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Croatian white grape variety Maraština: first taste of its indigenous mycobiota

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30 Abstract

31

32 The indigenous vineyard mycobiota contribute both to wine quality and vineyard sanitary status. Wines made
33 from same grape variety but from different geographical locations are appreciated for their diversity. Because
34 no information on indigenous mycobiota of Croatian grapevines is available, the aim of the present study was
35 to start filling this knowledge gap by characterizing the indigenous mycobiota of Maraština variety. The use
36 of metataxonomic approach has enabled the identification of 25 different fungal genera present on Maraština
37 grape berries collected from 11 vineyards located within the Croatian coastal winegrowing region of Dalmatia
38 (northern Dalmatia, Dalmatian hinterland, central and southern Dalmatia). The substantial regional and local
39 scale differences in their distribution were observed, thus supporting the concept of microbial terroir. Overall,
40 *Aureobasidium* was the dominant genus followed by *Cladosporium* and *Metschnikowia*. *Botrytis* and
41 *Plenodomus* were associated with the vineyards located in central Dalmatia, whereas *Pichia* was associated
42 with northern Dalmatia vineyards. The largest abundance of *Buckleyzyma*, *Cladosporium*, *Eremothecium*,
43 *Fusarium*, *Papiliotrema*, and *Rhodotorula* was observed in Dalmatian hinterland. Moreover, data suggested
44 that climate conditions and soil type partially influenced the distribution of fungal communities. The local-
45 scale differences emerged also for the physicochemical characteristics of fresh musts. The high malic acid
46 content supported the development of *Metschnikowia*, and inhibited *Fusarium* growth, whereas a positive
47 correlation between *Erysiphe* and pH values was observed. *Sporobolomyces* and *Cystobasidium* were
48 negatively associated with high glucose concentration. The revealing of Maraština indigenous mycobiota
49 provided information on the members of fungal community negatively influencing the grapevine sanitary
50 status as well as those which could be employed in disease biocontrol. The presence of autochthonous yeasts
51 belonging to genera *Hanseniaspora*, *Metschnikowia*, *Lachancea*, *Pichia* and *Hyphopichia* could confer
52 possible improvements to sensory characteristics of wine.

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54 **Keywords:** Maraština, indigenous mycobiota, microbial terroir, metataxonomic approach, grapevine,
55 Dalmatia, *Aureobasidium*

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58 1. Introduction

59

60 *Vitis vinifera* L., native to southern Europe and western Asia, as well as other *Vitis* L. species are grown
61 worldwide mostly for wine production (Pancher et al., 2012). Despite the fermentation of wine being strictly
62 correlated to the conversion of sugar into ethanol, it is a complex procedure that starts in the vineyard and ends
63 with the consumption (Bokulich et al., 2014). The indigenous vineyard mycobiota, including yeasts and other
64 fungal communities, contribute both to wine quality and vineyard sanitary status. Yeast colonizing grape
65 berries produce various compounds that can exert positive or even detrimental effects on the wine quality and
66 aroma complexity (Capozzi et al., 2015). The grape berry surface is dominated by non-*Saccharomyces* yeasts,
67 including basidiomycetous oxidative species from the genera *Filobasidium*, *Cryptococcus* and *Rhodotorula*;
68 ascomycetous oxidative or weakly fermentative species from the genera *Aureobasidium* (yeast-like fungus),
69 *Hanseniaspora*, *Candida*, *Metschnikowia*, *Debaryomyces*, *Pichia*, and *Lachancea* as well as fermentative
70 species from the genera *Saccharomyces*, *Torulaspora*, *Zygosaccharomyces*, *Dekkera/Brettanomyces*,
71 *Schizosaccharomyces*, and *Saccharomycodes* (Setati et al., 2015). The grapes mycobiota also include fungal
72 obligate parasites such as *Plasmopara viticola* and *Erysiphe necator*, responsible for downy and powdery
73 mildew, respectively, as well as saprophytic moulds including *Botrytis cinerea*, causing grey rot, and other
74 ubiquitous genera such as *Aspergillus*, *Cladosporium* and *Penicillium*, responsible for various grape rots or
75 ochratoxin production (Barata et al., 2012). However, the surface of grape berries is an unstable habitat for
76 microorganisms whose composition and the abundance are mainly driven by grape variety, the vineyard
77 geographical position, local and regional climate (temperature, precipitation, relative humidity), soil, growth
78 stage of the berries, health status of the grapevine, and the viticultural management practices (organic or
79 commercial vineyard) (Milanović et al., 2013; Zhu et al., 2021). The vineyard mycobiota have been extensively
80 studied using traditional culture-dependent methods that might miss up to 95% of the community due to low
81 frequency or the presence of viable but non-culturable cells (Taylor et al., 2014). By contrast, metataxonomic
82 methods can reveal larger microbial diversity than other fingerprinting methods, thus playing a fundamental
83 role in the assessment of the grape microbiome (Rantsiou et al., 2020; Stefanini & Cavalieri, 2018).

Vineyards in Croatia cover about 25,000 ha and include 197 cultivars, among which 103 are considered indigenous (Maletic et al, 2015). Croatian wine-growing zones are divided into continental (eastern and western) and coastal region. The latter, including Istria/Kvarner and Dalmatia (northern Dalmatia, Dalmatian hinterland, central and southern Dalmatia) is located along the coast of Adriatic Sea and is characterized by Mediterranean climate (Regulation EU No 1308/2013). In contrast to the continental region, where native cultivars represent only a small fraction, in the coastal region, especially in central and southern Dalmatia, native cultivars are grown in more than 90% of the vineyards. Although the most cultivated white variety in Dalmatia is Trbljan (9.5%, 495 ha), followed by Kujunduša (6.3%, 328 ha), Maraština (4.6%, 242 ha) and Pošip (4.3%, 227 ha) (Voncina et al., 2011), Maraština is the second (after Pošip) most important variety for wine sector due to its capacity for producing high quality wines. Maraština (synonyms Rukatac, Malvasia del Chianti, Malvasia binca lunga) is characterized by small- to medium-sized grapes of a golden yellow colour with small, brown spots, thick skin and the grapes tightly packed in bunches. Maraština is considered an autochthonous Croatian white variety, although Šimon et al. (2007) reported its high similarity with the Italian variety Malvasia del Chianti and the Greek variety Pavlos. By contrast, Crespan et al. (2009) reported just seven of the 11 simple sequence repeat loci of Maraština overlapping with Malvasia del Chianti. Wines made from the same grape variety but from different geographical regions are appreciated for their differences in aroma, flavour, taste, and quality, thus leading to their higher price and market demand (van Leeuwen & Seguin, 2006). The fungal communities have been proposed as contributing to the concept of wine terroir; therefore, understanding fungal composition and dynamics among different vineyards or winegrowing regions is of great importance in the wine-making process (Alexandre, 2020). To the best of our knowledge, no report on indigenous mycobiota of Croatian grapevine cultivars is available. Hence, the aim of the present study was to employ a culture-independent metataxonomic approach to give the first insight into the fungal communities associated with Croatian white grapevine cultivar Maraština as influenced by geographical position of the vineyards located within the Croatian coastal winegrowing region of Dalmatia, including sub-regions of northern Dalmatia, Dalmatian hinterland, and central and southern Dalmatia. Correlations between the mycobiota composition and climate data, vineyard soil type and physicochemical characteristics of fresh musts were also calculated.

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112 2. Materials and methods

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115 2.1 Grape sampling

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117 Healthy and undamaged vines were used for the collection of the grape berry samples from 10 commercial
118 vineyards and the germplasm repository of native varieties cultivated at the Institute for Adriatic Crops and
119 Karst Reclamation in Split as part of the Croatian National Collection. The vineyards were located along the
120 Croatian coast in the winegrowing subregions of northern Dalmatia [Smilčić (S), Nadin (Polača) (N),
121 Stankovci (Z), Vukšić (V)], Dalmatian hinterland [Okalj (O)], and central and southern Dalmatia [Institute for
122 Adriatic Crops and Karst Reclamation in Split (IJK-RB), Kaštela (VP), Dračevica (DR), Prapatna 1 (P),
123 Prapatna 2 (B), Kruševo (K)] as shown in Figure 1. The vineyards DR, P, B and K are situated in the island of
124 Korčula. The air distance between the northernmost (S) and the southernmost vineyard (located on island
125 Korčula) is 177 km. The detailed information, including the global positioning coordinates, altitude, the
126 plantation year, soil type, row distance per vine and the trellis system for each vineyard, is reported in Table
127 1.

128 On 11th, 12th and 16th September 2021, a total of 11 technologically mature samples of Maraština grapes were
129 collected in biological triplicate. In detail, the experimental plan consisted of three randomized blocks in the
130 middle of each vineyard. A block was formed by one row of vines. The sample for each block was composed
131 of nine well-exposed bunches collected from three different vines from the beginning, middle and end of the
132 row. Only healthy and undamaged grapes (around 3 kg per vineyard) were harvested using sterile scissors,
133 placed in sterile bags, and transported to the laboratory in a cool bag. Once in the laboratory, 200 berries from
134 different parts of the grape bunches (top, centre, and bottom) were aseptically cut off by scissors and
135 immediately transferred in a refrigerator to the Polytechnic University of Marche (Ancona, Italy) for
136 microbiological analyses. The remaining berries were pressed by hand and homogenized manually to obtain
137 fresh must for physicochemical analyses.

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139 2.2 Climate data

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Climate data collected from the nearest meteorological station for each vineyard (Table 1) were obtained from the Croatian Meteorological and Hydrological Service. The average (Tav), maximum (Tmax) and minimum (Tmin) temperature (°C) as well as the average daily precipitation (Dp) (mm) for each winegrowing sub-region are reported in Supplementary Table 1.

2.3 Physicochemical analyses of fresh must

Standard physicochemical parameters were determined according to the International Organisation of Vine and Wine reference methods for wine analysis (OIV, 2021) in a laboratory accredited according to HRN EN ISO/IEC 17025 at Institute for Adriatic Crops and Karst Reclamation (Split, Croatia). The content of total soluble solids, TSS (°Brix), was measured using a refractometer (Hi 96814, Hanna Instruments, USA). The pH was measured using a pH meter Titrino 718 (Metrohm, Switzerland) and total acidity (TA) was determined by titrating the samples with 0.1 M sodium hydroxide solution to reach a pH end-point of 7. A FTIR Lyza 5000 Wine analyser (Anton Paar GmbH, Austria) was used to determine the following oenological parameters of the fresh musts: glucose (g/L), fructose (g/L), malic acid (g/L), tartaric acid (g/L) and yeast assimilable nitrogen, YAN [mg/L (N)]. Concentrations of D-glucose and D-fructose were confirmed by using an enzymatic test K-FRUGL (Megazyme, Ireland). Also, concentrations of malic acid and tartaric were confirmed by using the enzymatic tests for L-malic acid and tartaric acid (Megazyme, Ireland).

2.4 DNA extraction and sequencing

A total number of 33 fresh grape berry samples (three biological replicates for each of 11 vineyards) were crushed at 260 rpm by a Stomacher 400 Circulator machine (VWR International PBI, Milan, Italy) for 5 min. The 1.5 mL aliquots of the obtained homogenates were centrifuged at 16 000 g for 10 minutes to pellet the microbial cells that were then used for the extraction of the total microbial DNA using an E.Z.N.A. soil DNA kit (Omega Bio-tek, GA, USA). The quantity and the purity of the extracted DNA were checked by a Nanodrop ND 1000 (Thermo Fisher Scientific, Wilmington, DE, USA).

168 A metataxonomic approach was applied to study the mycobiota composition of Maraština grapes collected
169 from 11 geographical locations within three Dalmatian winegrowing subregions. The 26S rRNA gene of the
170 extracted DNA was amplified by using the primers NL4R (5'-GGTCCGTGTTTCAAGACGG-3') and LS2-
171 MF (5'-GAGTCGAGTTGTTTGGGAAT-3') following the procedure previously described by Mota-Gutierrez
172 et al. (2019). The PCR products were purified, tagged, and pooled following the Illumina Sequencing Library
173 Preparation guidelines. An Illumina MiSeq platform with V2 chemistry was used to generate 250-bp paired-
174 end reads. After sequencing, the obtained raw files (*fastq*) were processed by QIIME2 software as described
175 by Bolyen et al. (2019). *Cutadapter* was used to trim the sequence adapters and primers, and DADA2 algorithm
176 (Callahan et al., 2016) was used to eliminate low quality reads. The DADA2 denoise paired plug-in of QIIME2
177 was implemented to remove chimeric sequences and join sequences shorter than 300 bp. The manually build
178 database for the mycobiota was used for the taxonomy classification using the QIIME feature-classifier plugin
179 against SILVA database implemented in Mota-Gutierrez et al. (2019). BLAST suite tools were used to confirm
180 the taxonomic assignment. Data generated by sequencing were deposited in the National Center for
181 Biotechnology Information (NCBI) Sequence Read Archive (SRA) and are available under the BioProject
182 Accession Number PRJNA851272.

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184 2.5 Statistical analyses

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186 The diversity script of QIIME2 was used for alpha and beta diversity indices calculation. In R environment,
187 the differences between alpha diversity parameters and Amplicon Sequence Variants (ASVs) relative
188 abundance were evaluated by non-parametric Kruskal Wallis test. Bray–Curtis distance matrix was used
189 to perform PERMANOVA by the “vegan” package in R environment.

190 Principal Component Analysis (PCA), using the function *dudi.pca* of R, was used to analyse the differences
191 of ASVs. Spearman correlation analysis between fungal ASVs and physicochemical parameters of fresh
192 Maraština must was performed through the package *psyc* of R, and only the significant associations ($P < 0.05$)
193 are shown in the plots drawn by the *corr.plot* function of R.

194 One-way analysis of variance (ANOVA) was used to evaluate differences in physicochemical characteristics
195 of the samples collected from different vineyards by Tukey-Kramer’s Honest Significant Difference (HSD)

test and the one-tailed t-test (level of significance 0.05) using the JMP software version 11.0.0 (SAS Institute Inc., Cary, NC). Furthermore, prior to PCA, the entire data set related to physicochemical characteristics of fresh musts was subjected to factor analysis to examine whether there was a need to include all the data. The decision on the data inclusion was based on factor loading of ≥ 0.7 (Topić Popović et al., 2021), and only the ratio glucose/fructose was considered a variable that would not greatly affect the qualitative distribution of harvest locations. This data set was used to perform the PCA using statistical software for Excel, XLStat 2014, using the Varimax rotation and presented in a form of a distance biplot.

3. Results and discussion

3.1 Characterization of indigenous mycobiota

The indigenous grapevine microbial communities together with other biological and physical factors play a crucial role in shaping the organoleptic characteristics of wine. Consequently, wines produced from the same grapevine cultivar but in different geographical regions can be recognized for their different sensory characteristics, which in cases of specific regions may lead to increased consumer's acceptance and significant economic returns (Stefanini & Cavalieri, 2018). Because fungi are reported to have greater impact on wine sensory attributes than bacteria (Liu et al., 2020), the current study focused on indigenous mycobiota associated with the Croatian grapevine cultivar Maraština, thus laying a foundation for research on the composition of fungal communities of Croatian grapevine cultivars. The high-throughput sequencing methods revealed the local distribution patterns of microbial communities throughout different world winegrowing regions, showing a strong correlation between local microbial terroir and wine organoleptic characteristics (Li et al., 2022). To verify whether this pattern could be applied to Maraština, 33 grape berry samples collected from 11 vineyards located along the Croatian coastal area were subjected to metataxonomic analysis. A total of 14,007,462 paired reads were obtained by sequencing. After quality filtering, a total of 146,485,613 reads were used, with an average value of 44,389 reads/sample, and a mean sequence length of 375 bp. *Alternaria*, *Aureobasidium*, *Cladosporium*, *Filobasidium*, *Hanseniaspora* and *Metschnikowia* were ubiquitous and characterized by high relative abundance (Figure 2, Supplementary Table 2). *Aureobasidium* was the dominant ASV, with the

relative abundance ranging between 19.7% (vineyard O, Dalmatian hinterland) and 94.6% (vineyard VP, central and southern Dalmatia), followed by *Cladosporium* varying from 3.6% (vineyard VP) to 47.6% (vineyard O), and *Metschnikowia* with relative abundance between 0.03% (vineyards IJK-RB and B, central and southern Dalmatia) and 33.3% (vineyard Z, northern Dalmatia). *Aureobasidium* is commonly found on the surface of grape berries at all stages of maturation, probably due to its high tolerance to different environmental conditions and high antagonistic activity against plant pathogens due to production of volatile organic compounds and antimicrobials (Galli et al., 2021). Moreover, it has been reported to have a positive role on mycotoxin biocontrol and to produce valuable industrial enzymes such as amylases, proteases, pectinases, β -glucosidase, lipases, cellulases, xylanases and mannanases, with some of them very useful for the improvement of wine quality and aroma (Bozoudi & Tsaltas, 2018). In the present study, for most of the samples, the relative abundance of *Aureobasidium* was inversely proportional with the relative abundance of the *Cladosporium*. The latter genus is considered ubiquitous but particularly frequent in geographical zones with mild Mediterranean climates such as Dalmatia, exerting negative influence on wine quality by diminishing aroma, flavour, and colour (Briceno & Latorre, 2008). The highest relative abundance of ASVs ascribed to genus *Metschnikowia* were detected in vineyards Z, DR, and O, showing distribution of this genus within different winegrowing regions. *Metschnikowia* is one of the most explored genera in oenology, frequently used in mixed fermentations with the aim to improve the organoleptic profile of wines by modulating the synthesis of secondary metabolites. It has also been reported that *Metschnikowia pulcherrima* has the strong antimicrobial activity against spoilage yeasts and fungi as well the ability to decrease the concentration of ochratoxin, thus making this species useful in the winemaking (Vicente et al., 2020). Moreover, *M. pulcherrima* showed the ability to decrease the ethanol concentration, which is particularly important for wines produced in regions characterized by warm climate (Vaquero et al., 2021). The last genus commonly present in Maraština samples with the relative abundances >10% (vineyards N and O) was *Hanseniaspora*, comprising the most abundant yeasts found in vineyards able to increase the concentration of acetate esters contributing to positive fruity aroma, as well as sulfur-containing compounds and higher concentration of alcohols (Capozzi et al., 2015). Finally, samples collected from the vineyard IJK-RB were characterized by the highest relative abundance (23.7%) of *Quambalaria*, known as plant pathogenic fungal genus (Narmani & Arzanlou, 2019).

252 *Botrytis*, *Buckleyzyma*, *Cryptococcus*, *Cystobasidium*, *Didymella*, *Eremothecium*, *Hyphopichia*, *Penicillium*,
 253 *Pichia*, *Plenodomus* and *Sporobolomyces* were detected in less than 50% of the samples with the low relative
 254 abundance (<1%). *Eremothecium* and *Plenodomus* were identified only in O and IJK-RB vineyards,
 255 respectively, whereas *Botrytis*, causing grey rot, was present only in vineyards located in central and southern
 256 Dalmatia (IJK-RB, DR, B, and K).
 257 Samples collected from the vineyards IJK-RB, DR and Z showed the highest Shannon diversity index ($P < 0.05$,
 258 Figure 3). Bray–Curtis distance matrix showed a significant separation between samples according to
 259 vineyards (PERMANOVA, $p = 0.001$).
 260 The PCA analysis confirmed a separation of the samples based on their mycobiota composition (Figure 4). In
 261 detail, the samples collected from IJK-RB and DR vineyards, both from central and southern Dalmatia
 262 subregion, were well separated from the other samples. The samples from VP and N vineyards, although from
 263 different winegrowing regions, clustered together. These findings suggest a local-scale effect of the
 264 distribution of fungal ASVs, confirming the concept of microbial terroir. Indeed, several ASVs were associated
 265 with different locations; *Aspergillus*, *Cryptococcus*, *Cystobasidium*, *Erysiphe*, *Filobasidium* and *Plenodomus*
 266 showed higher relative abundance in samples collected from IJK-RB vineyard ($P < 0.05$), whereas
 267 *Cladosporium*, *Fusarium* and *Rhodotorula* showed the highest relative abundance in samples collected from
 268 the O vineyard located in Dalmatian hinterland ($P < 0.05$, Supplementary Figure 1). Even though the genus
 269 *Fusarium* comprises numerous harmless species of filamentous fungi, some of them can cause grapevine wilt
 270 disease or even produce the mycotoxins (Desjardins, 2006; Gonzalez & Tello 2011). The *Rhodotorula* genus
 271 is frequently detected and isolated from grape berries, probably due to its ability to produce biofilms on berry
 272 surfaces (Lederer et al., 2013). Although some species from this genus can enhance the wine aroma complexity
 273 due to β -glucosidase and α -L-arabinofuranosidase activity, they are rarely used in wine production (Hu et al.,
 274 2016; Martínez et al., 2006). Samples belonging to vineyard Z located in northern Dalmatia winegrowing
 275 subregion were characterized by the highest relative abundance of *Metschnikowia* and *Pichia* ($P < 0.05$,
 276 Supplementary Figure 1). Different wine related species of the latter genus are reported to produce enzymes
 277 that positively influence wine organoleptic characteristics. Moreover, they can produce antimicrobial
 278 compounds, thus showing high potential for reducing the growth of wine spoilage microorganisms. However,
 279 only *Pichia kluyveri* strains are commercially available as a starter culture (Vicente et al., 2021). Finally, the

280 highest abundance of the *Lachancea* was detected in the vineyard V from the same winegrowing subregion
 281 ($P < 0.05$, Supplementary Figure 1). Members of this genus have been found in various habitats, with *Lachancea*
 282 *thermotolerans* as a key species in wine fermentation processes principally due to its ability to reduce pH
 283 through lactic acid production, thus giving pleasant acidity to wine (Porter et al., 2019).
 284 Considering the winegrowing subregions of northern Dalmatia, central and southern Dalmatia, and Dalmatian
 285 hinterland as the main factor influencing the distribution of the ASVs, the significant separation of the samples
 286 was observed ($P < 0.05$, Figure 5). Indeed, the one tailed t-test (Supplementary Table 3) indicated that the
 287 highest relative abundance of ASVs ascribed to *Botrytis* and *Plenodomus* was detected in the vineyards located
 288 in central and southern Dalmatia, whereas the ASVs ascribed to *Pichia* were characteristic for the samples
 289 collected from northern Dalmatia. Finally, Dalmatian hinterland subregion was characterized by the highest
 290 relative abundance of *Buckleyzyma*, *Cladosporium*, *Eremothecium*, *Fusarium*, *Papiliotrema* and *Rhodotorula*,
 291 whereas the abundance of *Hanseniaspora* and *Metschnikowia* was the lowest in central and southern Dalmatia.
 292 It has been suggested that the structure of grapevine microbial communities partly depends on climate
 293 conditions both inside and between vineyards, but it remains unclear which climate factor has the greatest
 294 impact (Liu et al., 2019). Here, due to lack of meteorological data for each single vineyard, the average values
 295 of air temperatures (average, maximum and minimum) ($^{\circ}\text{C}$) and daily precipitations (mm) (Supplementary
 296 Table 1) were estimated only for the winegrowing subregion and were correlated with metataxonomic analysis
 297 results. The highest average (T_{av}) and minimum (T_{min}) air temperatures as well as daily precipitations (Dp)
 298 were correlated with *Aspergillus*, *Aureobasidium*, *Botrytis*, *Cryptococcus*, *Cystobasidium*, *Didymella*,
 299 *Eremothecium*, *Erysiphe*, *Penicillium*, *Plenodomus*, *Sporobolomyces* and *Quambalaria*, all associated with
 300 central and southern Dalmatia (Figure 6). Regarding *Aureobasidium*, Chalvantzi et al. (2021) reported its
 301 positive correlation with net precipitation amounts in different Greek vineyards. Furthermore, *Filobasidium*
 302 and *Alternaria* were well correlated with the maximum temperature values (T_{max}).
 303 Soil has been proposed to be a possible natural source of microbial communities associated with grapevines,
 304 thus making the wind-blown soil dust the principal vector for their distribution (Zarraonaindia et al., 2015). In
 305 the current study, the correlation between the vineyard soil type and fungal ASVs present on grape berries was
 306 calculated. As reported in Table 1, the vineyards were planted on different soil types including brown soil on
 307 limestone, red soil, loam, sand, reclaimed karst and brown soil. Samples collected from grapevines planted on

308 brown soil on limestone were well separated from the other samples (Figure 7) principally due to the highest
309 relative abundance of *Alternaria*, *Aspergillus*, *Botrytis*, *Didymella*, *Erysiphe*, *Plenodomus* and *Quambalaria*
310 ($P<0.05$, Supplementary Figure 2), all known for their negative influence on the grapevine sanitary status.
311 Moreover, samples collected from grapevines grown on brown soil were characterized by the presence of
312 *Metschnikowia* and *Cladosporium* ($P<0.05$, Supplementary Figure 2).

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314 3.2 Physicochemical characteristics of fresh musts

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316 The physicochemical analyses were performed to characterize the fresh grape musts obtained from the
317 collected samples. The results, expressed as the average values coupled with standard deviations are reported
318 in Table 2. The pH values ranged between 3.3 (vineyards Z and O) and 3.6 (vineyards IJK-RB, P, K, S), which
319 is comparable with the results commonly reported in the literature for fresh grape must samples (Unluturk &
320 Atilgan, 2015). Grape musts are mainly composed of water (70-80%), carbohydrates (15-25%) with glucose
321 and fructose commonly present in equal amounts (1:1 ratio), plus several organic acids (Granato et al., 2016).
322 Glucose/fructose ratio in Maraština samples was about 1, with glucose concentration ranging from 92.3 g/L
323 (vineyard DR) to 118.1 g/L (vineyard K) and fructose concentration between 77.4 g/L (vineyard DR) and
324 115.7 g/L (vineyard O). The TSS (°Brix) content in Maraština samples was between 18.1 (vineyard DR) and
325 23.4 (vineyard V), which, according to OIV (1990) indicated the stage of technical maturity (14–25 °Brix).
326 Even if the TSS level usually determines the grape price, it does not always correspond to the best overall
327 maturity. Indeed, it has been reported that in cultivars such as Merlot and Chardonnay a concentration of 24 to
328 25 °Brix possibly establishes the upper limit beyond which an additional increase of TSS is associated mainly
329 with deterioration and dehydration of the berries (Bondada et al., 2017; Tillbrook & Tyerman, 2008). After
330 sugars, organic acids such as malic, tartaric, acetic, citric, succinic, and lactic acid are the most abundant solids
331 in grape musts directly impacting the flavour, colour and wine stability (Eyduran et al., 2015). The
332 concentration of organic acids is directly linked to TA which commonly ranges between 0.40 and 7.0 g/L in
333 fresh grape musts (Granato et al., 2016). The Maraština samples were characterized by TA values ranging
334 from 3.7 g/L (vineyard K) to 6.4 g/L (vineyard IJK-RB), which is in line with the results previously reported
335 for Maraština sampled and analysed during three consecutive years (2009-2011) (Preiner et al., 2013). Malic

336 and tartaric acids account for 70-90% of the total acids. The concentration of tartaric acid, responsible for taste
337 and the wine biological stability, is relatively constant during ripening and independent of climate conditions,
338 thus making it characteristic of a grape cultivar (Ribereau-Gayon et al., 2006). Conversely, the concentration
339 of malic acid is variable depending on several factors, including climate conditions, soil type, sunlight exposure
340 and grape variety (Granato et al., 2016). Here, the concentration of tartaric acid in fresh Maraština musts ranged
341 from 2.3 g/L (vineyard K) to 4.0 g/L (vineyard O), and that of malic acid from 0.2 g/L (vineyards B and VP)
342 to 0.9 g/L (vineyard DR). These values are lower than those previously reported for the same cultivar during
343 the 2009-2011 triennial (average concentration of 4.92 g/L for tartaric and 1.21 g/L for malic acid, Preiner et
344 al., 2013). A negative correlation between malic acid and high air temperatures has been reported previously
345 (Conde et al., 2007).

346 One of the most important parameters for wine fermentation is nitrogen (N) availability because it is essential
347 for the metabolism of yeast cells. Different N sources such as amino acids, ammonium, and small peptides are
348 present in must, but not all of them can be used by yeasts. The content of YAN in grape musts is commonly
349 between 50 and 450 mg/L, although a minimum of 140 mg/L has been established as crucial to prevent stuck
350 or sluggish fermentations (Verdenal et al., 2021). Only samples collected from VP vineyard (142 mg/L)
351 satisfied the minimum acceptable YAN concentration, whereas the lowest YAN value was registered for the
352 samples collected from DR vineyard (90.7 mg/L) (Table 2).

353 The PCA was used to assess the distribution of samples collected from different vineyards based on the results
354 of physicochemical analysis, including primary parameters such as acidity (TA, malic and tartaric acids), sugar
355 concentration (glucose, fructose), and analytical data such as TSS (°Brix) and pH, as well as secondary
356 parameters such as YAN (Figure 8). The samples collected from IJK-RB, VP and O vineyards grouped
357 together due to their high YAN and tartaric acid concentration, similar to the samples collected from vineyard
358 Z. Even though the concentration of tartaric acid in fresh musts obtained from the samples collected in vineyard
359 DR was not significantly lower than in the samples from the IJK-RB, VP and O vineyards, the high
360 concentration of malic acid in the DR samples caused a separation into the second quadrant, opposite to sugar-
361 related parameters (glucose and fructose) that were low at 92.3 g/L and 77.4 g/L, respectively. The P samples
362 were distinguished from all the other samples mainly due to their relatively high concentration of malic acid
363 and low YAN concentration. The last group containing samples collected from vineyards V, B, S, N and K

364 was positively correlated with pH and fructose concentration. Interestingly, samples from the same
365 winegrowing region were scattered among different PCA quadrants, thus indicating that local conditions such
366 as climate, soil and vineyard practices may impact the physicochemical characteristics of fresh grape musts
367 and consequently organoleptic characteristics of resulting wines. This was further confirmed by the fact that
368 the samples DR, K, B and P, geographically close to each other (all on Korčula island) were distributed in all
369 four quadrants.

370 During ripening, grapes undertake various physiological and biochemical modifications that may influence the
371 mycobiota of grape berries (Conde et al., 2007). The availability of nutrients such as sugars, organic and amino
372 acids is undoubtedly an important factor shaping the fungal ecology on grapes. Prakitchaiwattana et al. (2020)
373 have recently demonstrated the presence of nutrients on grape surfaces, with their concentration increasing
374 during ripening, which was associated with more abundant fungal population. Given these premises, the
375 correlation analysis between mycobiota and principal physicochemical parameters of fresh Maraština musts
376 was performed. As shown in Figure 9, concentration of malic acid was associated positively with the relative
377 abundance of *Metschnikowia* and negatively with that of *Fusarium*. Indeed, the relative abundance of
378 *Fusarium* in the analysed samples followed the opposite trend compared to *Metschnikowia*. Some non-
379 *Saccharomyces* wine yeasts are assumed to metabolize malic acid, especially *M. pulcherrima*, decomposing
380 around 10% of malic acid during fermentation (Vicente et al., 2020). Regarding negative correlation between
381 *Fusarium* and malic acid, a similar result was recently reported by Lv et al. (2021), whereby malic acid had a
382 significant inhibitory effect on the occurrence of *Fusarium* wilt in faba bean. Finally, the relative abundance
383 of *Erysiphe* showed a positive correlation ($P<0.05$) with pH, whereas the relative abundance of
384 *Sporobolomyces* and *Cystobasidium* was negatively ($P<0.05$) associated with glucose concentration (g/L)
385 (Figure 9). The species from the latter two genera may represent a source of biocontrol agents effective in
386 regulation of different grapevine diseases (Patanita et al., 2022).

387

388 4. Conclusions

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390 The current study aimed to fill a knowledge gap on indigenous mycobiota associated with the Croatian
391 grapevine cultivar Maraština, hence laying a foundation for further research on the composition of microbial

communities related to Croatian grapevines. The high-throughput metataxonomic analysis revealed a significant regional as well as local scale differences in fungi distribution, thus further supporting the concept of microbial terroir. The climate conditions and the vineyard soil type as well as the physicochemical characteristics of fresh musts (such as pH and the concentrations of malic acid and glucose) partly contributed to local distribution patterns of fungal communities. *Aureobasidium* dominated the surface of Maraština grapes followed by *Cladosporium*, *Metschnikowia*, *Hanseniaspora*, *Alternaria* and *Filobasidium*. The knowledge of Maraština indigenous mycobiota provided a basis for examining the role of *Aureobasidium*, *Cryptococcus*, *Cystobasidium*, *Metschnikowia*, *Pichia*, *Sporobolomyces* and *Vishniacozyma* in grapevine disease biocontrol and wine quality. Of special interest are the yeasts from the genera *Hanseniaspora*, *Metschnikowia*, *Lachancea*, *Pichia* and *Hyphopichia* because they are known for their potential positive contributions to organoleptic characteristics of wine. To preserve the role of the microbial terroir, future research will be oriented toward isolation and oenological characterization of indigenous Maraština non-*Saccharomyces* yeasts for their potential as wine starter cultures.

405

406 **CRedit authorship contribution statement**

407

408 **Vesna Milanović:** Conceptualization, Investigation, Formal analysis, Writing - Original Draft; **Federica**
409 **Cardinali:** Investigation, Formal analysis; **Ilario Ferocino:** Investigation, Formal analysis, Writing - Review
410 & Editing; **Ana Boban:** Investigation, Formal analysis; **Irene Franciosa:** Investigation, Formal analysis;
411 **Jasenka Gajdoš Kljusurić:** Formal analysis; Writing - Review & Editing; **Ana Mucalo:** Investigation;
412 **Andrea Osimani:** Validation, Visualization, Resources; **Lucia Aquilanti:** Visualisation, Resources;
413 **Cristiana Garofalo:** Resources; Writing - Review & Editing; **Irena Budić-Leto:** Conceptualization,
414 Resources, Writing - Review & Editing, Supervision, Project administration, Funding acquisition.

415

416 **Declaration of Competing Interest**

417

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

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Impact of native non-*Saccharomyces* wine yeast on wine aromas (WINE AROMAS).

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Figure captions

Figure 1. Position of the vineyards located along the Croatian coastal area in the winegrowing subregions of central and southern Dalmatia [Institute for Adriatic Crops and Karst Reclamation at Split (IJK-RB), Kaštela (VP), Dračevica (DR), Prapatna 1 (P), Prapatna 2 (B), Kruševo (K)], Dalmatian hinterland [Oklaj (O)], and northern Dalmatia [Smilčić (S), Nadin (Polača) (N), Stankovci (Z), Vukšić (V)].

Figure 2. Relative abundance (%) of fungal genera detected in Maraština grape samples collected from 11 different vineyards located along the Croatian coastal area in the winegrowing subregions of central and southern Dalmatia [Institute for Adriatic Crops and Karst Reclamation at Split (IJK-RB), Kaštela (VP), Dračevica (DR), Prapatna 1 (P), Prapatna 2 (B), Kruševo (K)], Dalmatian hinterland [Oklaj (O)], and northern Dalmatia [Smilčić (S), Nadin (Polača) (N), Stankovci (Z), Vukšić (V)].

Figure 3. Boxplot showing the alpha diversity index (Shannon index and observed ASVs) for Maraština grape samples.

The samples are labelled as indicated in Figure 1.

Figure 4. Principal component analysis (PCA) showing a separation of the samples collected from 11 vineyards located along Croatian winegrowing region of Dalmatia based on their mycobiota composition.

PC1 = 15.52%; PC2 = 13.47%; Significance = 0.001.

The samples are labelled as indicated in Figure 1.

616 **Figure 5.** Principal component analysis (PCA) showing grouping of the samples based on their mycobiota
617 composition according to winegrowing subregions of northern Dalmatia, Dalmatian hinterland, and central
618 and southern Dalmatia.

619 PC1 = 15.52%; PC2 = 13.47%; Significance = 0.002.

620

621 **Figure 6.** Principal component analysis (PCA) showing distribution of the samples based on their mycobiota
622 composition according to winegrowing subregion and climate data.

623

624 **Figure 7.** Principal component analysis (PCA) showing grouping of the samples based on their mycobiota
625 composition according to vineyard soil type.

626 PC1 = 15.52%; PC2 = 13.47%; Significance = 0.001.

627

628 **Figure 8.** Principal component analysis (PCA) showing grouping of the samples based on Yeast Assimilable
629 Nitrogen (YAN) vs measured parameters indicated as significant after Factor analysis.

630

631 **Figure 9.** Correlation analysis between fungal ASVs and physicochemical parameters of fresh Maraština must
632 (only significant associations are shown, $P < 0.05$). The colour intensity and the circle dimension represent the
633 degree of correlation where red dots represent a negative degree of correlation and blue dots a positive degree
634 of correlation.