as the core mycobiota detected at the initial phase of fermentation. By contrast, *Saccharomyces cerevisiae* took over the dominance starting from the middle stage of fermentation. The wine's phenolic compounds were revealed by high-performance liquid chromatography, with tyrosol being the most abundant. *Rhodotorula babjevae* and *Botrytis cinerea* showed a positive correlation with *p*-hydroxybenzoic acid, gentisic acid, caffeic acid and cinnamic acid, while demonstrating a negative correlation with protocatechuic acid and chlorogenic acid. *Heterophoma novae-verbascicola* exhibited the opposite behaviour regarding the same phenolic compounds. The concentration of lactic acid was positively correlated with *B. cinerea* and negatively correlated with *Het. novae-verbascicola*. These findings serve as a foundation for in-depth investigations into the role of autochthonous grape mycobiota in phenolic transformation during spontaneous fermentation, potentially leading to the production of high-quality wines with unique *terroir* characteristics. Future studies should aim to explore the specific role played by individual yeast isolates in the formation of phenolic compounds.

1. Introduction

The grape must provide a fertile environment for a variety of yeast species. Indigenous grape mycobiota plays a major role in spontaneous alcoholic fermentation by producing metabolites that affect wine composition. The mycobiota of grapes is primarily influenced by the health status of the grape and can vary within the same variety based on the vineyard practices, geographical position, soil type and climate, thereby impacting the diversity of wines produced from the same grape variety (Chalvantzi et al., 2021; Milanović et al., 2022). The grape surfaces and, consequently, the grape musts are characterised by high fungal biodiversity (Wang et al., 2015; Shimazu et al., 1984), mainly comprising non-*Saccharomyces* species such as *Hanseniaspora uvarum* (*Kloeckera apiculata*), *Metschnikowia pulcherrima* (*Candida pulcherrima*), *Candida stellata* or newly described *Candida zemplinina*, along with various species from *Cryptococcus*, *Debaryomyces*, *Hansenula*, *Issatchenkia*, *Kluyveromyces* and *Pichia* genera. Furthermore, basidiomycetous oxidative species from the genera *Filobasidium*, *Cryptococcus* and *Rhodotorula*, as well as weakly fermentative species from the *Aureobasidium* genus (Kántor & Kačániová, 2015; Milanović et al., 2022), are frequently found on the grape surface. These yeasts significantly contribute to the complexity of the wine's composition by producing various metabolites during the early stages of spontaneous fermentation (Francesca et al., 2016; Gschaedler, 2017; Liu et al., 2023; Luzzini et al., 2021; Xu et al., 2020).

Among metabolites, phenols represent a relevant chemical class related to different aspects of wine production that significantly affect wine quality, being responsible for its sensorial properties (taste and

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colour), uniqueness and health benefits (Budić-Leto et al., 2008, 2017). Phenolic compounds are extracted from different parts of the grapes during the vinification process and undergo transformation into intermediate products or final metabolites through enzymatic and biochemical reactions (Lukić et al., 2017). Furthermore, many factors, including yeasts involved in the fermentation process, may affect the phenolic content of wine (Downey et al., 2006; Morata et al., 2019). Most studies that investigated the impact of phenolic compounds on human health were related to red wines, principally due to their high content of quercetin, resveratrol and flavan-3-ols. By contrast, only a recent study performed by Boronat et al. (2019) demonstrated a positive impact of tyrosol from white wine on human health. The first studies of yeast's impact on the phenolic profile highlighted the correlation between yeast strain and chromatic properties (Caridi et al., 2004), as well as anthocyanin concentration in wines (Medina et al., 2005). Indeed, yeast metabolism may lead to different metabolic precursors during must fermentation, influencing the formation of proanthocyanins and oligomeric compounds (Escott et al., 2018). Grieco et al. (2019) reported that the application of selected indigenous yeasts can increase the content of stilbene and flavonols (quercetin, myricetin and kaempferol) in wine. Some studies pointed out that the presence of enzymes produced by the autochthonous mycobiota can impart the biochemical reactions of phenols during fermentation, thus altering their composition in wine (Belda et al., 2017; Claus & Mojsov, 2018). Several studies have examined the phenolic composition of Croatian red wines fermented using commercial yeast strains (Jagatić Korenika et al., 2021; Lukić et al., 2017). However, despite their importance, to the best of our knowledge, no studies on the effect of indigenous mycobiota on the phenolic composition of Croatian wines, especially the Maraština variety, have been performed. Maraština is an indigenous Croatian grape variety known for its golden-yellow grapes with thick skin and small brown spots, holding a significant position among white grape cultivars in the Adriatic coastal region. Maraština has the potential for the production of high-quality monovarietal and dessert wines. Maraština wines are distinguished by a pronounced yellow colour, body, viscosity, astringency and tannin content, which play a crucial role in shaping their unique sensory profile. Additionally, young Maraština wines exhibit a prominent aroma, featuring fruity notes of strawberry, lemon, apple, banana and prune, complemented by subtle honey undertones (Boban et al., 2022; Budić-Leto et al., 2020). Examining the correlation between indigenous mycobiota and phenolic compounds in wines, especially those obtained from autochthonous grape varieties such as Maraština, helps reveal the unique *terroir* of a specific region (Alexandre, 2020; Milanović et al., 2023). Accordingly, the present study represents the first effort to discover the possible correlation between the autochthonous mycobiota of Maraština grapes and the phenolic composition and physicochemical parameters of Maraština wines produced by spontaneous fermentation. To this end, grape musts were obtained from grapes harvested in seven vineyards located within the Croatian winegrowing region of Dalmatia, including both the Northern Dalmatia (ND) and Central and Southern Dalmatia (CSD) sub-regions. The relative abundance of fungal species at the initial, intermediate and final phases of fermentation was determined by a metataxonomic approach. This study is relevant for both local winemakers seeking to express their distinct regional identity and for international consumers interested in experiencing diverse wine profiles. Overall, these aspects contribute to ensuring the market competitiveness of the wines obtained.

2. Materials and methods

2.1. Grape sampling and spontaneous fermentation

In September 2021, samples of Maraština grapes were harvested from seven vineyards located in the coastal winegrowing region of Dalmatia, Croatia. These samples were collected in three separate biological repetitions, with each repetition consisting of approximately 3 kg of grapes with a glucose/fructose ratio equal to 1. The vineyards labelled N, V and Z were located in the winegrowing sub-region of ND, and grapes were harvested on the 16th of September (Fig. 1). The vineyards B, D, K and P were located on the island of Korčula, in the winegrowing sub-region of CSD, and grapes were harvested on the 11th of September (Fig. 1). The vineyards in the ND sub-region were positioned at higher elevations and predominantly on brown soil with a limestone base. Conversely, the vineyards in the CSD sub-region were established on reddish-brown soil. All seven observed vineyards had a north–south row orientation, used a bilateral cordon trellis system and applied the same ampelographic interventions. After harvesting, the Maraština grapes were destemmed and crushed. The obtained grape musts were sulfated with potassium metabisulfite to a total SO_2 concentration of approximately 50 mg/L. After the musts were separated and cold-stabilised at 4 °C for 24 h, they were distributed into 500 mL Erlenmeyer flasks closed with porous cellulose sterile caps, shielded from light using aluminium foil and left to ferment spontaneously at a temperature between 18 °C and 20 °C. The seven experimental wines (named by the vineyard: B, D, K, N, P, V and Z) were produced in biological triplicates. For the metataxonomic analysis of the native mycobiota, 2 mL aliquots were collected from each sample in sterile conditions at the following phases defined based on sugar concentration (glucose + fructose, g/L): the beginning (~211.0 g/L), middle (100.0 g/L) and end of the alcoholic fermentation (<2.0 g/L). The basic physicochemical parameters and phenolic content of the resulting wines were also determined.

2.2. Basic physicochemical analysis of Maraština wines

The basic wine parameters, including ethanol (vol%), sugar content (°Oe), malic acid (g/L), lactic acid (g/L), glycerol (g/L), total acidity (g/L), and tartaric acid (g/L), were analysed by an FTIR Lyza 5000 Wine (Anton Paar GmbH, Graz, Austria). The measurement of volatile acidity in wines and the calibration of FTIR were performed in accordance with the standard physicochemical OIV methods for wine in a laboratory accredited according to ISO IEC 17025. By contrast, calibration for malic, lactic and tartaric acid, glucose and fructose data was done by enzymatic methods with L-malic acid, D-/L-lactic acid, tartaric acid and D-fructose/D-glucose assay kits (Megazyme, Wicklow, Ireland).

2.3. DNA extraction and amplicon-based sequencing

The 2 mL aliquots of each sample from the beginning, middle and final phases of fermentation were centrifuged at 14,000 × *g* for 10 min. The supernatants were discarded, and the pellets containing microbial cells were used for the extraction of the total microbial DNA using an EZNA soil DNA extraction kit (Omega Bio-Tek, GA, USA). The quantity and purity of the extracted DNAs were determined by a Nanodrop ND 1000 (Thermo Fisher Scientific, Wilmington, DE, USA), followed by the amplification of the 26S rRNA gene as previously detailed by Milanović et al. (2022). A library of 26S rRNA of fungal DNA was constructed using MiSeq Reagent Kit V3 (2 × 250 bp) (Illumina, San Diego, USA) as described by Mota-Gutierrez et al. (2019). The G PCR products were purified by means of an Agencourt AMPure kit (Beckman Coulter, Milan, Italy). The resulting products were tagged with sequencing adapters using the Nextera XT library preparation kit (Illumina Inc., San Diego, CA), according to the manufacturer's instructions. Sequencing was performed using a MiSeq Illumina instrument (Illumina) with V3 chemistry, which generated 2 × 250 bp paired-end reads. MiSeq Control Software, V2.3.0.3, RTA, v1.18.42.0 and CASAVA, v1.8.2, were used for the base-calling and Illumina barcode de-multiplexing processes.

2.4. Bioinformatic analysis

A total of 4,828,558 raw reads were produced by the 26S rRNA amplicon-based sequencing of 63 samples collected during spontaneous fermentation. To obtain Amplicon Sequence Variants (ASVs), the raw

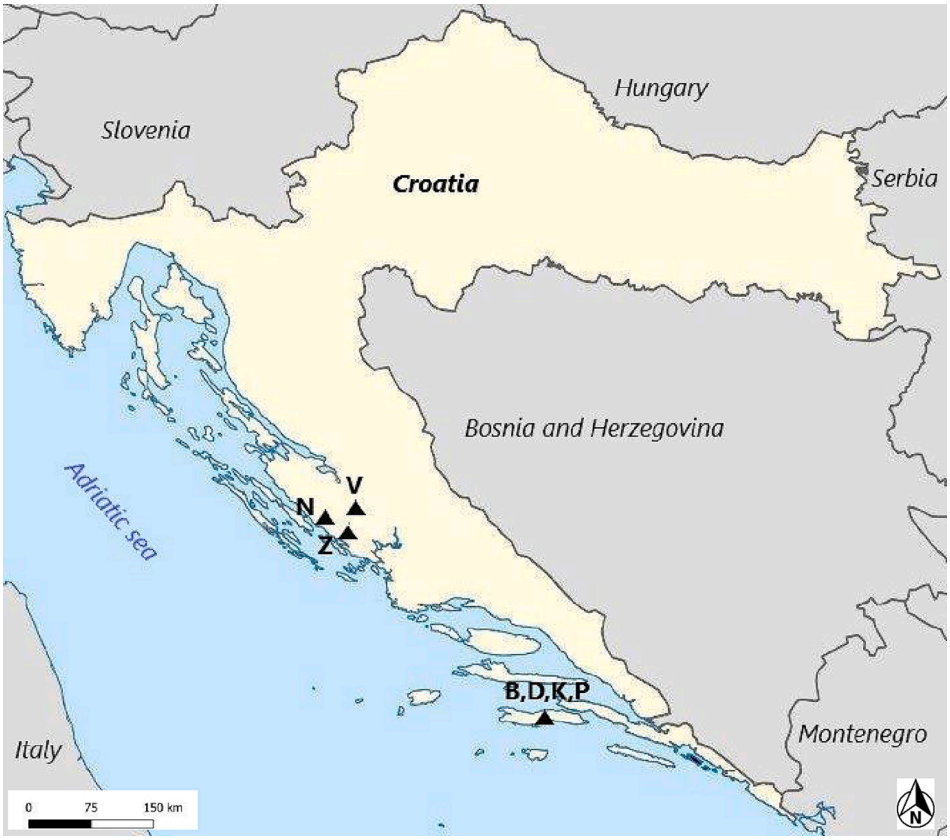


Fig. 1. Geographical locations of the Maraština vineyards B, D, K and P from the Central and Southern Dalmatia (CSD) and N, V and Z from the Northern Dalmatia (ND) winegrowing sub-regions.

reads were analysed with the *DADA2* package (Callahan et al., 2016) in an R environment (R version 4.1.1; http://www.r-project.org). The pipeline previously described was followed for raw read filtering [*truncLen = c(250,250); trimLeft = c(36,36); maxEE = c(2,2); minLen = c(50,50); truncQ = 6*], paired-end merging [minOverlap = 20] and chimaera removal (Botta et al., 2022). All parameters not reported for filtering or merging steps are intended as default *DADA2* settings.

Taxonomy was assigned with a 99 % sequence similarity through the Bayesian classifier method (Wang et al., 2007) by matching fungal ASVs to an internal database of 26S rRNA (Mota-Gutierrez et al., 2019), thus double-checking for species-level assignment at 100 % similarity by using the BLASTn suite (https://blast.ncbi.nlm.nih.gov). ASVs with uncertain classification (to the class taxon rank) and matching (>99 % similarity) with grapes' genomes were removed from the frequency tables. Two samples collected from the beginning stages of fermentation in two vineyards were excluded from the analysis due to their low library quality. Finally, a total of 2,795,924 paired-end reads (an average of 44,380 reads per sample) were used to construct the ASV frequency table.

ASVs were aligned with the *DECIPHER* package, and an unrooted phylogenetic tree was constructed with the *phangorn* package (Schliep, 2011; Wright, 2016). Alpha-diversity metrics and weighted UniFrac beta-diversity distance were calculated with *phyloseq* and *picante* packages (Kembel et al., 2010; McMurdie and Holmes, 2013); the rarefaction limit was set to the lowest number of sequences or samples.

Sequencing data were deposited at the Sequence Read Archive of the National Centre for Biotechnology Information under the BioProject accession number PRJNA940632.

2.5. High-performance liquid chromatography analysis

The high-performance liquid chromatography (HPLC) analysis of the phenolic compounds was conducted using a Shimadzu Nexera LC-40 HPLC system (Shimadzu, Kyoto, Japan), equipped with a UV–Vis detector. The separation was done using a Phenomenex C18 reverse-phase chromatography column (250 mm length, 4.6 mm width and particle size 5 μm; Phenomenex, Torrance, CA, USA). To create a calibration curve, six concentration levels of phenolic standard solutions were injected in triplicate into the HPLC system using an autosampler. Sample elution was performed at a flow rate of 1 mL/min and a temperature of 30 °C with solvent A consisting of ultra-pure water/85 % o-phosphoric acid (Sigma-Aldrich, St. Louis, MO, USA) at 99.8:0.2 v/v and solvent B, HPLC-grade acetonitrile (VWR International, Radnor, PA, USA). The total run time was 52 min using a concentration gradient as follows: initially 96 % A; 16 min 85 % A; 37.5 min 85 % A; 39 min 60 % A; 49 min 60 % A; 50 min 96 % A and 52 min 96 % A. Validation parameters of the method that evaluate data quality and confidence, such as the limit of detection, the limit of quantification, linearity, repeatability, accuracy and intra-day precision, are reported here for the first time (Supplementary Tables S1–S3).

2.6. Phenolic compounds, standard solutions and sample preparation

Standard solutions were prepared for 19 studied phenolic compounds, such as gallic acid, protocatechuic acid, tyrosol, *p*-hydroxybenzoic acid, catechin hydrate, chlorogenic acid, gentisic acid, caffeic acid, syringic acid, epicatechin, epigallocatechin gallate, vanillin, *t*-*p*-coumaric acid, *t*-o-coumaric acid, resveratrol, quercetin, cinnamic acid and naringenin purchased from Sigma-Aldrich and 2,6-dimethoxy benzoic acid, ferulic acid and sinapic acid obtained from Fluka (Fluka Analytical, Buchs, Switzerland). Phloroglucinol (Sigma-Aldrich) was used as an internal standard (Supplementary Fig. 1; Supplementary Fig. 2). Standard solutions were prepared using HPLC-grade methanol (VWR International, Radnor, PA, USA) to make solutions for all standard

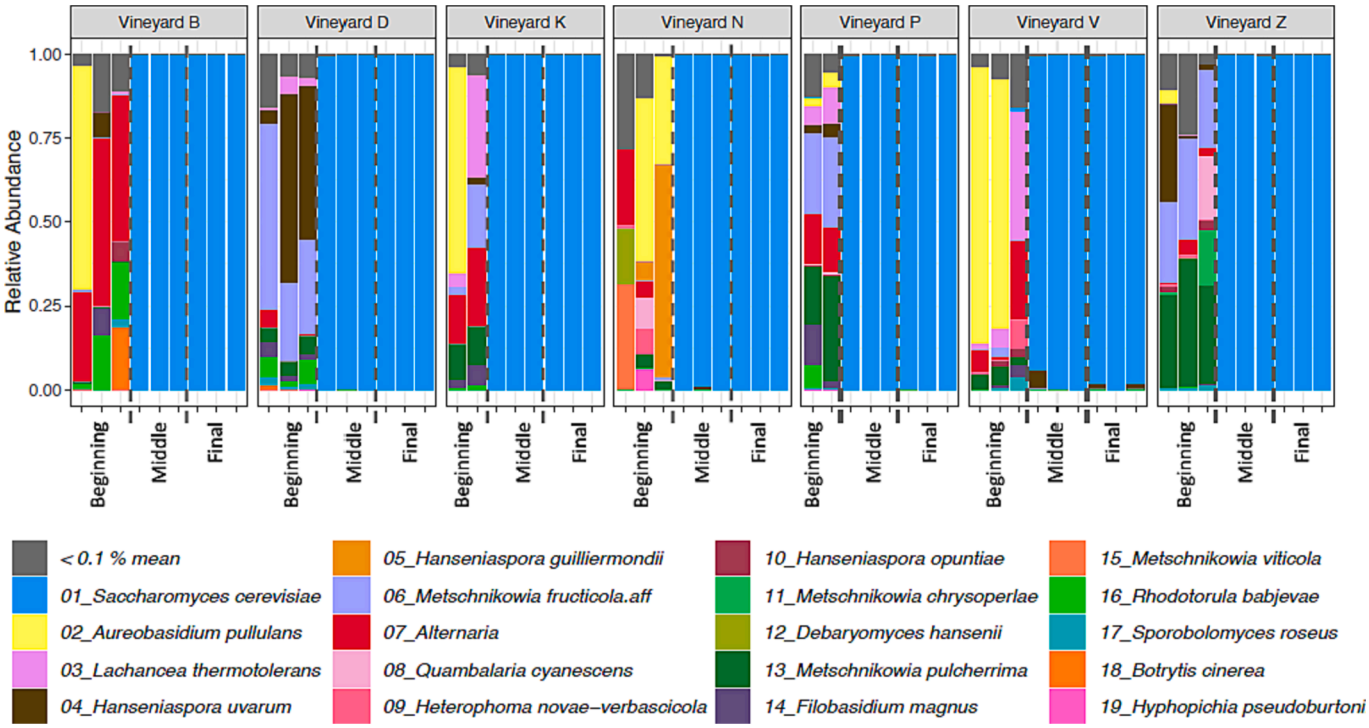


Fig. 2. Mycobiota composition and distribution. Stacked bar plots showing mycobiota composition (relative abundance) at the genus or species taxa ranks, with colour coding keys. Samples are grouped following the temporal sampling order in each vineyard (coded as B, D, K, N, P, V and Z). Taxa are sorted and enumerated (from 1 to 19) in the legend from the most to the least abundant; only taxa with an average abundance > 0.1 % have been displayed.

plots 0.05–5 mg/L, except tyrosol, with a concentration range of 2.5–50 mg/L. Wine samples for the analysis were filtered through 0.45-μm-pore size PTFE filters prior to analysis, and 10 μL of the sample was injected into the system. All samples were analysed in duplicate.

2.7. Statistical analysis

Statistical elaboration for phenol compounds and oenological parameters was performed with IBM®SPSS® Statistica for Windows, version 23.0 (SPSS Inc., Chicago, IL, USA). The data analyses were subjected to the analysis of variance (ANOVA) test coupled with post hoc multiple comparisons using Tukey's range. Principal component analysis (PCA) was generated by using the metabolomics data analysis programme MetaboAnalyst v.5.0. (https://www.metaboanalyst.ca) (accessed on 14 July 2023) created at the University of Alberta, Canada.

Unless otherwise stated, statistical analyses and data plotting for metataxonomic analysis were performed in the R environment (R version 4.1.1; https://www.r-project.org). Data normality and homogeneity were checked by means of the Shapiro–Wilk W test and Levene's tests, respectively. Variation and differences between multiple groups were assessed with one-way ANOVA (coupled with Tukey's post hoc test) and Kruskal–Wallis's test (coupled with pairwise Wilcoxon's test) for parametric and non-parametric data, respectively. Pairwise comparisons were alternatively performed with Wilcoxon and *t*-tests according to data normality.

Principal coordinate analysis (PCoA) was used to visualise beta-diversity distance (weighted UniFrac). Significant effects of different vineyard origins on the fungal community variations were evaluated with permutational multi-variate ANOVA (*adonis* function based on 999 permutations and Bray–Curtis dissimilarity distances). To identify species and/or genera that were specifically abundant in a given vineyard, the Indicator Species Analysis was conducted using the *multi-patt* function and verified with *strassoc–signassoc* functions in the package *indispecies* (De Cáceres and Legendre, 2009). Significant associations between taxa and vineyards were visualised in a bipartite network-constructed Gephi suite (version 0.10.0; https://gephi.org).

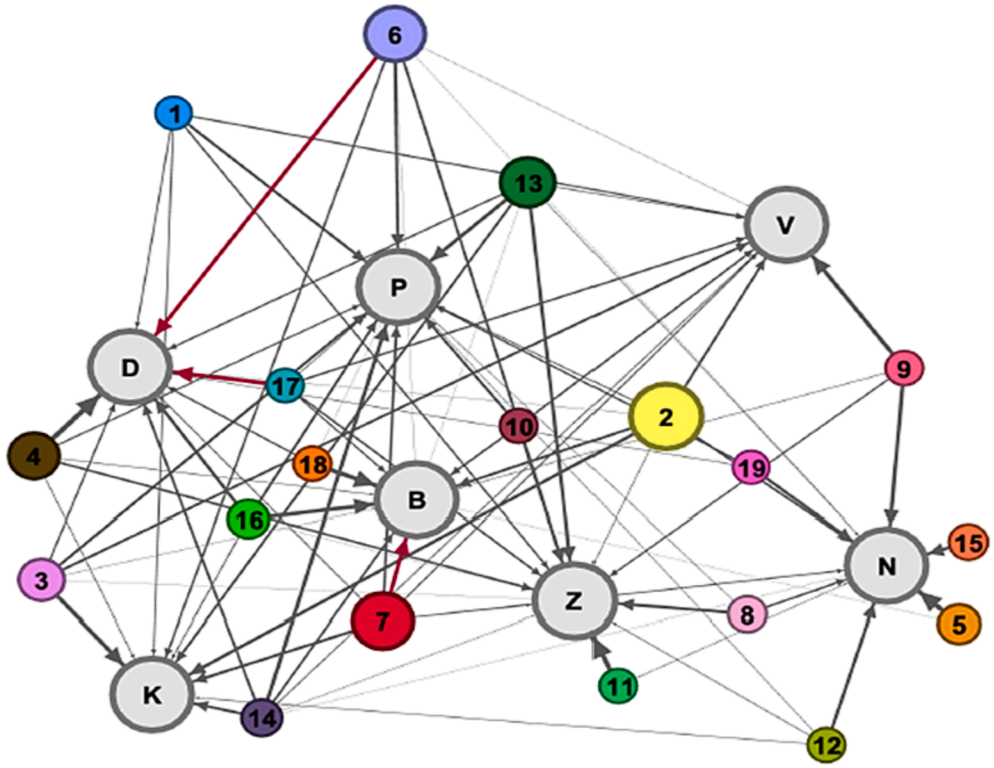
Correlation analysis between fungal taxa at the species or genus level and chemical compounds (phenols, organic acid and alcohol) was performed by means of Spearman's rank correlation.

3. Results and discussion

3.1. Composition and distribution of mycobiota during spontaneous fermentation

A total of 213 unique ASVs were detected in the 61 samples analysed and thus collapsed into 95 different taxa at the species or genus level, of which the first 19 represented cumulatively > 70 % of relative abundance in all samples: *Saccharomyces cerevisiae*, *Aureobasidium pullulans*, *Lachancea thermotolerans*, *H. uvarum*, *Hanseniaspora guilliermondii*, *Metschnikowia fructicola*, *Alternaria*, *Quambalaria cyanescens*, *Heterophoma novae-verbascicola*, *Hanseniaspora opuntiae*, *Metschnikowia chrysoperlae*, *Debaryomyces hansenii*, *M. pulcherrima*, *Filobasidium magnus*, *Metschnikowia viticola*, *Rhodotorula babjevae*, *Sporobolomyces roseus*, *Botrytis cinerea* and *Hyphopichia pseudoburtonii* (Fig. 2). *M. pulcherrima*, *M. fructicola*, *H. uvarum* and *S. cerevisiae* represented the core mycobiota detected during fermentations. However, *S. cerevisiae* took over the dominance of the mycobiota from the middle stage in all fermentations, representing, in most cases, the only taxa present. This was expected since ethanol produced during alcoholic fermentation inhibits the growth of non-*Saccharomyces* yeasts, which is mainly attributed to their oxidative and weak fermentative metabolism (Bokulich et al., 2016). Moreover, the dramatic reduction of biodiversity has been evident from observing the alpha-diversity metrics (observed species, Shannon's index, phylogenetic diversity) and the distribution of the samples in the biplot of PCoA performed on the beta-diversity distance matrix (Supplementary Fig. 3).

In relation to the mycobiota uniformity from the second sampling point, we focused on the beginning stage of fermentation, and we detected the significant associations (Indicator Species Analysis; multi-



1 – *S. cerevisiae*
2 – *A. pullulans*
3 – *L. thermotolerans*
4 – *H. uvarum*
5 – *H. guillermondii*
6 – *M. fructicola*
7 – *Alternaria*
8 – *Q. cyanescens*
9 – *Het. novae-verbascicola*
10 – *H. opuntiae*
11 – *M. chrysoperlae*
12 – *D. hansenii*
13 – *M. pulcherrima*
14 – *F. magnus*
15 – *M. viticola*
16 – *R. babjavae*
17 – *S. roseus*
18 – *B. cinerea*
19 – *H. pseudoburtonii*

Fig. 3. Bipartite network shows the distribution of taxa (genus or species rank) in the seven vineyards. Taxa (coloured nodes) are unidirectionally connected with arrows (edges) to the vineyards (B, D, K, N, P, V and Z) if detected at the beginning of fermentation; red edges highlight the significant association between taxa and vineyard (Indicator Species Analysis: multi-part statistics; R > 0.4 and $p < 0.05$). Nodes are made proportional to taxa abundances at the fermentation beginning (log Transformed); for taxa, numeric code and colour refer to the colour coding keys of graph A. The network layout was constructed using the ForceAtlas2 algorithm, with edge thicknesses directly proportional to the association strength.

patt statistic: R > 0.4 and $p < 0.05$) between ASVs and vineyards, which were displayed in a bipartite network (Fig. 3).

Among the core taxa, *M. fructicola* was significantly associated with vineyard D, whereas *Alternaria* and *S. roseus* were indicators of B and D vineyards, respectively. Moreover, *H. guilliermondii* was exclusively detected in vineyard N. These results are consistent with other studies

Table 1
Concentration of basic physicochemical parameters (mean ± standard deviation, n = 3) for seven experimental wines and Maraština musts. The vineyards B, D, K and P belong to the Central and Southern Dalmatia sub-regions, and N, V and Z belong to the Northern Dalmatia sub-region.

	Physicochemical parameter	Vineyard							S
		B	D	K	N	P	V	Z	
MUST	°Oe	100.33 ± 1.53^{d}	75.67 ± 1.15^{a}	101 ± 1^{d}	95.67 ± 2.52^{c}	89.67 ± 2.08^{b}	95.67 ± 0.58^{c}	91.00 ± 1.73^{b}	*
	Total acidity (g/L)	3.85 ± 0.15^{a}	5.72 ± 0.67^{c}	3.70 ± 0.27^{a}	3.73 ± 0.07^{a}	4.14 ± 0.34^{ab}	4.97 ± 0.24^{bc}	5.05 ± 0.27^{bc}	*
	Glucose/fructose	1.10 ± 0.10^{a}	1.20 ± 0.00^{a}	1.00 ± 0.10^{a}	1.00 ± 0.00^{a}	1.00 ± 0.10^{a}	1.00 ± 0.10^{a}	1.10 ± 0.10^{a}	ns
	Lactic acid (g/L)	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	ns
	Malic acid (g/L)	0.67 ± 0.37	1.55 ± 0.41	0.88 ± 0.20	1.03 ± 0.07	0.80 ± 0.61	0.81 ± 0.10	1.11 ± 0.00	ns
	Tartaric acid (g/L)	2.78 ± 0.68	2.77 ± 0.86	2.33 ± 0.24	2.99 ± 0.38	2.41 ± 0.25	2.95 ± 0.46	3.59 ± 0.00	ns
WINE	Alcohol (vol%)	13.94 ± 0.87^{b}	9.90 ± 1.12^{a}	13.97 ± 0.15^{b}	13.30 ± 0.46^{b}	12.12 ± 0.80^{b}	13.22 ± 1.21^{b}	12.47 ± 1.37^{b}	*
	Lactic acid (g/L)	0.63 ± 0.06^{abc}	0.81 ± 0.07^{bc}	0.55 ± 0.06^{ab}	0.61 ± 0.07^{abc}	0.86 ± 0.09^{c}	0.53 ± 0.05^{ab}	0.35 ± 0.25^{a}	*
	Total acidity (g/L)	6.07 ± 0.12^{c}	4.95 ± 0.19^{a}	6.03 ± 0.15^{c}	5.57 ± 0.25^{bc}	5.80 ± 0.44^{c}	6.63 ± 0.06^{c}	5.10 ± 0.10^{ab}	*
	Volatile acidity (g/L)	0.53 ± 0.06	0.50 ± 0.00	0.47 ± 0.06	0.43 ± 0.06	0.40 ± 0.00	0.43 ± 0.06	0.43 ± 0.06	ns
	Malic acid (g/L)	0.87 ± 0.03	1.33 ± 0.40	1.23 ± 0.09	1.26 ± 0.28	1.11 ± 0.14	1.36 ± 0.23	1.41 ± 0.14	ns
	Glycerol (g/L)	6.10 ± 0.26	6.07 ± 0.15	6.37 ± 0.06	6.23 ± 0.51	5.83 ± 0.75	6.83 ± 0.25	6.30 ± 0.00	ns

Concentrations within rows with different letters (^{a,b,c}) are significantly different at $p < 0.05$. Abbreviations: S, signification; *, significant differences; ns, no significant differences.

where authors highlighted the species from the genera *Hanseniaspora* and *Metschnikowia* as the most abundant non-*Saccharomyces* yeasts in the initial stage of fermentation (Albergaria and Arneborg, 2016; Raymond Eder et al., 2017). Previous studies reported the presence of *Metschnikowia*, *Candida*, *Lachancea*, *Torulaspora* and *Hanseniaspora* genera after 7 days of spontaneous fermentation (Franco et al., 2021), with the latter genus identified even at the end of spontaneous fermentation (Shimizu et al., 2023).

3.2. Physiochemical parameters of Maraština wines and their correlation with mycobiota

The basic physiochemical compounds of Maraština must and wines are reported in Table 1.

The ethanol concentration in Maraština wine D (9.90 vol%) was notably lower than that in other wines, with the highest concentration observed in the wine produced from grapes harvested in the K vineyard (13.97 vol%). Thus, the observed correlations with the mycobiota (Fig. 4) are not reliable. Despite variations in grape must sugar content, notably, all the grapes were harvested with a glucose/fructose ratio equal to 1. In terms of total acidity, statistically significant differences were observed in grape musts and in the resulting wines. The must obtained from grapes harvested in vineyard D was characterised by a significantly higher concentration (5.72 g/L) than the musts obtained from vineyards B, K and N (<3.85 g/L). Conversely, wine D had the lowest concentration of total acidity (4.96 g/L), which statistically differs from wines B, K, P, V and N (>5.50 g/L). The concentration of lactic acid in wines was comprised between 0.35 (vineyard Z) and 0.86 g/L (vineyard P), whereas lactic acid was absent in all grape musts. Lactic acid was correlated positively with *B. cinerea* and negatively with *Het. novae-verbascicola* present in the grape musts (Fig. 4). The study conducted by Wang et al. (2016) revealed that wild *B. cinerea* isolates can moderately metabolise both D- and L-malic acids, whereas L-lactic acid is not metabolised. Moreover, the positive correlation observed between *B. cinerea* and lactic acid concentrations in our study suggests the potential for *B. cinerea* to produce lactic acid. This is supported by previous research (Shimazu et al., 1984), which demonstrated that *B. cinerea* can produce L-lactic acid from L-tartaric acid, especially at a pH level of 3.5, and can also accumulate pyruvic, acetic and D-glyceric acids. However, the observed correlation may also result from indirect factors, such as conditions promoting both *B. cinerea* growth and the production of lactic acid by other microorganisms, principally lactic acid bacteria and yeast *L. thermotolerans*. Even if the latter species was identified in the analysed musts, no significant correlation with the lactic acid concentration emerged. This suggests that, whereas *B. cinerea* might contribute to lactic acid production, further investigation is necessary to clarify the

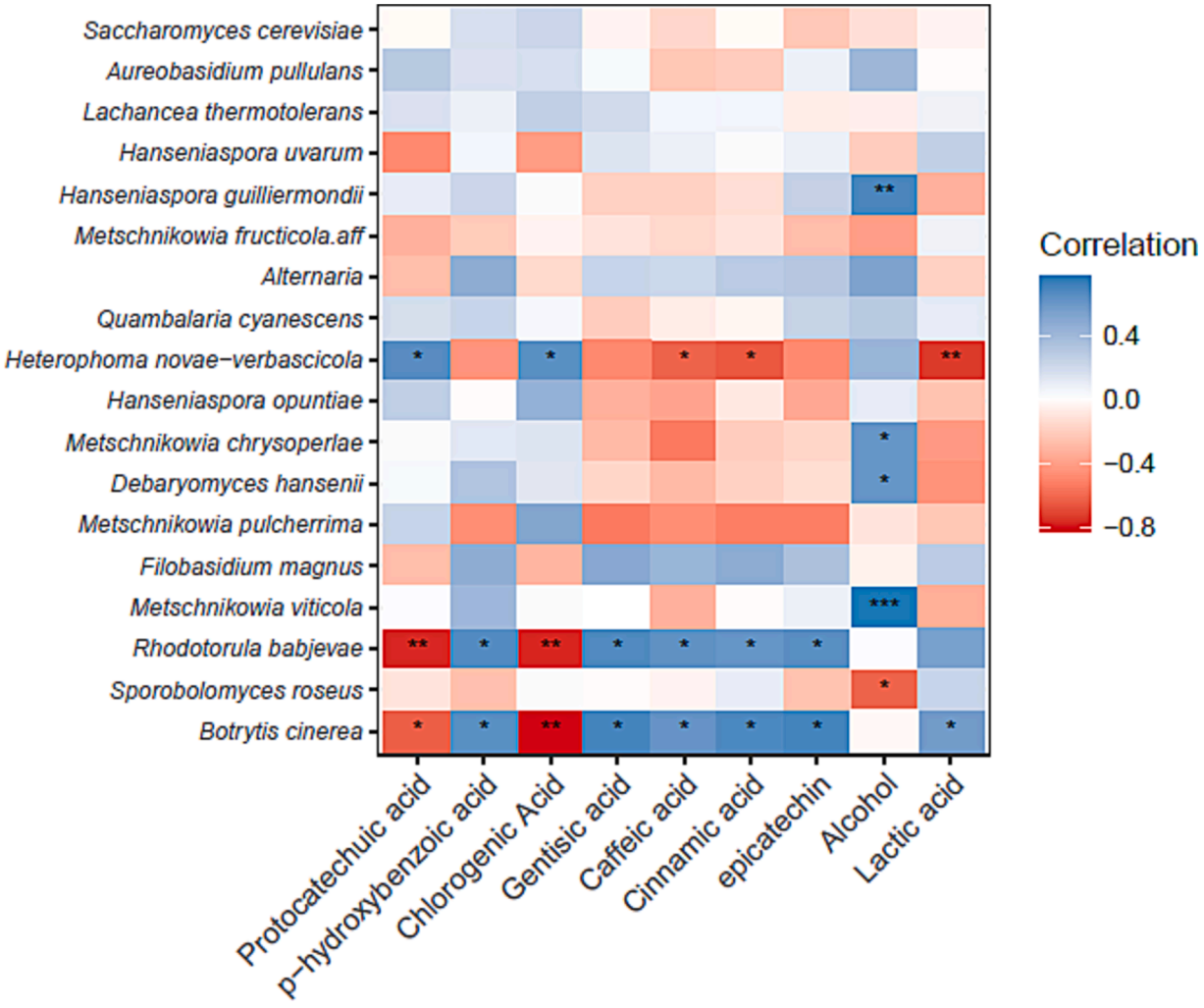


Fig. 4. Correlation between metataxonomic and chemical compounds. Tile plots showing the existing pairwise correlations between phenols, organic acids, alcohol and fungal ASVs (merged at the species/genus level). Taxa are ordered from the more abundant to the less abundant. Colours represent the level of Spearman's Rho correlation (from − 1 to 1; caption), and significant positive and negative correlations are highlighted with asterisks (*p*-value [FDR adjusted]: * = <0.05; ** = <0.01; *** = <0.001).

precise source of lactic acid in this context.

3.3. Phenolic profile of Maraština wines and its correlation with mycobiota

The phenolic profile of seven Maraština wines consisted of 21 compounds (Table 2) sorted by chemical classes. In general, different yeasts can affect the phenolic content of wine through biochemical, biological and physical mechanisms (Lee et al., 2019). The physical mechanism considers the capacity of yeast cells to adsorb phenolic compounds (Morata et al., 2016). During fermentation, autolysed and dead yeast cells can still have β-glucosidase activity, which can impact wine phenols (Pérez-Serradilla and De Castro, 2008).

Tyrosol, known as a phenolic antioxidant compound, was the most abundant phenol detected in Maraština wines under study in concentrations (22.48–47.09 mg/L) higher than those previously detected in different Italian white wines (1.10–6.00 mg/L) and Chardonnay (21.60 mg/L) (Minussi et al., 2003). The concentration of this component, which did not show statistical differences among vineyards, could be a notable characteristic of the Maraština variety. Notably, this compound is related to the Mediterranean diet and is rarely investigated in wines (di Benedetto et al., 2007). Furthermore, Lukić et al. (2017) reported that different pre-fermentation treatments do not considerably impact the concentration of tyrosol, thus suggesting grape juice as a primary source of this compound derived from the amino acid tyrosine. The conversion from tyrosine to tyrosol is a chemical reaction catalysed by enzymes involved in the Erlich pathway of yeasts, as recently examined in a study including *S. cerevisiae* and *Escherichia coli* (Ruan et al., 2022).

The Maraština wines obtained by spontaneous fermentation showed higher concentrations of hydroxybenzoic acids than of hydroxycinnamic acids, which is not in line with the results reported by Lukić et al. (2019) for commercial Maraština wines.

Seven hydroxybenzoic acids were identified and quantified in Maraština wines by HPLC-UV/Vis: *p*-hydroxybenzoic acid (0.29–1.86 mg/L), gentisic acid (1.63–2.57 mg/L), gallic acid (1.12–1.89 mg/L), syringic acid (0.40–0.97 mg/L), sinapic acid (0.10–0.18 mg/L) and 2,6-dimetoxybenzoic acid (0.30–0.70 mg/L), among which protocatechuic acid was the most abundant phenolic acid (4.14–9.59 mg/L). Lukić et al. (2019) found that the concentration of hydroxybenzoic acids, especially *p*-hydroxybenzoic acid, was a good marker for differentiating between monovarietal wines made from the white grape varieties Pošip and Maraština. Among the six hydroxycinnamic acids determined in our study, caffeic acid had the highest concentration (0.64–5.06 mg/L), especially evident in wines produced from the grapes collected from vineyards located on Korčula Island (CSD). The concentration of ferulic acid (0.02–0.09 mg/L) obtained by spontaneous fermentations is extremely similar to data previously reported for the commercially produced Maraština (0.07 mg/L) (Lukić et al., 2019) and Chardonnay (0.05 mg/L) wines (Jandera et al., 2005). Although phenolic acids in wine derive from grape berries, concentrations of phenolic acids in wine could be influenced by the enzymatic activity of the yeasts (Monagas et al., 2005; Zhang et al., 2021). According to the means of Spearman's rank correlation between the initial mycobiota composition and phenolic compounds, very few significant correlations emerged (Fig. 4). *R. babjevae* and *B. cinerea* were correlated negatively with protocatechuic acid and chlorogenic acid and positively with *p*-hydroxybenzoic acid, gentisic acid, caffeic acid and cinnamic acid. The unexpected positive correlation between *B. cinerea* and caffeic acid, despite its known laccase activity that typically oxidises caffeic acid (Zimdars et al., 2017), could be attributed to the variable physicochemical and kinetic properties of fungal laccases, which may exhibit reduced activity under certain conditions. This polymorphism is not only evident between fungal species but also within the same species under different conditions (Quijada-Morin et al., 2018; Zouari et al., 1987). The less active laccase under specific conditions might allow some caffeic acid to remain unoxidised, resulting in the observed positive correlation. Additionally, the complex enzymatic reactions during fermentation could involve interactions with other enzymes or

Table 2
Concentration (mg/L) of phenolic compounds (mean ± standard deviation, n = 3) identified and quantified in seven Maraština wines by HPLC-UV/Vis.

Phenol	RT (min)	Concentration (mg/L)							S
		B	D	K	N	P	V	Z	
Hydroxybenzoic acid									
Protocatechuic acid	12.895	4.48 ± 0.39^{a}	4.14 ± 1.24^{a}	4.97 ± 0.39^{a}	7.37 ± 0.32^{b}	5.77 ± 0.96^{a}	9.59 ± 0.40^{c}	5.51 ± 0.50^{a}	*
p-Hydroxybenzoic acid	18.079	0.84 ± 0.16^{b}	0.48 ± 0.15^{ab}	0.69 ± 0.09^{ab}	0.52 ± 0.29^{ab}	1.86 ± 0.34^{c}	0.47 ± 0.05^{ab}	0.29 ± 0.13^{a}	*
Gentisic acid	20.518	2.41 ± 0.33^{ab}	2.55 ± 0.27^{b}	1.92 ± 0.13^{ab}	1.68 ± 0.53^{a}	2.57 ± 0.28^{b}	1.98 ± 0.19^{ab}	1.63 ± 0.08^{a}	*
Gallic acid	7.670	1.41 ± 0.26^{ab}	1.89 ± 0.18^{b}	1.48 ± 0.14^{ab}	1.42 ± 0.21^{ab}	1.12 ± 0.08^{a}	1.54 ± 0.16^{ab}	1.42 ± 0.15^{ab}	*
Syringic acid	21.470	0.97 ± 0.46	0.60 ± 0.01	0.96 ± 0.40	0.72 ± 0.11	0.72 ± 0.25	0.44 ± 0.18	0.40 ± 0.25	ns
Sinapic acid	36.034	0.10 ± 0.03	0.18 ± 0.00	0.10 ± 0.04	0.20 ± 0.06	0.15 ± 0.04	0.12 ± 0.08	0.10 ± 0.05	ns
2,6-Dimetoxybenzoic acid	31.387	0.38 ± 0.37	0.29 ± 0.19	0.30 ± 0.19	0.18 ± 0.08	0.7 ± 0.66	0.18 ± 0.10	0.27 ± 0.33	ns
Hydroxycinnamic acid									
p-Coumaric acid	30.008	0.05 ± 0.02^{a}	0.2 ± 0.02^{b}	0.18 ± 0.03^{b}	0.08 ± 0.02^{a}	0.23 ± 0.04^{b}	0.04 ± 0.01^{a}	0.05 ± 0.02^{a}	*
Ferulic acid	35.436	0.02 ± 0.00^{a}	0.02 ± 0.00^{a}	0.09 ± 0.01^{b}	0.09 ± 0.01^{b}	0.04 ± 0.01^{a}	0.03 ± 0.01^{a}	0.03 ± 0.02^{a}	*
Chlorogenic acid	18.381	0.15 ± 0.02^{ab}	0.08 ± 0.03^{a}	0.22 ± 0.04^{b}	0.21 ± 0.02^{b}	0.16 ± 0.01^{ab}	0.42 ± 0.09^{c}	0.25 ± 0.02^{b}	*
Caffeic acid	20.748	3.47 ± 1.26^{bc}	5.06 ± 0.69^{c}	3.01 ± 1.12^{bc}	2.59 ± 0.89^{ab}	5.16 ± 0.86^{c}	0.64 ± 0.38^{a}	0.56 ± 0.12^{a}	*
Cinnamic acid	45.922	0.61 ± 0.12	0.62 ± 0.24	0.50 ± 0.25	0.42 ± 0.21	0.75 ± 0.20	0.28 ± 0.18	0.21 ± 0.05	ns
o-Coumaric acid	42.717	2.61 ± 0.18	1.69 ± 2.23	3.61 ± 0.39	3.68 ± 0.53	3.00 ± 0.15	2.90 ± 0.41	2.07 ± 0.74	ns
∑Phenolic acids		17.50 ± 0.57^{b}	17.81 ± 2.27^{b}	18.01 ± 1.55^{b}	19.17 ± 0.49^{b}	22.23 ± 0.76^{c}	18.64 ± 1.21^{b}	12.78 ± 1.01^{a}	*
Catechin	18.176	1.02 ± 0.45^{b}	0.13 ± 0.01^{a}	0.57 ± 0.05^{ab}	0.41 ± 0.22^{a}	0.36 ± 0.05^{a}	0.10 ± 0.06^{a}	0.08 ± 0.07^{a}	*
Epigallocatechin gallate	23.537	1.00 ± 0.16^{bc}	0.99 ± 0.09^{bc}	0.82 ± 0.03^{ab}	1.01 ± 0.06^{bc}	0.72 ± 0.05^{a}	1.11 ± 0.10^{c}	0.88 ± 0.04^{abc}	*
Epicatechin	22.005	1.26 ± 0.94	1.24 ± 1.23	0.53 ± 0.13	0.48 ± 0.14	1.06 ± 0.18	0.14 ± 0.05	0.10 ± 0.06	ns
∑Flavan-3-ols		3.28 ± 1.48^{b}	2.36 ± 1.31^{ab}	1.91 ± 0.20^{ab}	1.90 ± 0.23^{ab}	2.14 ± 0.18^{ab}	1.35 ± 0.09^{ab}	1.06 ± 0.08^{a}	ns
Naringenin	47.418	0.12 ± 0.04^{ab}	0.12 ± 0.04^{ab}	0.08 ± 0.04^{ab}	0.19 ± 0.01^{bc}	0.28 ± 0.04^{c}	0.05 ± 0.06^{a}	0.06 ± 0.02^{a}	*
Quercetin	44.831	0.61 ± 0.29	0.37 ± 0.03	0.50 ± 0.19	0.62 ± 0.06	0.41 ± 0.12	0.43 ± 0.17	0.34 ± 0.19	ns
∑Flavonols		0.72 ± 0.30	0.49 ± 0.02	0.59 ± 0.23	0.81 ± 0.07	0.69 ± 0.16	0.48 ± 0.23	0.40 ± 0.20	ns
Resveratrol	43.747	0.10 ± 0.01^{ab}	0.08 ± 0.03^{ab}	0.10 ± 0.04^{ab}	0.14 ± 0.03^{b}	0.06 ± 0.03^{a}	0.06 ± 0.02^{a}	0.04 ± 0.01^{a}	*
∑Stilbene		0.10 ± 0.01^{ab}	0.08 ± 0.03^{ab}	0.10 ± 0.04^{ab}	0.14 ± 0.03^{b}	0.06 ± 0.03^{a}	0.06 ± 0.02^{a}	0.04 ± 0.01^{a}	*
Vanillin	26.931	0.56 ± 0.45	0.04 ± 0.00	0.02 ± 0.02	0.03 ± 0.01	0.06 ± 0.01	0.07 ± 0.03	0.05 ± 0.03	ns
∑Phenolic aldehyde		0.56 ± 0.45	0.04 ± 0.00	0.02 ± 0.02	0.03 ± 0.01	0.06 ± 0.01	0.07 ± 0.03	0.05 ± 0.03	ns
Tyrosol	16.662	25.1 ± 2.15	29.93 ± 5.78	47.09 ± 3.72	29.81 ± 4.86	22.48 ± 1.63	27.94 ± 24.82	27.49 ± 4.95	ns
∑Phenolic alcohol		25.1 ± 2.15	29.93 ± 5.78	47.09 ± 3.72	29.81 ± 4.86	22.48 ± 1.63	27.94 ± 24.82	27.49 ± 4.95	ns

Concentrations within rows with different letters are significantly different at $p < 0.05$. Abbreviations: RT, retention time; S, significance; *, significant difference; ns, no significant difference.

compounds inhibiting laccase activity and preserving caffeic acid. Furthermore, a positive correlation was observed between *Het. novae-verbascicola* and protocatechuic and chlorogenic acids, as well as a negative correlation between this species and caffeic and cinnamic acids. Uzkuç et al. (2020) found that spontaneous fermentation increases the concentrations of gallic, coumaric and syringic acids in wines compared to sequential fermentation. This could be explained by the influence of native grape mycobiota on these phenolic compounds.

Epicatechin (0.10–1.26 mg/L), catechin (0.08–1.02 mg/L) and epigallocatechin gallate (0.88–1.11 mg/L), identified in Maraština wines, belong to the flavan-3-ols phenol classes, which are reported to have good antioxidant activity (Hornedo-Ortega et al., 2020) and positive health benefits (Fernandes et al., 2017) in white wines. In addition to the negative correlations of epicatechin with the *Metschnikowia* genus, *B. cinerea* and *R. babjevae* species were positively correlated. As a pathogen species, *B. cinerea* could impact the synthesis of epicatechin in grapes due to the plant's defence mechanism (Muganu and Paolocci, 2013). Flavonols are another important subclass of phenolic flavonoids known for their antioxidant properties and their beneficial health effects when present in white wine (Fernandes et al., 2017). Naringenin (0.05–0.28 mg/L) and quercetin (0.34–0.62 mg/L) are the only two flavanols identified in Maraština wines, but no correlation with grape mycobiota was found (Fig. 4). Among 21 phenolic compounds, resveratrol was the only stilbene found in Maraština wines (0.06–0.14 mg/L). Several studies have shown that different yeasts (e.g. *M. pulcherrima* and *S. cerevisiae*) have a significant influence on the level of resveratrol in wine (Clare et al., 2005; Vacca et al., 1997). However, no correlation between resveratrol and the autochthonous Maraština mycobiota was

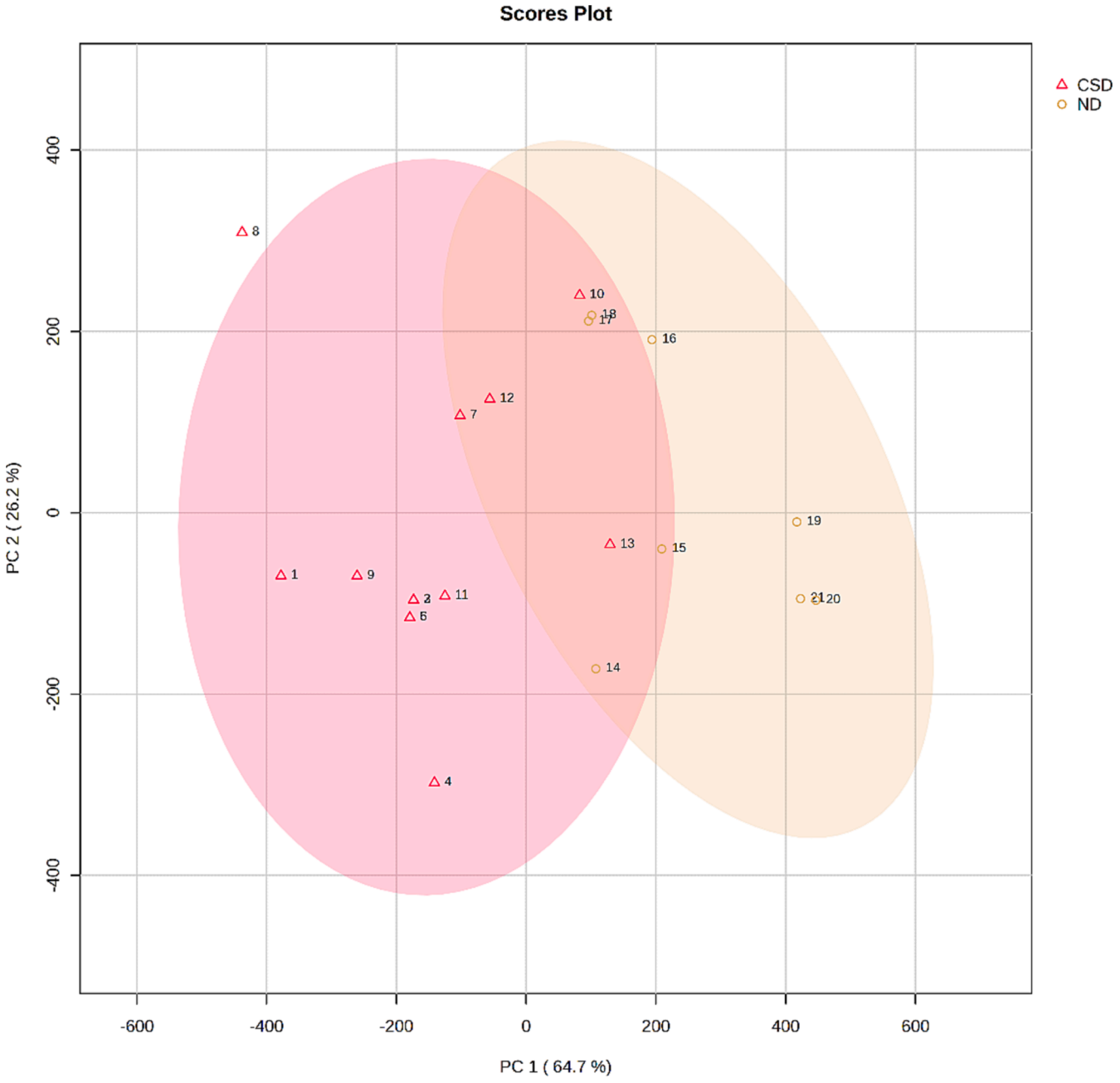


Fig. 5. Principal component analysis (PCA) of the phenolic composition of Maraština wines from the Central and Southern Dalmatia (CSD) and Northern Dalmatia (ND) winegrowing sub-regions. The dataset comprises 12 phenolic components that showed significant statistical differences among the various wines. Sample marks: 1–3 (wine D), 4–6 (wine P), 7–9 (wine B), 10–12 (wine K), 13–15 (wine N), 16–18 (wine Z) and 19–21 (wine V).

observed in this study.

3.4. Impact of the vineyard site on the phenolic profile

Numerous studies have shown that soil type, climate, training systems, canopy management and cultural practices strongly impact shoot growth, yield per vine and the chemical composition of the berries (Del Barrio-Galán et al., 2021; Urvieta et al., 2021). In the present study, the vineyards of two winegrowing sub-regions encompass a geographically restricted zone characterised by comparable climatic, pedological and agrobiological conditions. In addition to grape mycobiota, these conditions facilitate the production of wine that exhibits distinct characteristics specific to each sub-region. The PCA performed on the HPLC reduced data set allowed for a good separation of Maraština wines obtained from two winegrowing sub-regions. In a projection of 12 statistically significant phenolic compounds that defined the principal components PC1 and PC2, the first two principal components explained 90.4 % of the variability (Fig. 5). PC1 accounted for 64.47 % of the total variability, whereas PC2 accounted for 26.20 % of the variability. The wines originating from vineyards B, D, K and P, belonging to the CSD sub-region, demonstrated clear distinctions when compared to the wines produced in the ND sub-region's vineyards N, V and Z. Notably, the wines from the ND sub-region tended to have positive PC1 values. Wines from the CSD sub-region displayed a higher concentration of detected phenolic compounds in this study. The CSD sub-region had higher temperatures and greater overall precipitation. These findings contradicted previous studies conducted by Bonada et al. (2015) and Urvieta et al. (2021). They established a positive correlation between phenolic composition and climatic conditions characterised by lower temperatures and precipitation.

4. Conclusion

Among the 95 different taxa identified during the spontaneous alcoholic fermentation of fresh Maraština musts, *M. pulcherrima*, *M. fructicola*, *H. uvarum* and *S. cerevisiae* represented the core mycobiota. Our results reveal significant correlations between the autochthonous mycobiota members present at the initial fermentation phase and the phenolic compounds observed in Maraština wines. Furthermore, wines from the CSD sub-region had a higher concentration of total phenolic compounds, likely due to their higher temperatures and greater precipitation. Overall, these findings contribute to a more profound understanding of the various species' roles in phenolic transformation during spontaneous fermentation. Such insights have the potential to enhance the production of high-quality wines with distinctive *terroir* characteristics.

CRediT authorship contribution statement

Ana Boban: Writing – original draft, Methodology, Investigation, Formal analysis. **Vesna Milanović:** Writing – original draft, Visualization, Resources, Investigation, Formal analysis, Conceptualization. **Maja Veršić Bratinčević:** Formal analysis, Writing – original draft. **Cristian Botta:** Writing – original draft, Validation, Formal analysis. **Ilario Ferrocino:** Writing – original draft, Investigation, Formal analysis. **Federica Cardinali:** Investigation, Formal analysis. **Stipe Ivić:** Investigation. **Giorgia Rampanti:** Formal analysis. **Irena Budić-Leto:** Writing – review & editing, Visualization, Supervision, Resources, Project administration, Investigation, 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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2024.114072.

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