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ORIGINAL RESEARCH ARTICLE

A catalogue of cultivable yeasts from the microbiota of grape berries cv. Vinhão and Loureiro

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ABSTRACT

The relevance of non-*Saccharomyces* native yeasts for improving the complexity of wine aroma is increasingly recognised, besides their use in a range of biotechnological applications. Previously, we used a metagenomic approach to assess the effects of vineyard calcium sprays on the microbiota of grape berries cv. Vinhão and Loureiro. In this study, we aimed at assembling a catalogue of the cultivable yeasts from grape berries of these cultivars and to investigate the direct effect of calcium and other elicitors on the isolated taxa. Seventeen unique colony morphologies were identified, and the sequencing of the region spanning the rDNA internal transcribed spacers or the rDNA D1/D2 domain revealed that they are distributed into 13 different genera. Although the cultivar and vintage influenced the taxa abundance and diversity, *Aureobasidium pullulans* and *Hanseniaspora uvarum* were the most abundant species, ubiquitously populating the fruits together with *Pichia terricola*, *Rhodotorula glutinis*, *Starmerella bacillaris* and *Vishniacozyma heimaeyensis*. The rarest genera comprised *Candida*, *Trichosporon* and *Zygoascus*. Analysis of yeast growth in the presence of specific elicitors revealed that *Pichia* spp. are the most sensitive to exogenous calcium, while *Sporobolomyces* roseus is the most sensitive to copper exposure. Moreover, *A. pullulans*, *H. uvarum* and *S. bacillaris* displayed the highest sensitivity to osmotic stress. In turn, *Wickerhamomyces anomalus*, *Metschnikowia pulcherrima*, *P. fermentans*, *P. kluyveri* and *P. kudriavzevii* showed mild resistance to ethanol, unlike the remaining non-*Saccharomyces* yeasts. Correlation networks expressing the overall yeast performance showed that *H. uvarum* and *S. bacillaris* share the strongest positive interspecies correlation, followed by the pairs *P. fermentans–P. kluyveri*, *B. albus–R. glutinis* and *A. pullulans–V. heimaeyensis*. The strongest negative correlations were found for the pairs *P. kluyveri–S. roseus*, *P. terricola–W. anomalus*, *P. kudriavzevii–V. heimayensis*, *P. kluyveri–V. heimayensis*, and *A. pullulans–P. kluyveri*. This study exposed microbial niches with a predictably common response to abiotic stress.

KEYWORDS: calcium, copper, cultivable yeasts, *Hanseniaspora*, interspecies correlations, osmotic stress, *Vitis vinifera*

INTRODUCTION

Grape berries harbour a complex microbial community that is composed of filamentous fungi, yeasts and bacteria, which diversity and abundance are affected by the fruit ripening stage, pedoclimatic factors, viticultural practices and other parameters that modulate fruit integrity (Barata *et al*., 2012; Loureiro *et al*., 2012; Martins *et al*., 2012; Belda *et al*., 2017; Bisson *et al*., 2017; Drumonde-Neves *et al*., 2021). Beneficial microorganisms involved in crucial functions such as plant nutrition and resistance to biotic and abiotic stresses coexist with harmful ones that cause diseases, and their balance greatly determines fruit health (Belda *et al*., 2017). The indigenous microflora plays an important role in winemaking and its beneficial effects on wine properties have increasingly been established (Barbe *et al*., 2001; Renouf *et al*., 2005; Barata *et al*., 2012; Bisson *et al*., 2017; Drumonde-Neves *et al*., 2021). Thus, unveiling the indigenous microbial community associated with particular grape varieties from specific locations could represent an important source of distinctive metabolites and bring out the authenticity terroir of each region (Pinto *et al*., 2015; Bokulich *et al.,* 2016; Lappa *et al.,* 2020). The use of postharvest microbiota as an early predictor of wine chemical composition potentially poses a new paradigm for quality control of agricultural products (Bokulich *et al.,* 2016).

Different studies based on culture-independent methods have explored the microbial communities associated with wine grapes and the effects of the various factors detailed above on their composition (Pinto *et al*., 2015; Belda *et al*., 2017; Mezzasalma *et al*., 2017). Both organic and conventional practices may negatively impact the grape berry microflora, depending on factors such as pedoclimatic conditions and grape cultivar (Cordero-Bueso *et al*., 2011; Martins *et al*., 2012; Milanović *et al*., 2013; Agarbati *et al*., 2019a). In line with these observations, a delay in the progress of alcoholic fermentation was observed in musts from vines cv. Vinhão treated with the copper-based fungicide Bordeaux mixture, an outcome explained by the wide fungicide spectrum of copper (Martins *et al*., 2015). Metabarcoding approaches showed that calcium-based supplements used to improve grape berry firmness also modulate the surface microbiota from cv. Vinhão and cv. Loureiro berries (Martins *et al*., 2020b; Martins *et al*., 2021a), with a consequent shift in the volatile profile of wines produced by spontaneous fermentation (Martins *et al*., 2020c).

Although culture-independent methods provide a fast and accurate characterisation of whole microbial communities, the study of the cultivable microbiota, especially the resident yeasts of ripe berries, has gained increasing attention, given the possibility of propagating and using these yeast species in a range of oenological and biotechnological applications (Fleet *et al*., 2002; Renouf *et al*., 2005; Comitini *et al*., 2017). Species like *Dekkera*/*Brettanomyces* spp. are responsible for wine spoilage (Silva *et al*., 2004), but other non-*Saccharomyces* yeasts have been progressively used as starters for improving the complexity of wine aroma (Comitini *et al*., 2017; Jolly *et al*., 2014). In addition, the potential of these yeasts as biocontrol agents of grapevine fungal diseases has been increasingly explored (Raspor *et al*., 2010; Cordero-Bueso *et al*., 2017; Solairaj *et al*., 2020).

Early studies dedicated to the grape berry cultivable yeasts did not provide a visual catalogue with species traits, although for almost two decades, they have been very useful in the identification of yeast species through a written description of their phenotypes (Pallmann *et al*., 2001; Renouf *et al*., 2005). Because many species can only be detected by direct identification techniques (without platting) such as PCR-DGGE, a few studies combine both culture-dependent and culture-independent isolation methods for the characterisation of grape berry resident yeasts (Stringini *et al*., 2008; Agarbati *et al*., 2019b; Drumonde-Neves *et al*., 2021). In the present study, we aimed at assembling a visual catalogue of grape berry cultivable yeasts, with the collected information about the phenotype of each species, complementing recent studies where a culture-independent metabarcoding approach was used to characterise the surface microbiota of berries cv. Vinhão and Loureiro (Martins *et al*., 2021a). The microflora was collected from berries exposed to the same microclimate and farming system, further allowing the elucidation of the contribution of the cultivar and vintage to the cultivable yeast profile. The culture-dependent approach enabled us to study, in controlled conditions, the effect of specific stresses on the performance of isolated native species, namely, calcium, copper, ethanol and osmotic stress. While, as reported above, calcium and copper have been used as active principles of vineyard treatments which may affect berry microbiota (Martins *et al*., 2015; Martins *et al*., 2021a), ethanol is one of the main elicitors exerting selective pressure over the yeast consortium throughout the fermentation process, and high osmotic pressure constitutes a stress factor at the beginning of the wine-making process (Jolly *et al*., 2014). The growth of each isolated taxon in the presence of the selected elicitors was used to establish correlation networks that exposed interspecies interactions, allowing the identification of niches with a common response to abiotic stress.

MATERIALS AND METHODS

1. Grape berry cultivable microbiota collection

Healthy and undamaged grape berries were collected at the mature stage (E-L 38; Coombe, 1995), in two consecutive vintages (2019 and 2020), from grapevines cv. Vinhão and cv. Loureiro grown in adjacent vineyard parcels and exposed to the same microclimate in a commercial farm of the Portuguese DOC region 'Vinhos Verdes' located in the Minho region, in the north of Portugal [coordinates: N41°28'28'' latitude, W8°34'59'' longitude, 165 m altitude]. Loureiro and Vinhão are the most prominent white and red wine grape cultivars, respectively, in this region and have been a targeted of studies regarding the effect of copper and calcium-based supplements on grape berry biochemistry and microbiota (Martins *et al*., 2014; Martins *et al*., 2020a; Martins *et al*., 2020b; Martins *et al*., 2021a; Martins *et al*., 2021b).

Plants were oriented from west southwest to east/northeast, spaced at 2.2 m between rows, 1.0 m along the row and trained on a vertical shoot position trellis system, uniformly pruned on a unilateral Royat cordon, and subject to the same routine phytosanitary treatments with Topaze® and Ridomil Gold® R WG, according to the suppliers' instructions. Four independent pools of 20 grape berries randomly collected from 5 bunches each were crushed in sterile vessels, and 10-fold serial dilutions of the grape juice were platted in Wallerstein Differential agar (WL) medium, as originally described (Pallmann *et al*., 2001). Plates were incubated at 24 °C for 5 days. Although it is not possible to culture yeast from an environment without imposing a selection based on the type of medium used or temperature of growth (Bisson *et al*., 2017), this approach has proven highly effective in retrieving a high diversity of yeasts from grape berries (Pallmann *et al*., 2001).

2. Isolation and identification of unique colony types

Unique colony types were counted for four biological replicates per cultivar and restreaked to fresh WL medium, as detailed previously (Pallmann *et al*., 2001). After an initial macro and micromorphological inspection of an average of 100 isolates per replicate, species identification was performed for 75 selected isolates by amplification and sequencing of ITS regions, following an adaptation of previously described methods (Esteve-Zarzoso *et al*., 1999; Stringini *et al*., 2008). Briefly, colony PCRs were performed using fresh cell mass of pure yeast cultures transferred to PCR tubes containing 15 µl of 2× Master Mix (NZYTaq II 2x, NZYTech, Lda. Genes and Enzymes), 0.1 µM of each primer (ITS1: 5′-TCCGTAGGTGAACCTGCGG-3′; ITS4: 5′-TCCTCCGCTTATTGATATGC-3′) and nuclease-free water to a final volume of 30 µl. The following cycler conditions were used: 15 min at 95 °C, 35 cycles of 30 s at 94 °C, 30 s at 55.5 °C and 1 min at 72 °C, and a final step of 10 min at 72 °C. The amplification of DNA fragments was confirmed in 1 % (w/v) agarose gels and the purification of PCR products was performed by washing in 4 volumes of 75 % (v/v) isopropanol. Samples were centrifuged at 14,000 *g* for 30 min and the pellets were air-dried for 30 min and suspended in 10 µl of nuclease-free water. Samples were Sanger-sequenced by a specialised service provider (Eurofins Genomics). Sequences were queried onto BLASTn (nucleotide BLAST—basic local alignment search tool) of NCBI (National Center for Biotechnology Information) and organism identification was based on the result with the highest score (sequence of the best match, determined through values of query cover, percentage identity and E value). For sequences without a satisfactory match at the species level, amplification and sequencing of the divergent D1/D2 domain at the LSU rRNA gene were performed using the primers NL-1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and NL-4 (5'-GGTCCGTGTTTCAAGACGG-3'), and the cycler conditions described above (Kurtzman and Robnett, 1991). Representative sequences used for the identification of each yeast strain were deposited in GenBank (NCBI) under accessions OM044591 to OM044592 and OM049507 to OM049521. The yeast strains were preserved as glycerol stocks in the Microbial Collection of the Biology Department of the University of Minho (Portugal).

The relative abundance of each taxon in each cultivar was then determined and results were expressed as bar graphs of the mean values found for four biological replicates in both vintages, plotted in Prism®6 (GraphPad Software, Inc.). For assembly of the visual yeast catalogue, isolated colonies of each species growing in solid WL medium were photographed with a Canon EOS 250D camera. Cells of each taxon were observed by differential interference contrast microscopy (DIC) in a Leica Microsystems DM-5000B microscope and photographed with a Leica DCF350FX digital camera.

3. Effect of specific elicitors on the growth of isolated yeasts

Yeast isolates growing into liquid YPD medium up to the mid-exponential phase were diluted to OD600 nm = 0.1 and cultivated for 14 h at 24 °C and 200 rpm in the absence (control) or in the presence of the following elicitors: $CaCl_2$ (0.2, 2, 10, 20, 40 g L^{-1}), $CuSO_4$ (250, 500, 1000 mg L^{-1}), PEG 6000 (5, 10, 20 % (w/v)), and ethanol (3, 6, 10 % (v/v) (Sigma Aldrich, Merck KgaA), in a final volume of 5 mL. The concentration range of each elicitor was optimised based on information from previous studies (Martins *et al*., 2014; Martins *et al*., 2015; Martins *et al*., 2020b; Martins *et al*., 2020c; Silambarasan *et al*., 2019). Specifically, 20 g L^{-1} $CaCl_2.2H_2O$ corresponds to the concentration of the calcium formulation applied in vineyards cv. Vinhão and cv. Loureiro to improve fruit firmness (Martins *et al*., 2020b), and 0.2 g L^{-1} $CaCl_2.2H_2O$ is the range of calcium concentrations found in musts and wines cv. Vinhão (Martins *et al*., 2020c); 1000 mg L^{-1} $CuSO_4.5H_2O$ is 20× lower than the concentration found in the formulation of Bordeaux mixture, a practically insoluble contact fungicide used in organic agriculture (Martins *et al*., 2014; Martins *et al*., 2015) and 250 mg L^{-1} $CuSO_4.5H_2O$ is 8× higher than the copper concentration detected in musts of berries treated with Bordeaux mixture (Martins *et al*., 2015); 5 to 20 % (w/v) PEG 6000 is the range used previously to assess the effects of osmotic stress in yeast growth (Silambarasan *et al*., 2019); 10 % (v/v) ethanol is the maximum value found in wines cv. Vinhão produced from berries of the same vineyard (Martins *et al*., 2015; Martins *et al*., 2020c), 6 % (v/v) is the range detected in the middle of fermentation and 3 % (v/v) in the early stages of fermentation. The growth of a native *Saccharomyces cerevisiae* strain isolated from wines cv. Vinhão from the same commercial vineyard (Martins *et al*., 2020c) was also assessed as a reference for comparison with the growth of the isolated non-*Saccharomyces* yeasts. Cell density was monitored in a Thermo Spectronic Genesys 20 spectrophotometer. Results were expressed as logarithmically transformed fold-change (treatment/control) of the mean of two biological replicates and visualised as heatmaps plotted on R software version 3.5.3 using the ComplexHeatmap package 1.18.1 on Bioconductor 3.9 (Martins *et al*., 2021a).

Hierarchical cluster analysis was performed to group yeast species according to their responses to each elicitor. The Student's *t*-test was used to statistically compare the yeast growth in the absence (control) or presence of each concentration of elicitor, and significant differences were generally marked with an asterisk indicating a maximum value of $P \leq 0.05$.

4. Network analysis

Correlations between yeast species were assembled using the MetScape plugin from Cytoscape version 3.8.1 (www.cytoscape.org; Cline *et al.*, 2007), according to their overall response to all tested elicitors, normalised to the respective control without treatment (Teixeira *et al.*, 2020). In the network diagram, generated using a perfuse force direct layout algorithm, node size is proportional to the correlation node strength. Positive ($\rho > 0.10$) and negative ($\rho < -0.10$) correlations were coloured in red and blue, respectively. MCODE plugin was used to obtain a series of metrical topological parameters of the network, allowing, on a mathematical basis, to unravel the denser areas and distinguish the subnetworks with strong cross-links (Teixeira *et al.*, 2020).

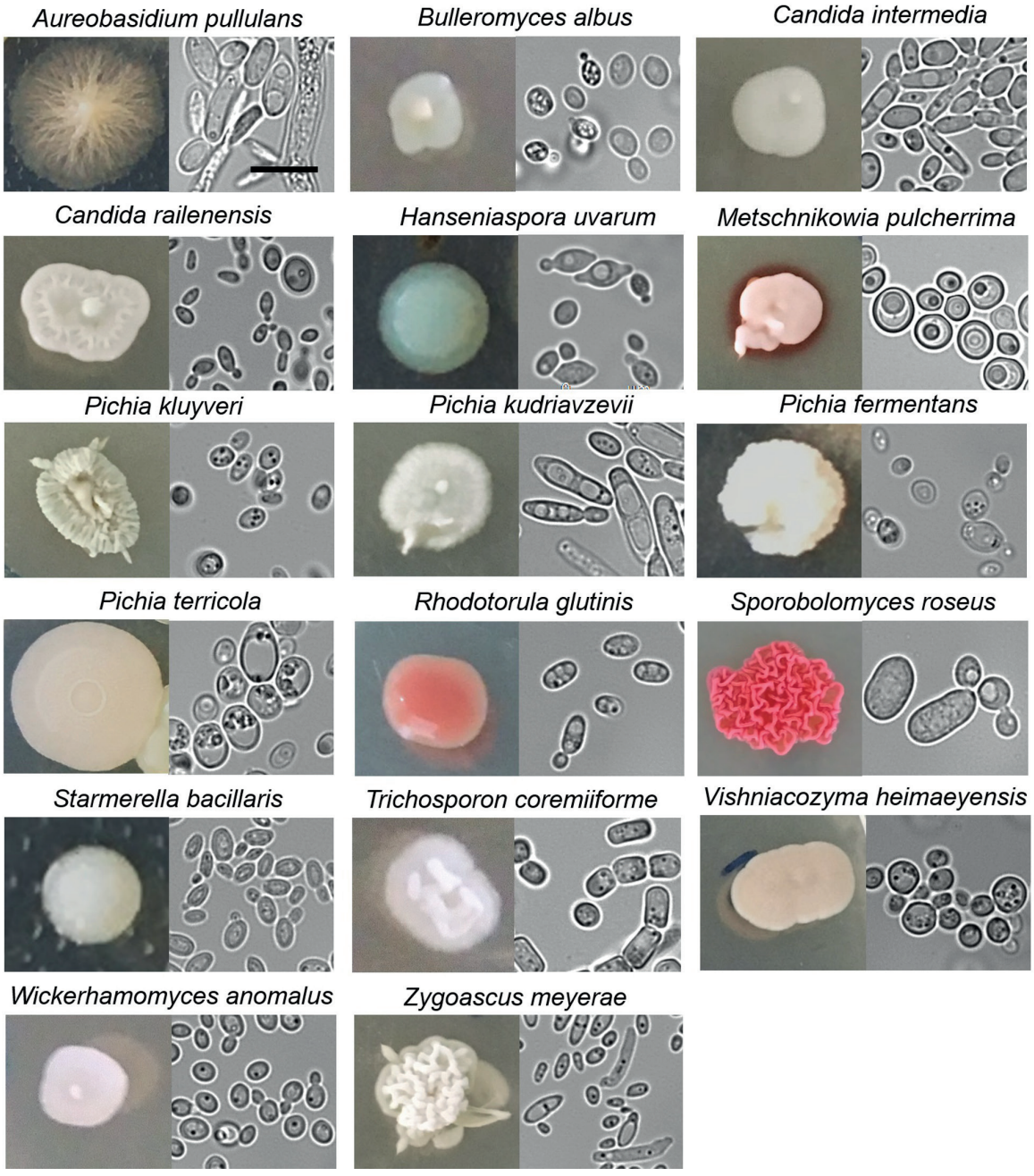


FIGURE 1. Catalogue of cultivable yeasts from grape berries cv. Vinhão and cv. Loureiro.

Colony morphology in WL medium (left) and cell phenotype (right: DIC microscopy, bar = 10 µm) are shown for each unique taxon.

RESULTS

1. Colony morphology and cell phenotype of cultivable yeasts and their relative abundance in berries cv. Vinhão and Loureiro

The culture-dependent method used in this study allowed us to estimate a total of 37,099 ± 5064 colony forming units (CFUs) per mL of grape juice of cv. Vinhão, and of 47,448 ± 11815 CFUs per mL of grape juice of cv. Loureiro, in four biological replicates per cultivar. The differential media distinguished taxa by colour and morphology, and several unique colony types were selected for the identification of each yeast species by ITS/LSU barcoding (Figure S1). This approach revealed the presence of 17 different taxa belonging to 13 genera (Figure 1). The sequences of representative strains selected for further phenotypic characterisation are detailed in Table 1.

Aureobasidium pullulans, a yeast-like fungus, presents itself in the form of cream-coloured colonies with radial filaments composed of cells disposed of in the form of conidia (Figure 1). *Bulleromyces albus* and *Candida intermedia* are characterised by forming smooth white colonies with a creamy appearance; the former species display oval cells composed of dense organelles evidenced by DIC microscopy, while the latter exhibits elongated cells with a large central vacuole and easily visible nucleus (Figure 1). *Candida railenensis* colonies are white, irregular and umbonate, composed of small oval cells with a visible nucleus. *Hanseniaspora uvarum* colonies are light green, with a creamy appearance and slightly raised at the centre; however, they could also be found with a white margin or with a white centre and a light green margin. Cells have a distinctive apiculate form, displaying bipolar budding. *Metschnikowia pulcherrima* colonies have a characteristic pale pink colour and were often surrounded by a reddish-brown halo. Cells are globose, with thick cell walls, and contain a large central droplet.

TABLE 1. Accession numbers of representative sequences used for identification of each yeast species and BLASTn results of the best match.

This study			NCBI BLASTn results	
GenBank accession #	Target rDNA region	Assigned species	Best match accession # and description	% Identity
OM049507	ITS	*Aureobasidium pullulans*	MT107050.1 *Aureobasidium pullulans* clone LHX3	99.27
OM049508	ITS	*Bulleromyces albus*	KX096662.1 *Bulleromyces albus* isolate RP247_2	99.21
OM049509	ITS	*Candida intermedia* (Clavispora clade)	KY495735.1 [*Candida*] *intermedia* strain AUMC 10767	98.31
OM049510	ITS	*Candida railenensis* (Kurtzmaniella clade)	KY102355.1 *Candida railenensis* culture CBS:8164	100.00
OM049511	ITS	*Hanseniaspora uvarum*	KY103552.1 *Hanseniaspora uvarum* culture CBS:2584	99.86
OM044591	D1/D2	*Metschnikowia pulcherrima*	KY108498.1 *Metschnikowia pulcherrima* culture CBS:2256	99.00
OM049512	ITS	*Pichia kluyveri*	KM982973.1 *Pichia kluyveri* strain YCH1204	99.51
OM049513	ITS	*Pichia kudriavzevii*	MT539198.1 *Pichia kudriavzevii* isolate BAL	99.77
OM049514	ITS	*Pichia fermentans*	HQ680960.1 *Pichia fermentans* isolate 17/4	99.51
OM049515	ITS	*Pichia terricola*	MN700642.1 *Pichia terricola* isolate MSU 06	100.00
OM049516	ITS	*Rhodotorula glutinis*	MN913570.1 *Rhodotorula glutinis* strain AD407	96.49
OM049517	ITS	*Sporobolomyces roseus*	KY611834.1 *Sporobolomyces roseus* strain AUMC 11233	98.00
OM049518	ITS	*Starmerella bacillaris*	KY076623.1 *Starmerella bacillaris* isolate IWBT-Y505	99.76
OM044592	D1/D2	*Trichosporon coremiiforme*	GU373784.1 *Trichosporon coremiiforme* strain BPC-M7	99.83
OM049519	ITS	*Vishniacozyma heimaeyensis*	KF036591.1 *Vishniacozyma heimaeyensis* strain CBS8933	99.58
OM049520	ITS	*Wickerhamomyces anomalus*	MF115993.1 *Wickerhamomyces anomalus* isolate HN1	99.32
OM049521	ITS	*Zygoascus meyerae*	MK352095.1 *Zygoascus meyerae* isolate 118	98.90

The colonies of the four different *Pichia* spp. found in berries are all creamy to white in colour but with distinctive morphology; *P. kluyveri* displays crateriform elevation with a distinctive pleated appearance, *P. kudriavzevii* and *P. fermentans* display pulvinate elevation and erose margins, and *P. terricola* displays a smooth circular form with entire margins and convex elevation. Cells of *P. kluyveri* and *P. fermentans* are ellipsoidal and smaller than those of the other *Pichia* species, the former exhibiting dense organelles and the latter a thick cell wall. *P. kudriavzevii* cells are elongated and contain multiple vacuoles, while *P. terricola* cells are ovoid and display a large central vacuole. *Rhodotorula glutinis* colonies exhibit a pink to coral-red glistening colour, with raised elevation and smooth texture. Cells have a slightly elongated oval shape and multiple vacuoles. *Sporobolomyces roseus* colonies also exhibit an intense pink to coral-red colour but have a distinctive irregular form, wrinkled surface and embossed curled margins. Cells have an elongated oval shape and often exhibit multiple budding. *Starmerella bacillaris* colonies often resembled *H. uvarum* colonies, with colours ranging from white to light green, a creamy appearance and convex elevation, composed of small ellipsoid cells with thick cell walls. Colonies of *Vishniacozyma heimayensis* and *Wickerhamomyces anomalus* display a creamy white colour with a flat surface and full margins. Cells of the former are round with dense organelles and thick cell walls, while cells of the latter are oval with an evidenced nucleus. *Trichosporon coremiiforme* colonies present a crateriform elevation, irregular form and slightly erose margins. Cells exhibit circular to rectangle shapes, appearing in the form of arthroconidia. *Zygoascus meyerae* colonies present a cream-coloured wrinkled surface, with pulvinate elevation and undulate smooth margins. Cells are ellipsoidal, with a large central vacuole and evident nucleus, appearing both in the form of budding cells, pseudohyphae and hyphae (Figure 1).

Only six species were ubiquitously found in grape berries regardless of the vintage or cultivar, specifically *Aureobasidium pullulans*, *Hanseniaspora uvarum*, *Pichia terricola*, *Rhodotorula glutinis*, *Starmerella bacillaris* and *Vishniacozyma heimaeyensis* (Figure 2). Rare species included *Bulleromyces albus*, *Candida intermedia*, *Candida railenensis*, *Sporobolomyces roseus, Trichosporon coremiiforme* and *Wickerhamomyces anomalus*, which were only detected in one vintage and in a specific cultivar. Among these, *B. albus*, *T. coremiiforme* and *W. anomalus* were exclusively found in berries cv. Loureiro, while the remaining were exclusive of berries cv. Vinhão, together with *Zygoascus meyerae* and *Pichia kudriavzevii* (Figure 2).

Following the association of specific macro and micro-morphological traits to each yeast species, it was possible to estimate the relative abundance of the yeast taxa in each cultivar from the total colony counts detailed previously. *Hanseniaspora uvarum* was the most abundant species found in berries of both cultivars, representing on average 34–43 % of the cultivable yeasts, followed by *Aureobasidium pullulans,* which was generally more represented in berries cv. Vinhão (25 %) than in cv. Loureiro (13 %) (Figure 3, Figure S2).

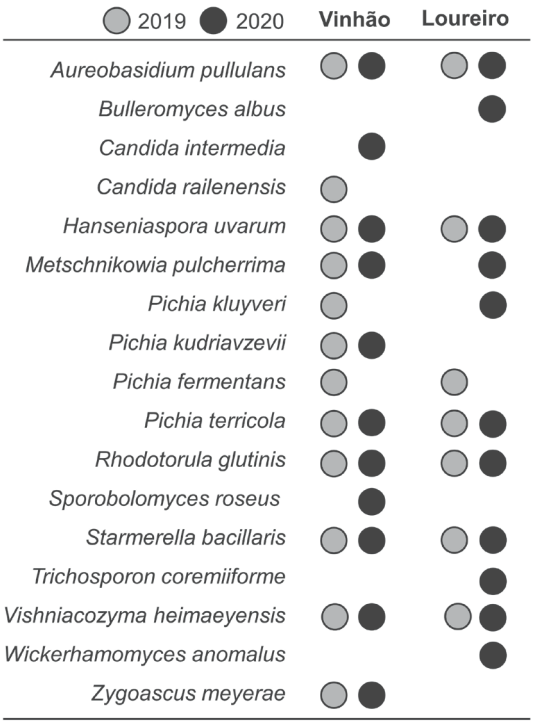


FIGURE 2. Detection of yeast taxa in grape berries cv. Vinhão and cv. Loureiro.

The presence of each taxon was mapped in each cultivar in two consecutive vintages.

Metschnikowia pulcherrima represented ~12 % of cultivable yeasts in both cultivars. Interestingly, *Aureobasidium* and *Metschnikowia* displayed a positive antagonistic effect against *Aspergillus niger* (Figure S3). *Starmerella bacillaris* also accounted for a significant portion of the cultivable yeasts of berries cv. Vinhão (11 %; Figure 3); in berries cv. Loureiro its rank was shared with *Rhodotorula glutinis* (6 %) and *Vishniacozyma heimayensis* (8 %). *Pichia* spp. were more abundant in berries cv. Vinhão (12 %) than in berries cv. Loureiro (4 %) being mostly represented by *P. kudriavzevii* and *P. terricola*. The remaining taxa represented < 6 % of the cultivable microbiota, being *Candida* spp., *T. coremiiforme* and *Z. meyerae,* the least present (Figure 3).

Most of the yeast genera are found in berries cv. Vinhão and cv. Loureiro, through this culture-dependent method, were also detected through a culture-independent method (Table S1), detailed in our previous study (Martins *et al*., 2021a). While some taxa, including *Aureobasidium pullulans* and *Pichia terricola,* were identified at the species level in both studies, other taxa such as *Metschnikowia pulcherrima*, *Rhodotorula glutinis* and several *Pichia* spp. were only identified through the culture-dependent approach used in the present study (Table S1).

2. Effect of specific elicitors on the growth of isolated yeasts and interspecies correlations

Analysis of the yeast biomass 14 h after treatment with selected elicitors revealed that *Pichia* spp. were the most sensitive to calcium, their growth being practically impaired in the presence of 40 g L^{-1} $CaCl_2$ (Figure 4).

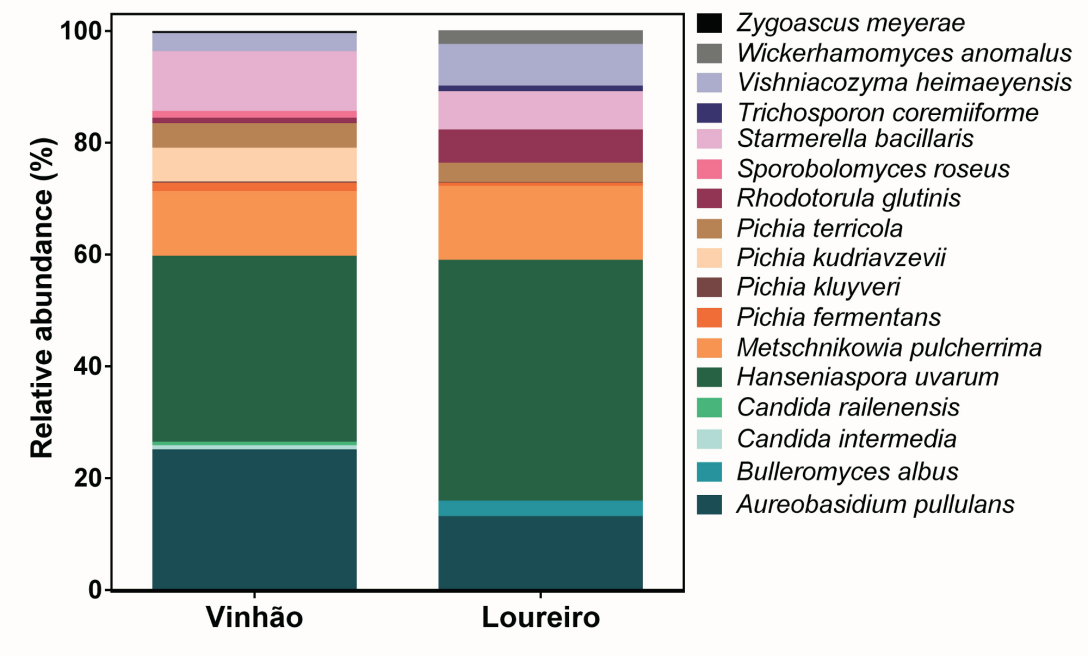


FIGURE 3. Relative abundance of cultivable yeast species in grape berries cv. Vinhão and cv. Loureiro.

Values are expressed as average percentages observed in two consecutive vintages.

In turn, *V. heimaeyensis* was the most tolerant to calcium because its growth remained unaltered at high $CaCl_2$ concentrations. Yeasts with mild tolerance to calcium comprised *R. glutinis*, *Z. meyerae*, *C. railenensis*, *B. albus*, *W. anomalus* and *S. roseus*. The remaining species were more sensitive to calcium, as their growth was affected at concentrations ≥ 10 g L^{-1}. The growth of all species was not negatively affected at concentrations ≤ 2 g L^{-1} $CaCl_2$. *S. roseus* was the most sensitive species to copper treatment, its growth being inhibited by 11–66 % in the presence of all concentrations tested (250–1000 mg L^{-1}; Figure 4). *A. pullulans*, *T. coremiiforme*, *P. terricola* and *P. kudriavzevii* displayed sensitivity to copper concentrations ≥ 1000 mg L^{-1}, together with a native *S. cerevisiae* strain isolated from cv. Vinhão wines used as a reference in these growth assays (see Material and Methods). The remaining species were considered tolerant to copper because their growth was not significantly affected by concentrations up to 1000 mg L^{-1}, an effect that was most evident for *M. pulcherrima* and *H. uvarum*. The most sensitive species to osmotic stress simulated in the present study by PEG 6000 was *A. pullulans*, its growth being strongly impaired by concentrations as low as 5 % (w/v) (Figure 4). The growth of *H. uvarum* and *S. bacillaris* was also severely affected (87–95 % inhibition) but only at PEG 6000 concentrations ≥ 20 % (w/v). In turn, the growth of *V. heimaeyensis* and *P. kluyveri* was impacted at concentrations ≥ 5 % (w/v) but to a lower extent (up to 65 % inhibition). The growth of *P. fermentans* and *C. railenensis* was significantly affected by PEG 6000 concentrations ≥ 10 % (w/v), while *C. intermedia*, *S. roseus*, *S. cerevisiae* and the remaining *Pichia* spp. were impacted only at concentrations ≥ 20 % (w/v). Species resistant to osmotic stress included *M. pulcherrima*, *Z. meyerae*, *W. anomalus*, *B. albus*, *T. coremiiforme* and *R. glutinis* (Figure 4). The growth of all non-*Saccharomyces* yeasts was significantly affected in the presence of ethanol concentrations ≥ 6 % (w/v). Yeasts displaying great sensitivity to 3 % (v/v) ethanol were grouped in a clade composed of *R. glutinis*, *P. terricola*, *A. pullulans* and *B. albus*. In turn, the species more tolerant to ethanol included *W. anomalus*, *P. fermentans*, *P. kluyveri*, *P. kudriavzevii* and *M. pulcherrima,* as their growth was not affected in these conditions (Figure 4); the first three species still displayed mild tolerance (30–42 % growth inhibition) in the presence of 6 % (v/v) ethanol, an effect identical to that found for *S. cerevisiae* in the presence of 10 % (v/v) ethanol.

Integrated analysis of the performance of the yeasts in response to all surveyed elicitors allowed the assembly of a correlation network highlighting interspecies relationships (Figure 5). The 17 different yeast taxa (nodes) were connected through 85 edges, 87 % of which corresponded to positive correlations (shown in red, Figure 5A). Each species exhibited correlations with at least 8 other taxa, with the exception of *P. fermentans,* which displayed fewer correlations with 6 other species. The strongest positive correlations were found for the pairs *H. uvarum–S. bacillaris*, *P. fermentans–P. kluyveri*, *B. albus–R. glutinis* and *A. pullulans–V. heimaeyensis*. In turn, the strongest negative correlations were observed for the pairs *P. kluyveri–S. roseus, P. terricola–W. anomalus, P. kudriavzevii–V. heimayensis*, *P. kluyveri–V. heimayensis,* and *A. pullulans–P. kluyveri* (Figure 5A). Two subnetworks of the main network could be distinguished (Figure 5B), the smallest one composed of *B. albus*, *C. intermedia*, *P. kluyveri*, *R. glutinis*, *S. cerevisiae*, *S. roseus* and *W. anomalus*, and the largest one composed of the remaining taxa, with the exception of *S. bacillaris* which was not attributed to a specific subnetwork.

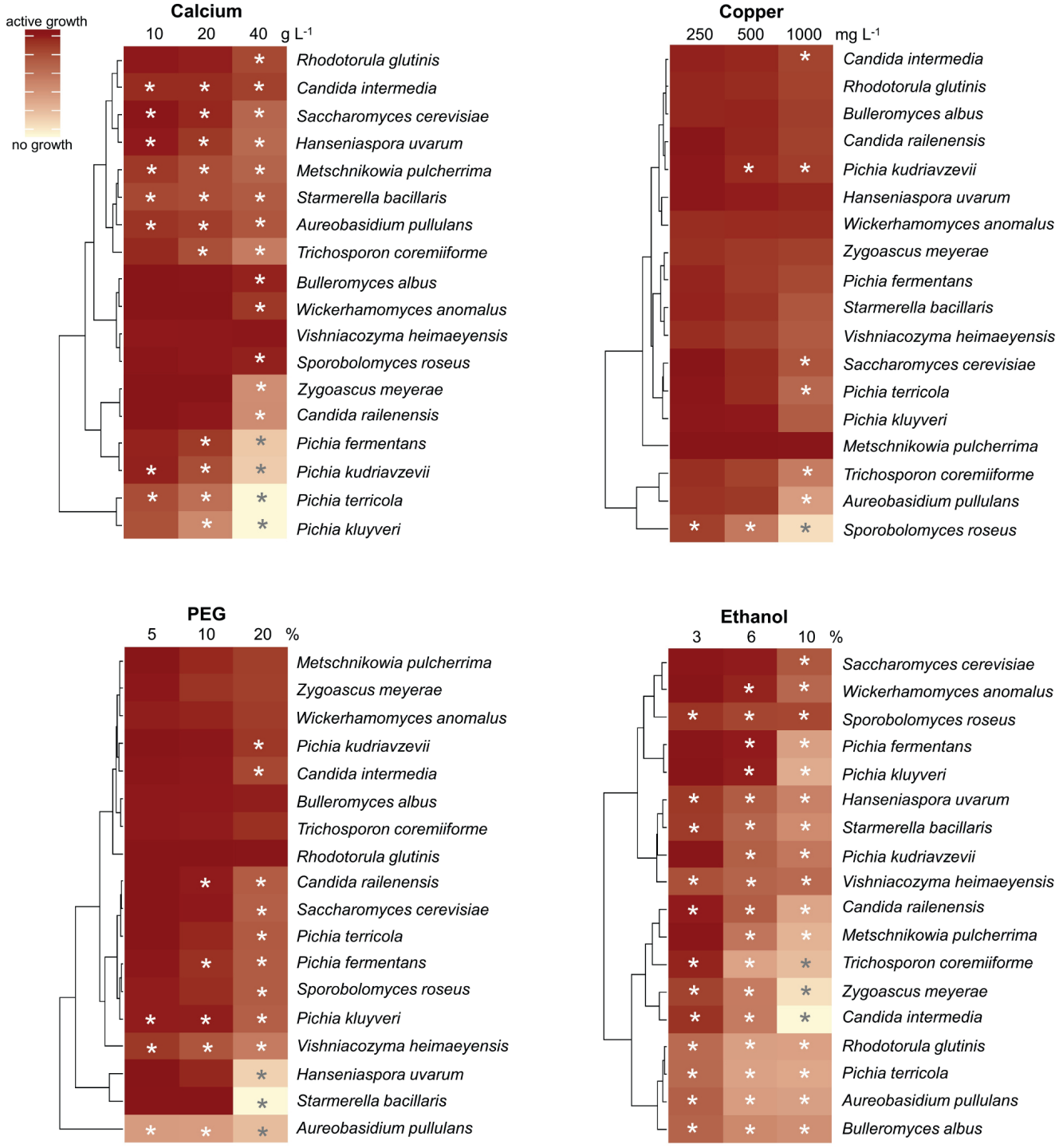


FIGURE 4. Heatmap and clustering of the growth of isolated yeasts in liquid YPD medium after 14 h of incubation in the presence of calcium ($CaCl_2.2H_2O$), copper ($CuSO_4.5H_2O$), PEG 6000 or ethanol.

For each concentration, values indicate the logarithmic transformed mean fold-change (treatment/control) and statistically significant differences between yeast growth in the absence (control) or presence of each elicitor concentration are marked with an asterisk indicating a maximum value of $P \leq 0.05$. Species that showed a similar response to each elicitor were clustered together. The growth of a native *Saccharomyces cerevisiae* strain isolated from wines cv. Vinhão was assessed as reference.

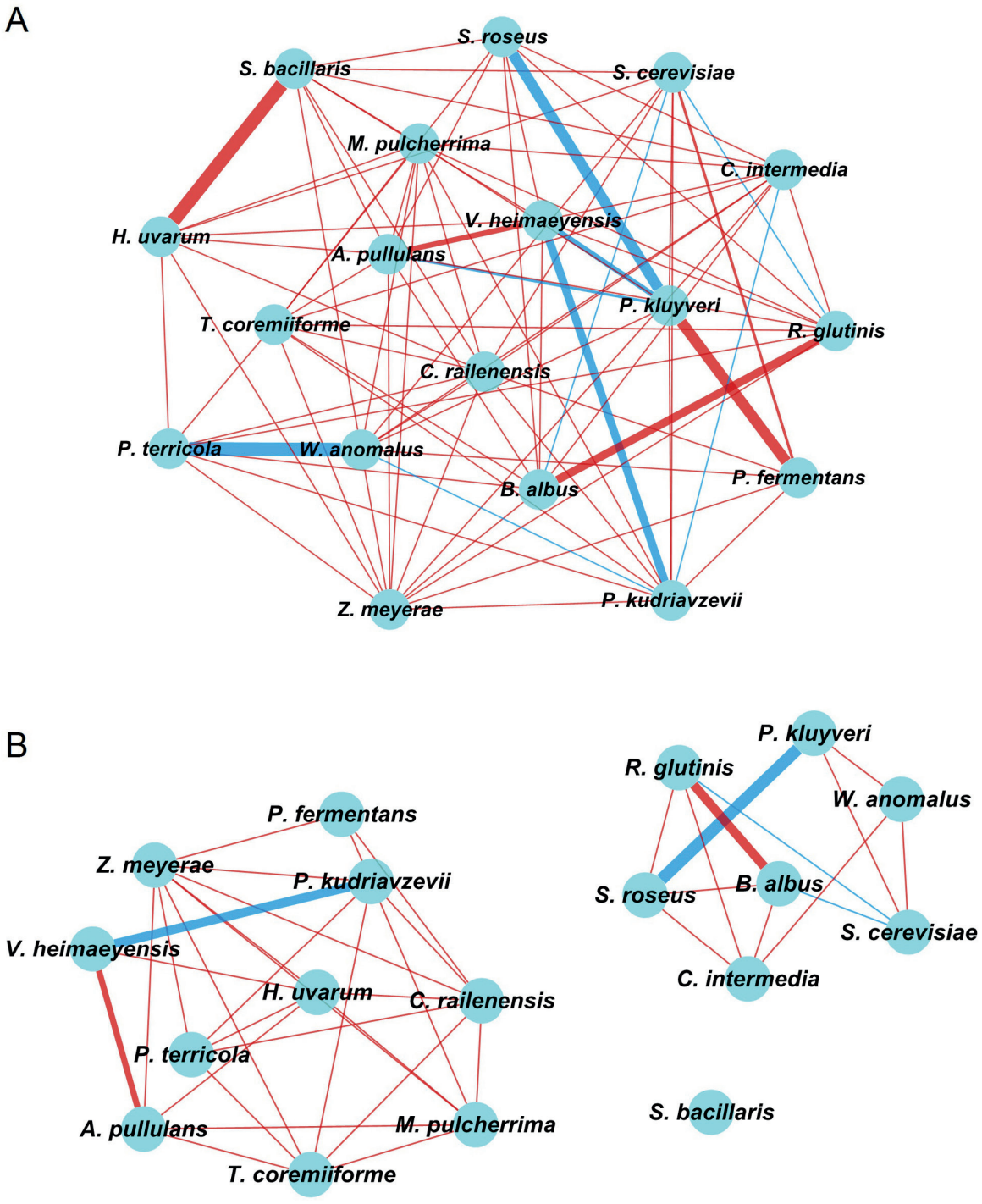


FIGURE 5. Correlation network (A) and intrinsic subnetworks (B) of cultivable yeast species found in grape berries cv. Vinhão and cv. Loureiro.

Node size is proportional to the correlation node strength (ns = |ρ|). Lines joining the nodes represent correlations with ρ > |0.10|; positive correlations are shown in red and negative correlations are shown in blue. Edge thickness is proportional to the correlation strength.

DISCUSSION

Seventeen different yeast taxa were successfully isolated from the microbiota of grape berries cv. Vinhão and Loureiro, a number that was expected according to previous classical reports (Pallmann *et al*., 2001; Renouf *et al*., 2005). The colony type in WL medium and the microscopic phenotype of yeast cells proved effective in the identification of yeast species, supporting previous studies (Pallmann *et al*., 2001). It is widely known that the cultivable taxa are largely outnumbered by the non-cultivable taxa because species that are in low abundance or unable to grow are not accounted for (Belda *et al*., 2017; Morgan *et al*., 2017). It is important to stress that the osmotic pressure of the grape juice following the crushing of the grape berries could contribute to species selection (Jolly *et al*., 2014), as well as the culture medium used for yeast growth. Thus, the use of other substrates (such as lysine medium that suppresses the growth of pitching yeasts) should be considered in further studies to increase the diversity of cultivable microbes. Although fungicide treatments may influence the microbiota profile (see Introduction), previous studies showed that the active principles of the fungicides used in the present study, metalaxil-M and penconazol, are not expected to significantly affect the grape microbial communities (Sapis-Domercq, 1980; Caboni and Cabras, 2010; Perazzolli *et al*., 2014). In particular, penconazol was shown to only minimally affect the richness and diversity of bacterial and fungal populations of the grapevine phyllosphere (Perazzolli *et al*., 2014), and although metalaxil-M was shown to modify the bacterial community of the pepper phyllosphere (Moulas *et al*., 2013), it was found to not influence the activity of several grape berry yeasts including *Hanseniaspora uvarum* (Sapis-Domercq, 1980; Caboni and Cabras, 2010).

Very few studies used culture-dependent methods as a complement to data obtained from culture-independent methods for characterisation of the microbiota (Stringini *et al*., 2008; Agarbati *et al*., 2019b). We recently used a metabarcoding approach to investigate the microbiota diversity of grape berries cv. Vinhão and cv. Loureiro (Martins *et al*., 2021a). In this approach, 174 fungi OTUs were detected, with Dothideomycetes and Leotiomycetes comprising the most abundant classes, and *Hanseniaspora*, *Aureobasidium*, *Vishniazcozyma*, *Sporobolomyces*, *Rhodotorula* and *Metschnikowia* incorporated the list of the top 15 most abundant genera. The present study thus complements the previous one, as the identification of several taxa at the species level was only possible through the culture-dependent approach. In addition, the retrieved microbial species can now be further explored.

Culture-dependent methods are usually laborious and time-consuming (Morgan *et al*., 2017), and the assembly of a visual catalogue of the cultivable microbiota will largely facilitate the initial identification of yeasts at genera or even at the species level, allowing the researcher to focus on the strains of interest in further studies. Performing the microbiota collection in different vintages also proved effective in the isolation of a greater diversity of species, supporting the influence of climatic conditions in the microbial community, as previously reported (Stefanini *et al*., 2017).

Many cultivable yeasts identified in the present study as colonisers of grape berries cv. Vinhão and cv. Loureiro have been detected in berries of other grape cultivars such as Merlot, Cabernet-Sauvignon, Cabernet Franc and Touriga Nacional where predominant genera included *Aureobasidium*, *Hanseniaspora*, *Candida*, *Metschnikowia*, *Pichia* and *Rhodotorula* (Pallmann *et al*., 2001; Renouf *et al*., 2005; Barata *et al*., 2012; Pinto *et al*., 2015; Stefanini *et al*., 2017; Agarbati *et al*., 2019a; Martins *et al*., 2021a). In addition, these non-*Saccharomyces* yeast species have also been isolated from other *Vitis* species, namely *V. labrusca*, *V. rotundifolia*, *V. amurensis* and *V. davidii* (Drumonde-Neves *et al*., 2021). Particularly, *A. pullulans* and *H. uvarum* are considered resident species in vineyards and the most abundant cultivable yeasts in grape berries regardless of the cultivar or farming system (Barata *et al*., 2012; Agarbati *et al*., 2019b); the latter was found to be the most cited in a recent review, in a total of 129 studies (Drumonde-Neves *et al*., 2021). *Aureobasidium* integrates the microbial consortium of various other fruits, including strawberry (Debode *et al*., 2013; Zhimo *et al*., 2021), blueberry (Ochmian *et al*., 2020), raspberry (Hamby *et al*., 2012), arbutus (Santo *et al*., 2012), sweet cherry (Hamby *et al*., 2012; Zhang *et al*., 2021), mandarin (Kumar *et al*., 2021), mango, lemon, plum, pear and apple (Vadkertiová *et al*., 2012; Koricha *et al*., 2019). Together with *Hanseniaspora* and *Metschnikowia* spp., pink yeasts from the genera *Rhodotorula* and *Sporobolomyces* were also found in strawberry, sweet cherry and arbutus fruit (Santo *et al*., 2012; Debode *et al*., 2013; Zhang *et al*., 2021; Zhimo *et al*., 2021). The detection of *Pichia* spp. was also reported in various fruits, including strawberry (Hamby *et al*., 2012; Vadkertiová *et al*., 2012; Koricha *et al*., 2019; Zhimo *et al*., 2021), while *Vishniacozyma* spp. was found in strawberry and mandarin fruit (Kumar *et al*., 2021; Zhimo *et al*., 2021).

The relative proportions of each yeast species are likely related to their resilience and the health status of the grape berry; *A. pullulans* and Basidiomycetes like *Bulleromyces*, *Sporobolomyces*, *Rhodotorula* and *Trichosporon* are the usual colonisers of sound berries as their oligotrophy allows them to survive in the harsh environment of the unblemished cuticle (Barata *et al*., 2012). In line with this observation, many species within these genera prevailed in grape berries treated with exogenous calcium, which increased fruit firmness and fitness by reducing skin cracking incidence (Martins *et al*., 2020b, 2021a), and the present study confirmed their tolerance to calcium treatments *in vitro*. Nonetheless, visually intact berries may still bear microfissures that increase nutrient availability, thus explaining the dominance of copiothrophic oxidative or weakly fermentative ascomycetous yeasts such as *Hanseniaspora*, *Candida*, *Pichia* and *Metschnikowia* (Barata *et al*., 2012). Thus, it is believed that the grape health status is the main factor affecting the microbial ecology of grapes and that the influence of abiotic, biotic and viticultural

factors is dependent on their primary damaging effect on the fruit (Barata *et al*., 2012). Copiotrophic strongly fermentative yeasts like *Saccharomyces*, *Torulaspora* and *Lachancea* are usually not detected in intact berries as they prosper at high sugar concentrations available in damaged fruits or during the winemaking process (Renouf *et al*., 2005; Barata *et al*., 2012; Pinto *et al*., 2015). Accordingly, none of these genera were isolated from grape berries in the present study.

The significant contribution of the grape cultivar to the berry microbiota diversity was reported in a few studies (Agarbati *et al*., 2019a; Martins *et al*., 2021a) and further explored in the present study, supporting the influence of traits such as fruit surface topology and nutrient availability, discussed above. Results are in line with those obtained by the culture-independent metabarcoding approach, showing a greater diversity of fungi taxa in berries cv. Vinhão than in cv. Loureiro (Martins *et al*., 2021a). Moreover, the greater abundance of *Hanseniaspora* and *Rhodotorula* spp. in berries cv. Loureiro than in cv. Vinhão is consistent in both studies, besides the exclusivity of *Trichosporon* sp. in the former cultivar, supporting the good complementary of culture-dependent and independent methods discussed previously. Our recent study showed a close correlation between the profile of triterpenoids and steroids of the cuticular waxes of berries cv. Vinhão and Loureiro and the composition of adjacent microbial communities (Martins *et al*., 2021a). This study highlighted that many of these compounds, among which oleanolic acid, are substrates for microbial growth and exposed the relevance of less explored metabolites such as stigmasterol and tremulone in the modulation of the microbiota profile. In addition, other metabolic differences between cultivars, found, for instance, in the profile of berry polyphenolics (Martins *et al*., 2020a; Martins *et al*., 2021b), likely modulate microbial taxa diversity, as these compounds have a myriad of functions, including antioxidant activity and stress response (Teixeira *et al*., 2013).

The present study exposed microbial niches within the cultivable microbiota, composed of species that are expected to present a similar response to abiotic stress. Interestingly, these expressed interspecies interactions were not linearly associated with the relative abundance of each taxon nor their oligotrophy, as discussed above. Rather, they showed that the metabolic activity of each taxon determines their performance which directly influences the structure of the microbial community. Novel results showing clear positive and negative correlations between the isolated taxa further support that the grape microbiota is modulated by the existence of synergistic and antagonistic relationships that have only recently begun to be explored (Bartle *et al*., 2019; Belda *et al*., 2020). Adding complexity to these correlations is the ability of many species of *Vishniacozyma*, *Bulleromyces* and *Trichosporon* to modulate the nutrient availability of the berry and, consequently, its microbial consortium (Raspor *et al*., 2010; Zhu *et al*., 2017; Oluwa, 2020). This complexity hampers the study of the effect of external factors such as the farming system on the microbiota composition. The unequivocally high sensitivity of *S. roseus* and *A. pullulans* to isolated copper exposure found in the present study parallels reports showing a negative influence of conventional treatments on fermenting yeasts in favour of oxidative yeasts such as *Aureobasidium pullulans* (Agarbati *et al*., 2019a). In turn, the high tolerance of *Metschnikowia pulcherrima* to copper is in line with previous studies (Vadkertiová and Sláviková, 2006). In any case, a strong correlation between the copper dose and the decrease in yeast biodiversity was reported and the yeast copper sensitivity was attributed to a greater copper adsorption efficiency (Martins *et al*., 2012; Comitini *et al*., 2017). Similar mechanisms may drive yeast resistance to calcium treatments, although field trials comprising the use of calcium supplements did not report a particular effect over weakly fermenting yeasts such as *Pichia* spp. (Martins *et al*., 2021a), results in the present study highlight the greater sensitivity of all species within this genus to the treatment, suggesting the existence of a specific metabolic response not shared by other yeasts that deserve further investigation. Calcium is a key secondary messenger in cells, regulating many physiological functions such as cell division, cell wall structure and stress response. Thus, its concentration in the cytosol is delicately balanced by a myriad of transporters and channels that can sequester it to internal storage, such as the vacuole (Tuteja and Mahajan, 2007). An inhibition of cell growth due to excess calcium was previously attributed to an overload of the mitochondrial matrix and consequent increase in reactive oxygen species, ultimately leading to apoptosis (Brookes *et al*., 2004). Results in the present study supported that the calcium toxicity experienced by *Pichia* spp. was not due to an osmotic effect, as this genus did not show great sensitivity to PEG 6000. In contrast, the great sensitivity of *A. pullulans*, *H. uvarum* and *S. bacillaris* to osmotic stress induced with PEG 6000 highlight the marked effects that drought can have on these species, suggesting these can be ideal markers for early detection of this type of abiotic stress in grapevine, an analysis most facilitated by their high abundance and ubiquitous presence in vineyards (Barata *et al*., 2012; Agarbati *et al*., 2019a). In turn, the extreme sensitivity of *A. pullulans* to this type of stress compared to the remaining yeasts likely contributes to its marked inhibition at the beginning of fermentation, where it is exposed to very high sugar concentrations (Pinto *et al*., 2015). Results further support that the increasing formation of ethanol during the fermentation process also contributes to this effect. In turn, the relative tolerance of *Metschnikowia*, *Pichia* spp. and *W. anomalus* to ethanol is in line with previous studies and likely associated with their ability to conduct the fermentation process and to produce ethanol, being detected in wines up to the end of fermentation (Díaz *et al*., 2013; Jolly *et al*., 2014; Padilla *et al*., 2018; Ivit *et al*., 2020).

Although commercial strains of non-*Saccharomyces* yeasts such as *M. pulcherrima* and *P. kluyveri* have recently been made available as pure starter cultures and in blends with *S. cerevisiae* (Morgan *et al*., 2017), the present study highlights the possibility of isolating and using native yeasts of a particular terroir for improving the oenological and biotechnological sectors at a regional scale.

Various studies showed that the metabolism of non-*Saccharomyces* yeasts affects the overall ethanol yield, alcoholic fermentation efficiency, biomass and by-products of the mixed fermentations (Comitini *et al*., 2017). For instance, strains of *S. bacillaris*, *H. uvarum*, *P. kluyveri*, *P. fermentans*, *M. pulcherrima* and *W. anomalus* were shown to improve wine quality by increasing their aromatic complexity following sequential or co-inoculation with *S. cerevisiae* (Jolly *et al*., 2014; Ivit *et al.*, 2020). The production of hydrolytic enzymes by many species, including *P. terricola*, *R. glutinis* and *S. pararoseus* can further influence the sensory and technological properties of the wine (Jolly *et al*., 2014; Varela and Borneman, 2017). Inoculations with *M. pulcherrima*, *W. anomalus*, *H. uvarum* and *Pichia* spp. are also being used as a strategy to produce wines with lower alcohol content, taking advantage of the different sugar utilisation pathways of these yeasts, which include respiration and glycerol–pyruvic metabolisms besides alcoholic fermentation, in contrast to *S. cerevisiae* which prefers the latter (Ivit *et al*., 2020).

The benefits of the culture of native yeasts from the berry surface also encompass their exploitation as biocontrol agents (Bleve *et al*., 2006; Cabañas *et al*., 2020). Preliminary results of the present study showing the ability of isolated *Aureobasidium* and *Metschnikowia* spp. to inhibit the growth of *A. niger* in vitro open good perspectives for the use of native yeasts in the combat of local pathogens. Future studies focused on directed phenomics in accordance with the desired applicability of the strains will lead to the valorisation of these natural resources at a regional scale. In particular, the evaluation of the performance of these strains in response to a combination of elicitors, such as calcium + PEG, will provide further insights into their resilience toward abiotic stress and expose their full biotechnological potential.

CONCLUSION

The present study rekindled the importance of characterising native cultivable yeasts of grape berries of specific regions for their exploitation in a range of oenological and biotechnological applications, primarily relevant at a regional scale. The significance of culture-dependent methods as essential complements to culture-independent methods for microbiota characterisation is reinforced, while the assembly of a visual catalogue of unique colony types poses a valuable tool for minimising the laborious practices inherent to the cultivation of native yeasts. The study further elucidates the contribution of the vintage and cultivar to taxa diversity, besides exposing interspecies relationships that aid the understanding of the responses of yeasts to environmental stimuli and their performance during fermentation.

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