Vineyard ecosystems are structured and distinguished by fungal communities impacting the flavour and quality of wine

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Abstract

The flavours of foods and beverages are formed by the agricultural environment where the plants are grown. In the case of wine, the location and environmental features of the vineyard site imprint the wine with distinctive aromas and flavours. Microbial growth and metabolism play an integral role in wine production from the vineyard to the winery, by influencing grapevine health, wine fermentation, and the flavour, aroma and quality of finished wines. The mechanism by which microbial distribution patterns drive wine metabolites is unclear and while flavour has been correlated with bacterial composition for red wines, bacterial activity provides a minor biochemical conversion in wine fermentation. Here, we collected samples across six distinct winegrowing areas in southern Australia to investigate regional distribution patterns of both yeasts and bacteria and how this corresponds with wine aroma compounds. Results show that soil and must microbiota distinguish winegrowing regions and are related to wine chemical profiles. We found a strong relationship between microbial and wine metabolic profiles, and this relationship was maintained despite differing abiotic drivers (soil properties and weather/ climatic measures). Notably, fungal communities played the principal role in shaping wine aroma profiles and regional distinctiveness. We found that the soil microbiome is a potential source of grape- and must-associated fungi, and therefore the weather and soil conditions could influence the wine characteristics via shaping the soil fungal community compositions. Our study describes a comprehensive scenario of wine microbial biogeography in which microbial diversity responds to surrounding environments and ultimately sculpts wine aromatic characteristics.

These findings provide perspectives for thoughtful human practices to optimise food and beverage flavour and composition through understanding of fungal activity and abundance.

Keywords: wine regionality, microbial biogeography, fungal diversity, climate, soil

Introduction

Regional distinctiveness of wine traits, collectively known as "*terroir*", can be measured by chemical composition and sensory attributes (Gonzálvez et al 2009, Pereira et al 2005, Robinson et al 2012), and this variation has been related to the physiological response of grapevines to local environments, such as soil properties (e.g., soil type, texture, nutrient availability), climate (temperature, precipitation, solar radiation), topography and human-driven agricultural practices (Gladstones 1992, van Leeuwen and Seguin 2006, Vaudour et al 2015). Wines made from the same grape cultivar but grown in different regions are appreciated for their regional diversity, increasing price premiums and the market demand (van Leeuwen and Seguin 2006). However, which factors from the vineyard to the winery drive wine quality traits have remained elusive.

Microbial biogeography contributes to the expression of wine regionality. Microorganisms, including yeasts, filamentous fungi and bacteria, originate in the vineyard and are impacted upon by the built environment (winery) and play a decisive role in wine production and quality of the final product (Barata et al 2012, Stefanini and Cavalieri 2018). The fermentative conversion of grape must (or juice) into wine is a complex and dynamic process, involving numerous transformations by multiple microbial species (Swiegers et al 2005). The majority of fermentations involve *Saccharomyces* yeasts conducting alcoholic fermentation (AF) and lactic acid bacteria (LAB) for malolactic fermentation (MLF), but many other species are present and impact the chemical composition of the resultant wine (Goddard 2008, Heard and Fleet 1985). Recent studies pose the existence of non-random geographical dispersion patterns of microbiota in grapes and wines (Bokulich et al 2014, Gayevskiy and Goddard 2012, Morrison-Whittle and Goddard 2018, Pinto et al 2015, Portillo and Mas 2016, Stefanini et al 2016, Taylor et al 2014). Few studies have explored the associations between microbial communities and wine chemical composition (Bokulich et al 2016, Knight et al 2015). Knight et al. (2015) showed empirically that regionally distinct *S. cerevisiae* populations drove different metabolites present in the resultant wines. Bokulich et al. (2016) suggested that wine metabolite profiles were more closely associated with bacterial profiles of wines undergone both AF and MLF. However, both numerically

and biochemically, yeasts dominate wine fermentation, so it is unclear why bacteria should be dominant.

The vineyard soil has long been attributed to fundamental wine characteristics and flavour. Vineyard soil provides the grapevine with water and nutrients and soil type and properties profoundly affect vine growth and development (van Leeuwen and Seguin 2006). Soil-borne microbiota associate with grapevines in a beneficial, commensal, or pathogenic way, and determine soil quality, host growth and health. For example, soil microbes can mineralize soil organic matter, trigger plant defence mechanisms, and thus influence flavour and quality of grapes and final wines (Müller et al 2016, Rillig et al 2017). Alternatively, serving as a reservoir for grapevine-associated microbiota, the soil microbiome can be recruited from the soil to the grapevine and transferred to the grape and must (Morrison-Whittle and Goddard 2018, Zarraonaindia et al 2015). Some of these microbes can influences the fermentation and contribute to final wine characteristics (Gilbert et al 2014, Stefanini and Cavalieri 2018). Geographic areas can constrain the vineyard soil microbiota (Burns et al 2015, Coller et al 2019, Knight et al 2019, Mezzasalma et al 2018, Zarraonaindia et al 2015), but whether the biogeographic patterns can link to resulting wine attributes has not been clearly shown.

Limited but increasing evidence reveals that environmental heterogeneity conditions microbial biogeography in wine production (recently reviewed by Liu et al (2019), Bokulich et al 2014, Burns et al 2015, Jara et al 2016, Martiny et al 2006, Zarraonaindia et al 2015), along with differentiations by geographic distances (Burns et al 2015, Martiny et al 2006, Miura et al 2017). Local climatic conditions significantly correlate with microbial compositions in grape musts, for example precipitation and temperatures that associate with the abundance of filamentous fungi (for example *Cladosporium* and *Penicillium* spp.) and ubiquitous bacteria (for example, the *Enterobacteriaceae* family) (Bokulich et al 2014), as well as yeast populations (particularly *Hanseniaspora* and *Metschnikowia* spp.) (Jara et al 2016). Dispersion of soil microbiota is driven by soil physicochemical

properties such as soil texture, soil pH, carbon (C) and nitrogen (N) pools (Burns et al 2015, Colle et al 2019, Zarraonaindia et al 2015), with some influences from topological characteristics (for example orientation of the vineyard) (Burns et al 2015, Portillo et al 2016). Soil microbiome/ bacteria may colonise grapes by physical contact (moving by rain splashes, dust, winds) (Zarraonaindia et al 2015) or migration through the plant (xylem/phloem) from the rhizosphere to the phyllosphere (Compant et al 2008, Mandl et al 2015). Insects help the movement and dispersal of microbes in the vineyard and winery ecosystem, for example, honey bees, social wasps and *Drosopholid* flies can vector yeasts among different niches (Goddard et al. 2010, Lam and Howell, 2015; Stefanini et al. 2012). The vineyard microbes enter the winery associated with grapes or must, so the effects of environmental conditions are finally reflected on microbial consortia in wine fermentation. How environmental conditions modulate microbial ecology from the vineyard to the winery and shape regional distinctiveness of wine is still largely unknown.

Here, we sampled microbial communities from the vineyard to the winery across six geographically separated wine-producing regions in southern Australia to provide insights into microbial biogeography, growing environments and wine regionality. We evaluated the volatile chemicals of wines made with Pinot Noir grapes to validate that these different regions have differently flavoured wines. Using next-generation sequencing (NGS) to profile bacterial and fungal communities, we demonstrate that the soil and must microbiota exhibit distinctive regional patterns, and this can be correlated to the wine metabolome. Network analysis supports the interaction of microbial community abundance with abiotic factors (weather and soil properties) to drive wine regionality both directly and indirectly. The important contributions arise from fungal diversity. We investigated a potential route of wine-related fungi from the soil to the grapes by isolating yeasts from the xylem/phloem of grapevines. Using vineyards, grapes and wine as a model food system, we have related the regional identity of an agricultural commodity to biotic components in the growing system to show the importance of conserving regional microbial diversity to produce distinctive foods and beverages.

Materials and methods

Sample sites and weather parameters

15 *Vitis vinifera* cv. Pinot Noir vineyards were selected in 2017 from Geelong, Mornington Peninsula (Mornington), Macedon Ranges, Yarra Valley, Grampians and Gippsland in southern Australia, ranging from 5 km to 400 km between vineyards (Supplementary Figure S1). In 2018, the sampling from the five vineyards in Mornington Peninsula (all < 20 km apart) was repeated to elucidate the influence of sampling year (vintage) on microbial patterns and wine profiles. Each site's GPS coordinates (longitude, latitude, altitude) were utilised to extract weekly weather data from the dataset provided by Australian Water Availability Project (AWAP). Variables were observed by robust topography resolving analysis methods at a resolution of 0.05° × 0.05° (approximately 5 km x 5 km) (Jones et al 2009). Retrospective weekly measurements for all vineyards were extracted for mean solar radiation (MS), mean high temperature (MHT), mean low temperature (MLT), maximum temperature (MaxT), minimum temperature (MinT), mean temperature (MT), mean precipitation, mean relative soil moisture, mean evaporation (soil + vegetation), and mean transpiration (MTrans) in growing seasons (October 2016/ 2017 - April 2017/ 2018).

Collection of soil and plant materials

In each vineyard, soil samples were collected from three sites at harvest March-April 2017 (n = 45) and 2018 (n = 15), at a depth of 0 - 15 cm, 30 – 50 cm distant from the grapevine. Comprehensive vineyard samples were collected from two vineyards 5 km apart in the 2018 vintage. The vineyards were managed by a single winery, and the viticultural management was very similar. Five replicate Pinot Noir vines in each vineyard were selected from the top, middle, and bottom of the dominant slope, covering topological profiles of the vineyard. For each grapevine, five different sample types were collected: soil (0 - 15 cm deep, root zone), roots, xylem/ phloem sap, leaves, and grapes were collected at harvest in March 2018. Xylem sap (n = 10) was collected from the shoots using a centrifugation method in aseptic conditions (López-Millán et al 2000). Details of xylem sap collection, nutrient composition analysis, and yeast isolation were provided in supplementary

materials. Samples (n = 50) were stored in sterile bags, shipped on ice, and stored at -80 °C until processing.

Longitudinal samples to study microbial abundance during fermentation were collected at five time points: must (destemmed, crushed grapes prior to fermentation), at early fermentation (AF, less than 10% of the sugar is fermented), at middle of fermentation (AF-Mid, around 50% of sugar fermented), at the end of fermentation (AF-End, following pressing but prior to barrelling), at the end of malolactic fermentation (MLF-End, in barrels). Grapes and must sampled in this study were fermented in the wineries following similar fermentation protocols of three days at a cool temperature (known as cold-soaking) followed by warming up the must, so fermentation could commence. Fermentations were conducted startedwithout addition of commercial yeasts and bacteria. Two fermentations from Grampians and Mornington did not complete and were excluded from analysis, giving wine samples from 13 vineyards in the 2017 vintage. Triplicate sub-samples from tanks or barrels were collected and mixed as composite samples (n = 90). All samples were shipped on ice and stored at -20 °C until processing.

Soil analysis

Edaphic factors were analysed to explore the effects of soil properties on wine-related microbiota and aroma profiles. Soil pH was determined in a 2:5 soil/water suspension. Soil carbon (C), nitrogen (N), nitrate and ammonium were analysed by Melbourne Trace Analysis for Chemical, Earth and Environmental Sciences (TrACEES) Soil Node, at the University of Melbourne. Total C and N were determined using classic Dumas method of combustion (Rayment and Lyons 2011) on a LECO TruMac CN at a furnace temperature of 1350°C (LECO Corporation, Michigan, USA). Nitrate and ammonium were extracted with 2 M KCl and determined on a Segmented Flow Analyzer (SAN++, Skalar, Breda, Netherlands) (Rayment and Lyons 2011).

Wine volatile analysis

Volatile compounds of wine samples (MLF-End) were collected to represent the wine aroma. Wine volatiles were determined using headspace solid-phase microextraction gas-chromatographic mass-

spectrometric (HS-SPME–GC/MS) method (Liu et al 2016, Zhang et al 2015) with some modifications. Analyses were performed with Agilent 6850 GC system and a 5973 mass detector (Agilent Technologies, Inc., CA, USA), equipped with a PAL RSI 120 autosampler (CTC Analytics AG, Switzerland). Briefly, 10 mL wine was added to a 20 ml glass vial with 2 g of sodium chloride and 20 μL of internal standard (4-Octanol, 100 mg/L), then equilibrated at 35 °C for 15 min and immersed by a polydimethylsiloxane/divinylbenzene (PDMS/DVB, Supelco) 65 μm SPME fibre for 10 min at 35°C with agitation. The fibre was desorbed in the GC injector for 4 min at 220 °C. Volatiles were separated on an Agilent J&W DB-Wax Ultra Inert capillary GC column (30 m × 0.25 mm × 0.25 μm), with helium carrier gas at a flow rate of 0.7 mL/min. The column temperature program was as follows: holding 40 °C for 10 min, increasing at 3.0 °C/min to 220 °C and holding at this temperature for 10 min. The temperature of transfer line of GC and MS was set at 240 °C. The ion source temperature was 230 °C. The electron impact (EI) at 70 eV scanning over a mass acquisition range of 35–350 *m/z*. Raw data were analysed with Agilent ChemStation Software for qualification and quantification (Liu et al 2015). Volatile compounds (n = 88) were identified in wine samples according to retention indices reference standards and mass spectra matching with NIST11 library. 13 successive levels of standard solution in model wine solutions (12% v/v ethanol saturated with potassium hydrogen tartrate and adjusted to pH 3.5 using 40% w/v tartaric acid) were analysed by the same protocol as wine samples to establish the calibration curves for quantification. Peak areas of volatile compounds were integrated via target ions model. The concentrations of volatile compounds were calculated with the calibration curves and used for downstream data analysis.

DNA extraction and sequencing

Genomic DNA was extracted from botanical and soil samples using PowerSoil™ DNA Isolation kits (QIAgen, CA, USA). DNA extraction of soil (0.25 g) and xylem sap (0.5 mL) samples was directly followed the kit protocol. Wine fermentation samples were thawed and recovered the biomass by centrifugation at 4,000 × *g* for 15 min, washed three times in ice-cold phosphate buffered saline (PBS) with 1% polyvinylpolypyrrolidone (PVPP) and centrifuged at 10,000 × *g* for 10 min (Bokulich et al 2014). The obtained pellets were used for DNA extraction following the kit protocol. For

192 grapevine samples, roots, leaves, grapes (removed seeds and stems), were ground into powder under
193 the protection of liquid nitrogen with 1% PVPP, and isolated DNA following the kit protocol
194 afterward. DNA extracts were stored at -20 °C until further analysis.
195
196 Genomic DNA was submitted to Australian Genome Research Facility (AGRF) for amplification and
197 sequencing. To assess the bacterial and fungal communities, 16S rRNA gene V3-V4 region and
198 partial fungal internal transcribed spacer (ITS) region were amplified using the universal primer pairs
199 341F/806R (Yu et al 2005) and ITS1F/2 (Gardes and Bruns 1993), respectively. The primary PCR
200 reactions contained 10 ng DNA template, 2× AmpliTaq Gold® 360 Master Mix (Life Technologies,
201 Australia), 5 pmol of each primer. A secondary PCR to index the amplicons was performed with
202 TaKaRa Taq DNA Polymerase (Clontech). Amplification were conducted under the following
203 conditions: for bacteria, 95 °C for 7 min, followed by 30 cycles of 94 °C for 30 s, 50 °C for 60 s, 72
204 °C for 60 s and a final extension at 72 °C for 7 min; for fungi, 95 °C for 7 min, followed by 35 cycles
205 of 94 °C for 30 s, 55 °C for 45 s, 72 °C for 60 s and a final extension at 72 °C for 7 min. PCR
206 products were purified, quantified and pooled at the same concentration (5 nM). This was followed by
207 300 bp paired-end sequencing on an Illumina MiSeq (San Diego, CA, USA).
208
209 Raw sequences were processed using QIIME v1.9.2 (Caporaso et al., 2010). Low quality regions (Q <
210 20) were trimmed from the 5′ end of the sequences, and the paired ends were joined using FLASH
211 (Magoč and Salzberg 2011). Primers were trimmed and a further round of quality control was
212 conducted to discard full length duplicate sequences, short sequences (< 100 nt), and sequences with
213 ambiguous bases. Sequences were clustered followed by chimera checking using UCHIME algorithm
214 from USEARCH v7.1.1090 (Edgar et al., 2011). Operational taxonomic units (OTUs) were assigned
215 using UCLUST open-reference OTU-picking workflow with a threshold of 97% pairwise identity
216 (Edgar 2010). Singletons or unique reads in the resultant data set were discarded, as well as the
217 chloroplast and mitochondrion related reads. Taxonomy was assigned to OTUs in QIIME using the
218 Ribosomal Database Project (RDP) classifier (Wang et al 2007) against GreenGenes bacterial 16S

219 rRNA database (v13.8) (DeSantis et al 2006) or the UNITE fungal ITS database (v7.2) (Kõljalg et al
220 2005), respectively.

221 **Data analysis**

222 Raw data are publicly available in the National Centre for Biotechnology Information Sequence Read
223 Archive under the bioproject PRJNA594458 (bacterial 16S rRNA sequences) and PRJNA594469
224 (fungal ITS sequences).

225 Microbial alpha-diversity was calculated using the Shannon index in R (v3.5.0) with the "vegan"
226 package (Oksanen et al 2007). One-way analysis of variance (ANOVA) was used to determine
227 whether sample classifications (e.g., region, fermentation stage) contained statistically significant
228 differences in the diversity after Bonferroni correction with the "ggsignif" package (Ahlmann-Eltze
229 2017). Principal coordinate analysis (PCoA) was performed to evaluate the distribution patterns of
230 wine metabolome and wine-related microbiome based on beta-diversity calculated by the Bray–Curtis
231 distance with the "labdsv" package (Roberts 2007). Permutational multivariate analysis of variance
232 (PERMANOVA) with 999 permutations was conducted within each sample classification to
233 determine the statistically significant differences with "adonis" function in "vegan" (Oksanen et al
234 2007).
235
236 Significant taxonomic differences of wine microbiome between fermentation stages were tested using
237 linear discriminant analysis (LDA) effect size (LEfSe) analysis (Segata et al 2011)
238 (https://huttenhower.sph.harvard.edu/galaxy/). The OTU table was filtered to include only OTUs >
239 0.01% relative abundance to reduce LEfSe complexity. This method applies the factorial Kruskal–
240 Wallis sum-rank test (α = 0.05) to identify taxa with significant differential abundances between
241 categories (using all-against-all comparisons), followed by the logarithmic LDA score (threshold =
242 2.0) to estimate the effect size of each discriminative feature. Significant taxa were used to generate
243 taxonomic cladograms illustrating differences between sample classes.
244

The co-occurrence/interaction patterns between wine metabolome and microbiota in the soil and must were explored in network analysis using Gephi (v0.9.2) (Bastian et al 2009). Only top 100 OTUs (both bacteria and fungi) based on the relative abundance were used to construct the network. A correlation matrix was calculated with all possible pair-wise Spearman's rank correlations between volatile compounds and OTUs. Correlations with a Spearman correlation coefficient $\rho \geq 0.8$ and a $p < 0.01$ were considered statistically robust and displayed in the networks (Junker and Schreiber 2008).

A Random forest supervised-classification model (Breiman 2001) was conducted to identify the main predictors of wine regionality among the following variables: must and soil microbial diversity (Shannon index), soil properties, and weather. The importance of each predictor is determined by evaluating the decrease in prediction accuracy (that is, increase in the mean square error (MSE) between observations and out-of-bag predictions) when the data is randomly permuted for the predictor. This analysis was conducted with 5,000 trees using the "randomForest" package in R (Liaw and Wiener 2002). The significance of the model and the cross-validated R^2 were assessed using the "A3" package (ntree = 5000) (Fortmann-Roe 2013). The structural equation model (SEM) (Grace 2006) was used to evaluate the direct and indirect relationships between must and soil microbial diversity, soil properties, climate and wine regionality. SEM is an *a priori* approach partitioning the influence of multiple drivers in a system to help characterize and comprehend complex networks of ecological interactions (Eisenhauer et al 2015). An *a priori* model was established based on the known effects and relationships among these drivers of regional distribution patterns of wine aroma to manipulate the data before modelling. Weather and soil properties were used as composite variables (both Random Forest and SEM) to collapse the effects of multiple conceptually-related variables into a single-composite effect, thus aiding interpretation of model results (Grace 2006). A path coefficient describes the strength and sign of the relationship between two variables (Grace 2006). The good fit of the model was validated by the χ2-test ($p > 0.05$), using the goodness of fit index (GFI > 0.90) and the root MSE of approximation (RMSEA < 0.05) (Schermelleh-Engel et al 2003). The standardised total effects of each factor on the wine regionality pattern were calculated by summing all direct and

indirect pathways between two variables (Grace 2006). All the SEM analyses were conducted using AMOS v25.0 (AMOS IBM, NY, USA).

SourceTracker was used to track potential sources of wine-related fungi within the vineyards (Knights et al 2011). SourceTracker is a Bayesian approach that treats each give community (sink) as a mixture of communities deposited from a set of source environments and estimates the proportion of taxa in the sink community that come from possible source environments. When a sink contains a mixture of taxa that do not match any of the source environments, that portion of the community is assigned to an "unknown" source (Knights et al 2011). In this model, we examined musts (n = 2) and vineyard sources (n = 50) including grapes, leaves, xylem sap, roots, and soils. The OTU tables were used as data input for modelling using the "SourceTracker" R package (https://github.com/danknights/sourcetracker).

Results

Chemical composition/ aroma profiles separate wines based on geographic location

Using GC-MS, we analysed the volatile compounds of Pinot Noir wine samples (MLF-End) representing distinct wine growing regions. These were finished wines, which are being prepared for sale to consumers and enabled the metabolite profiles to be compared directly to the microbial communities inhabiting the musts from which these wines were fermented. In all, 88 volatile compounds were identified in these wines. We used α- and β-diversity measures to elucidate wine complexity and regionality, respectively. In 2017, α-diversity varied with regional origins (ANOVA, $p < 0.001$), with higher Shannon indices (2.17 ± 0.05) were observed in the wines from regions of Mornington, Yarra Valley and Gippsland than others (1.94 ± 0.03) (Figure 1A). PCoA showed that 74.5% of the variance was explained by the first two principal coordinates, and that wines within regions grouped together (Bray–Curtis, ADONIS, $p < 0.001$) (Figure 1B). Overall, wine aroma profiles display highly significant regional differentiation across both vintages (ADONIS, $R^2 = 0.566$, $p < 0.001$) and the clustering patterns become more distinct and regression coefficients improve when comparing regional differences in 2017 (ADONIS, $R^2 = 0.703$, $p < 0.001$) (Supplementary Table S1).

Microbial ecology from the vineyard to the winery

To elucidate how microbial ecology drives regional traits of wine from the vineyard to the winery, 150 samples covering soils, musts and fermentations were collected to analyse wine-related microbiota. A total of 11,508,480 16S rRNA and 12,403,610 ITS high-quality sequences were generated from all the samples, which were clustered into 13,689 bacterial and 8,373 fungal OTUs with a threshold of 97% pairwise identity, respectively.

The dominant bacterial taxa across all soil samples were *Actinobacteria*, *Proteobacteria*, *Acidobacteria*, *Chloroflexi*, *Verrucomicrobia*, *Bacteroidetes*, *Gemmatimonadetes*, *Firmicutes*, *Planctomycetes* and *Nitrospirae* (Supplementary Figure S2A). Compared with bacteria, soil fungal communities were less diverse. *Ascomycota* was the most abundant and diverse phylum of fungi,

accounting for 72% of reads, followed by *Basidiomycota*, *Mortierellomycota*, *Chytridiomycota* and *Olpidiomycota* (Supplementary Figure S2B). The microbiome richness, α-diversity (Shannon index) varied significantly with regions for both bacteria and fungi (ANOVA, $p < 0.01$) (data not shown). Soil community structures widely varied across different grape growing regions, exerting significant impacts on both bacterial and fungal taxonomic dissimilarity (Bray–Curtis, ADONIS, $p < 0.001$) independent of vintage, with clearer differences within a single vintage (Supplementary Table S1). In 2017, soil fungal communities could discriminate growing regions (except Yarra Valley and Gippsland) (Figure 2B), whereas regional separation of bacteria was weaker with overlap between two or three regions (Figure 2A).

In grape musts, bacterial communities across six wine growing regions in both vintages consisted of ubiquitous bacteria *Enterobacteriales*, *Rhizobiales*, *Burkholderiales*, *Rhodospirillales*, *Actinomycetales*, *Sphingomonadales*, *Pseudomonadales*, *Saprospirales*, and *Xanthomonadales*, which do not participate in wine fermentations or spoilage (Barata et al 2012). The LAB *Lactobacillales*, responsible for malolactic fermentation, was present in low abundance (average = 0.4%) in the must (Figure 3A). Fungal profiles were dominated by filamentous fungi, mostly of the genera *Aureobasidium*, *Cladosporium*, *Botrytis*, *Epicoccum*, *Penicillium*, *Alternaria*, and *Mycosphaerella*, with notable populations of yeasts, including *Saccharomyces*, *Hanseniaspora*, and *Meyerozyma*, as well as the *Basidiomycota* genus *Rhodotorula* (Figure 3B). Pinot Noir musts exhibited significant regional patterns for fungal communities across vintages 2017 and 2018 (Bray–Curtis, ADONIS, $p < 0.001$) but no significant differences for bacterial communities across both vintages (Bray–Curtis, ADONIS, $p = 0.152$) (Supplementary Table S1). Within the 2017 vintage, both bacteria and fungi in the must showed distinctive structures and composition based on the region (Bray–Curtis, ADONIS, $p < 0.001$), with more distinct trend and improved regression coefficient for fungi ($R^2_{2017, 2018} = 0.292$, $R^2_{2018} = 0.565$) (Figure 3A, B) (Supplementary Table S1). Notably, the relative abundance of *Saccharomyces* yeasts varied significantly from 1.3% (Macedon Range) to 65.6% (Gippsland) between regions (ANOVA, $p < 0.01$) (Figure 3B). As the wine fermentation proceeded, fermentative

337 populations including yeasts and LAB grew and dominated, thus reshaping the community diversity
338 (Supplementary Figure S3A, B) and composition (Supplementary Figure S3C, D). Fungal species
339 diversity collapsed as alcoholic fermentation progressed (ANOVA, $p < 0.001$) (Supplementary Figure
340 S3B), while the impact of the fermentation on bacteria was less than fungi, with slight decreased
341 diversity at early stages and significant recovery at the end (ANOVA, $p < 0.05$) (Supplementary
342 Figure S3A). Linear discriminant analysis (LDA) effect size (LEfSe) analysis further identified
343 differentially abundant taxa (Kruskal–Wallis sum-rank test, $\alpha < 0.05$) associated with fermentation
344 stages (Figure 3). For fungal populations, *Dothideomycetes* (including *Aureobasidium* and
345 *Cladosporium*), *Debaryomycetaceae* (notably yeast *M. guilliermondii*), *Penicillium corylophilum* and
346 *Filobasidium oeirense* were significantly abundant in the grape must, with *Leotiomycetes*,
347 *Sarocladium* and *Vishniacozyma victoriae* in early fermentations (AF), *Saccharomycetes* yeasts
348 (notably *S. cerevisiae*) in mid fermentations (AF-Mid), and *Tremellales* at the end of fermentation
349 (AF-End) (Figure 3D). For bacterial communities, *Acidobacteriia* (spoilage), *Chloroflexi*,
350 *Deltaproteobacteria*, *Sphingobacteriia*, *Cytophagia*, *Planococcaceae* and *Rhizobiaceae* were
351 observed with higher abundances in the must, *Proteobacteria* (including *Burkholderiaceae* and
352 *Tremblayales*, spoilage) and *Micrococcus* in the AF, *Burkholderia* spp. in the AF-Mid,
353 *Rhodobacterales* and *Pseudonocardiaceae* in the AF-End, LAB *Leuconostocaceae* (notably
354 *Oenococcus*) in the MLF-End (Figure 3C). Regional differences in microbial profiles were not
355 significant in the finished wines (ADONIS, $R^2_{bacteria} = 0.149$, $p = 0.321$; $R^2_{fungi} = 0.109$, $p = 0.205$)
356 (Supplementary Table S1).

357

358 To uncover the impact of growing season (vintage) on wine regionality and related microbiota, five
359 vineyards in Mornington were sampled in 2017 and 2018 to compare within and between vintages.
360 Within these five vineyards alone, both microbial patterns and resulting wine aroma profiles see a
361 significant vintage influence (ADONIS, $p < 0.001$) (Supplementary Table S1). However, vintage only
362 weakly impacts microbial patterns and wine regionality, in particular an insignificant influence on
363 fungal patterns when comparing all must samples (ADONIS, $p = 0.066$) (Supplementary Table S1).

We used 2017 vintage data to further explore microbial biogeography and wine regionality in the following analyses.

Microbial patterns correlate to wine aroma profiling

Network analysis was used to explore connections between regional microbial and wine metabolic patterns. We selected 57 volatile compounds and 246 OTUs based on strong correlation coefficients (Spearman correlation coefficient, $\rho \geq 0.8$; $p < 0.01$), forming the co-occurrence patterns. These variables showed a sophisticated internal structure, consisting of 303 nodes and 610 edges (average degree = 4.026), with mostly positive correlations (90.66%) (Figure 4A). Both must (average degree = 3.791) and soil microbiota (average degree = 3.333) presented high connectivity with wine metabolites (Figure 4B, C). Fungal OTUs (n = 69) more densely connected with volatile compounds than bacteria (n = 42) in the soil (Figure 4B). This indicated that soil microbial communities, especially fungi, play an important role in wine regionality. The most densely connected volatile compounds were assigned to groups of monoterpenes (C18, *p*-cymene; C46, α-terpineol), phenylpropanoids (C71, benzaldehyde; C74, phenethyl acetate), and acetoin (C20) (Figure 4A). Correspondingly, high-connectivity nodes with these compounds were OTUs of bacterial taxa *Enterobacteriales*, *Rhizobiales*, *Burkholderiales* and *Xanthomonadales*, and fungal taxa *Penicillium*, *Rhodotorula* and *Neocatenulostroma*, from soil and must (Figure 4B, C). The group of esters, which are yeast-driven products (Swiegers et al 2005), were highly associated with fungal OTUs (in particular *Hypocreales*, *Alternaria*, *Cryptococcus and Mortierella*) from the vineyard soil, for example, ethyl hexanoate (C16) and ethyl dihydrocinnamate (C87) (Figure 4B). Fatty acids (including C47, 4-hydroxybutanoic acid; C77, octanoic acid) were mostly negatively connected with fungi (including *Acremonium*, *Penicillium*) from the must (Figure 4C).

Multiple drivers modify wine regionality in the vineyard

To disentangle the role of microbial ecology on wine regionality expression, we used Random Forest modelling (Breiman 2001) to identify important predictors (weather/ climatic conditions, soil properties, microbial diversity of soil and must) driving wine regionality, and structural equation modelling (SEM) (Grace 2006) to testify whether the relationship between microbial diversity and

391 wine regionality can be maintained when accounting for multiple drivers simultaneously. The
392 Random Forest model demonstrated that fungal diversities were major predictors of wine regionality.
393 Not surprisingly, must fungal diversity played an important role (increase in MSE) than soil (Figure
394 5). The SEM explained 94% of the variance found in the pattern of wine regionality (Figure 6A).
395 Weather drove wine aroma profiles directly (especially MT, MLT, MinT and MS) and indirectly by
396 powerful effects on soil and must microbial diversity, in particular strong effects on soil fungal
397 diversity (Figure 6A). Must fungal diversity had the highest direct positive effect on wine aroma
398 characteristics, with direct influences from soil fungal diversity (Figure 6A). Weather and climate
399 could impact soil nutrient pools primarily through MS, MLT, MinT, and MTrans. Soil properties
400 showed strong effects on soil microbial diversity and must bacterial diversity, but weak effects on
401 must fungal diversity (Figure 6A). Must bacterial diversity had a weak effect on wine aroma profiles,
402 as well as soil bacteria. Overall, must fungal diversity is the most important driver of wine
403 characteristics, followed by soil fungal diversity (Figure 6B), with influences from weather and soil
404 properties both directly and indirectly (Figure 6A).

405 **Source tracking wine-related fungi within vineyard**

406 As we showed in the SEM, soil fungal diversity had a direct positive influence on must fungal
407 diversity and thus indirectly contributed to wine aroma profiles (Figure 6). Given that soil is a
408 potential source of fungi associated with wine production (Morrison-Whittle and Goddard 2018), here
409 we attempt to uncover the mechanism whereby soil fungi could be transported from soil to the grapes.
410 We sampled fungal communities from grapevines and soil and hypothesised that the xylem/ phloem
411 was the internal mechanism to transport microbes. A total of 2,140,820 ITS high-quality sequences
412 were generated from soil and grapevine samples (grape, leaf, xylem sap, root), which were clustered
413 into 4,050 fungal OTUs with 97% pairwise identity. Using SourceTracker (Knights et al 2011), fungal
414 communities in the must were matched to multiple sources from the belowground to the aboveground.
415 Results showed that grape and xylem sap were primary sources of must fungi, with 32.6% and 41.9%
416 contributions, respectively (Figure 7A). Xylem sap showed similar fungal structure with must (Figure

S4A). Further source tracking revealed that the root and soil contributed 90.2% of fungal OTUs of xylem sap which contributed 67.9% of the fungi of grapes (Figure 7B, C).

Notably, *S. cerevisiae* were found shared between niches of soil, root, xylem sap, grape and must, with the highest (1.2%) and lowest (0.04%) abundance in the root and soil, respectively (Figure S4B). To explore whether xylem/phloem sap could be a translocation pathway of *S. cerevisiae*, we attempted to culture yeasts from the collected xylem sap. Chemical analysis of nutrient compositions showed that xylem sap contained nine carbohydrates (predominantly glucose, fructose and sucrose), 15 amino acids (mainly arginine, aspartic acid and glutamic acid) and six organic acids (primarily oxalic acid), which could be utilised as carbon sources and support yeast growth (Figure S4E, F, G) (Yadeta and Thomma 2013). No *S. cerevisiae* yeasts were isolated, but distinct isolates of the *Basidiomycota* yeasts of *Cryptococcus* spp. (primarily *C. saitoi*) and *Rhodotorula slooffiae* were found (Figure S4C). Similar species of yeasts were isolated when xylem/phloem sap was collected from grapevines grown in the glasshouse (Figure S4D).

Discussion

Microbial ecology can influence grapevine health and growth, fermentation, wine quality and style and flavour characteristics (Bokulich et al 2014, Gayevskiy and Goddard 2012, Knight et al 2015). Here we show that wine regionality is dependent on fungal ecology and is sensitive to local weather, climate and soil properties. A new mechanism to transfer fungi from the soil to grapes and must via xylem/ phloem sap was investigated. We systematically investigated the microbiome from the soil to wine to show that soil and grape must microbiota exhibit regional patterns and that these patterns can be translated into wine metabolites.

A microbial component of wine *terroir*

Biogeographic patterns are clearly present in the soil and grape must microbiota. The most abundant bacterial phyla in the soil were *Actinobacteria*, *Proteobacteria* and *Acidobacteria*, which are known dominant and ubiquitous in the vineyard soil worldwide (Burns et al 2015, Coller et al 2019, Fierer et al 2012, Zarraonaindia et al 2015). For fungi, the community composition and structure were more diverse within regions we studied and compared with studies in other major wine-producing areas in the world. Overall, we recovered 14 phyla, 30 classes, 65 orders, 125 families and 216 genera of fungi, recording a higher diversity than found in previous studies (Coller et al 2019, Knight et al 2019, Morrison-Whittle et al 2017). *Glomeromycota* and *Blastocaldiomycota* were reported as abundant phyla across vineyards in Marlborough, New Zealand (Morrison-Whittle and Goddard 2017). In our study, however, *Glomeromycota* was only recovered with low frequency in soils coming from Mornington and Macedon Ranges, and *Blastocaldiomycota* was not detected (data not shown). In the must, both principal fermentation drivers (*S. cerevisiae* and LAB) and innocent species (those that neither ferment nor spoil, such as *Enterobacteriales* and *Aureobasidium*) were present in different abundances among regions (Figure 3A, B, ANOVA, $p < 0.01$). The order *Lactobacillales*, representing LAB, was present 0.4% across regions, compared to 29.7% found in California, US (Bokulich et al 2014) and 14% in Catalonia, Spain (Portillo et al 2016).

458 Environmental factors (such as weather and climate) and geographic characteristics (in particular
459 latitude) drive microbial diversity and biogeography across various habitats, such as soil, ocean, and
460 plant ecosystems (Bokulich et al 2014, Fierer and Jackson 2006, Martiny et al 2006, Tedersoo et al
461 2014). In this study, we demonstrate that microbial biogeographic communities are distinct in both
462 vineyard soils and grape musts at the scale of 400 km and this is not dependent on the growing
463 season/ vintage. This aligns with previous studies on wine microbial biogeography and provide
464 further evidence for microbial *terroir* (reviewed by Liu et al. 2019; Bokulich et al 2014, Burns et al
465 2015, Morrison-Whittle and Goddard 2018, Taylor et al 2014). Soil bacteria can distinguish wine
466 growing regions, with impacts from soil properties (Figure 6A) and this is supported by previous
467 work in this field (Burns et al 2015, Mezzasalma et al 2018, Zarraonaindia et al 2015). An interesting
468 finding is that the must bacterial diversity is strongly influenced by soil properties, in particular C: N.
469 Previous work has shown that structures of must and soil communities are similar and some species
470 *Enterobacteriales* and *Actinomycetales* originate from the soil (Pinto et al 2015, Stefanini and
471 Cavalieri 2018). As C: N can be manipulated by composting and cover crops (Burns et al 2016) there
472 is an opportunity to manipulate wine microbiota by a focus on vineyard management (Liu et al 2019).
473 The bacterial microflora is recognised as important for plant growth processes more broadly (Berg et
474 al 2009), but fungal diversity beyond endosymbiotic mycorrhizae has not been systematically
475 investigated for grapevines. Here, we show that the soil fungal communities are distinct for a region.
476 Our modelling suggests that soil properties (C: N) and weather (MS) strongly affect soil fungal
477 diversity, which was in line with large-scale studies in which climatic (especially precipitation) and
478 edaphic factors (especially C: N) are the best predictors of soil fungal richness and community
479 composition (Lauber et al 2008, Tedersoo et al 2014). Must fungal diversity is also shaped by weather
480 and soil properties indirectly via the soil fungi to ultimately affect wine aroma profiles.
481
482 It is noteworthy that the drivers of microbial patterns change during wine fermentation. Microbial
483 diversity decreased as alcoholic fermentation proceeds, with a clear loss of microorganisms including
484 filamentous fungi, non-*Saccharomyces* yeasts (for example *M. guilliermondii*), spoilage bacteria
485 *Acidobacteria* and *Proteobacteria*, and other bacteria with unknown fermentative functions (for

example *Chloroflexi*), and thus the biogeographic trend was lost by the stage of MLF-End (Supplementary Table S1). This trend was observed more distinct in fungal communities compared with bacteria. This is not unexpected as it is clear that fermentation more strongly affects fungal populations compared with bacteria, due to increasing fermentation rate, temperature and ethanol concentration induced by *S. cerevisiae* growth (Bokulich et al 2012, Goddard 2008). In this case, fermentation conditions, such as chemical environments and interactions/competition within the community, reshape the microbial patterns. Despite the complex change of microbial ecosystem during fermentation, we show that biogeographic patterns in the must can be translated into regional metabolic profiling of wine. Our modelling indicates that the indirect influences of weather and soil properties via shaping soil and must microbial diversity are more powerful than the direct influences on wine aroma profiles (Figure 6). Co-occurrence networks demonstrate that soil and must microbiota closely correlate to the wine metabolome. These interactions also indicate some potential modulations of soil and must microbiota on wine chemistry, in particular that are numerically dominant taxa in the early fermentation. For example, bacteria *Enterobacteriales* positively correlated with some grape-derived volatile compounds, thus potentially enhancing variety aroma in wine (Swiegers et al 2005). *Enterobacteriales* have been also previously related to wine fermentation rate by Bokulich et al. (2016). Fungal genera abundance (for example *Penicillium*) negatively correlated with medium-chain fatty acid octanoic acid, which can contribute to mushroom flavour and also alter *Saccharomyces* metabolism (Bisson 1999). These microorganisms correlate with wine metabolites and/or can change chemical environments and fermentation behaviours and finally exert substantial effects on wine metabolite profiles (Swiegers et al 2015, Bokulich et al 2016). It is particularly important to inform farming practices to structure regional microbial communities that can benefit soil quality, hence, crop productivity.

Fungal communities distinguish wine quality and style

In grape musts, bacterial and fungal communities exhibit different responses to site-specific and environmental effects. Bacterial regional patterns were not as distinct as fungi, and significantly impacted by vintage (Supplementary Table S1). Although profound responses to soil properties (for

example C: N), affects wine fermentation, must bacteria show insignificant influences on wine aroma profiles (Figure 6). In contrast, fungal communities display discriminant distribution patterns at the regional scale and are consistent between the years studied in this work than the bacterial communities, aligning with results presented by Bokulich et al (2014). Soil fungal communities are less diverse than bacterial communities (Figure S2; Fierer et al 2017), but of more importance to resulting wine regionality (see Figure 4, 5, 6). Must fungi, in particular the fermentative yeasts, conduct alcoholic fermentations and provide aroma compounds to influence wine flavour (Swiegers et al 2005). As indicated by SEM, soil fungal communities are affected by local soil properties and weather and exert impacts on must fungal communities (Figure 6). Co-occurrence relationships between wine metabolome and soil microbiota highlight potential contributions from fungal taxa (Figure 4). One explanation is that grapevines filter soil microbial taxa selecting for grape and must consortia (Deveau et al 2018, Panke-Buisse et al 2015). Beyond fungi, plant fitness is linked strongly to the responses of soil microbial communities to environmental conditions (Lau and Lennon 2012). More sensitive responses of vineyard soil fungi might improve grapevine fitness to local environments thus benefiting the expression of regional characteristics of grapes and wines.

How could yeasts present in the soil be transported to the berry surface? Soil is a reservoir of grapevine-associated microbiome (Figure 7) and supported by results presented by Grady et al 2019, Mezzasalma et al 2018, Morrison-Whittle and Goddard 2018, Zarraonaindia et al 2015). As well as transporting water and minerals absorbed by roots to the photosynthetic organs, xylem sap is also a niche for microbes that can bear its nutritional environment (Yadeta and Thomma 2013). Here we investigated xylem sap as a conduit to shape the microbiota on the grape surface by enrichment and recruited by roots and transported by xylem sap to grape berries (Berg 2009, Compant et al 2008, Zarraonaindia et al 2015). No *S. cerevisiae* yeasts were cultured from collected sap in vines in the vineyard or in the glasshouse. We were able to isolate and identify yeasts belonging to *Cryptococcus* and *Rhodotorula* genera, which shows that this environment is not sterile and can potentially transport yeasts to the phyllosphere.

Fungi, while not as ecologically diverse as bacteria, are direct contributors to wine aroma. In the soil, fungi can distinguish wine regions in this study have relatively stable populations between years. While we were unable to find fermentative yeasts in the sap of grapevines, yeasts were present and thus may be transported in the grapevine as well as making their way to the phyllosphere through other mechanisms (water splashes, insect vectors). Previous studies have shown that fermentative yeasts are persistent in vineyards (Börlin et al 2016, Valero et al 2005) and can be transported by grapevines (Mandl et al 2015). We can thus conclude that fungi are the principal drivers of consistent expression of regionality in wine production.

Our study illustrates that microbes are absolute contributors to wine aroma and that this comes from the environment in which they are grown and provides a basis to explain how wine may have a distinctive flavour from a particular region. Fungi plays a crucial role in interrelating these biotic and abiotic elements in vineyard ecosystem and could be transported by the internal. Climate and soil properties profoundly drive microbial patterns from the soil to the grape must, which ultimately sculpt wine metabolic profiling. We do not yet know how grapevines recruit their microbiome to maximise physiological development and microbial diversity under local conditions.

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Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Figures

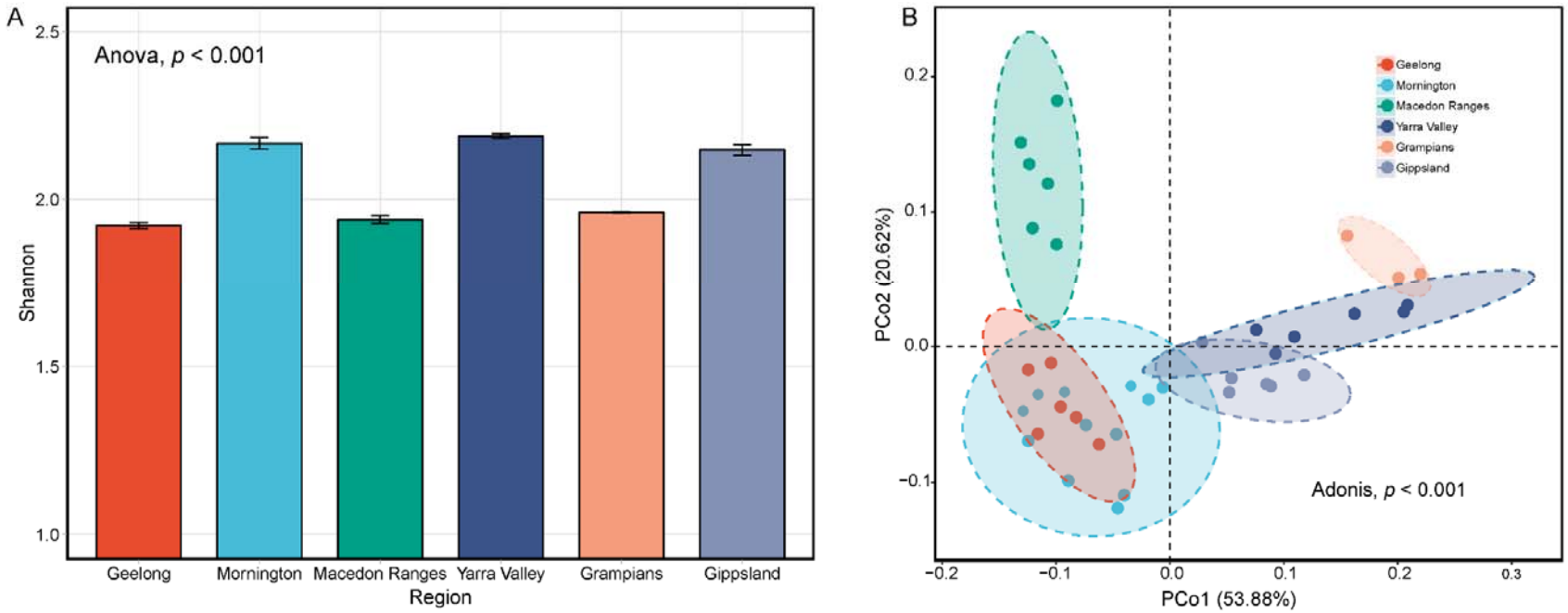

Figure 1 Wine metabolome shows regional variation across six wine growing regions. Shown are α-diversity (Shannon index) (A) and PCoA comparisons of Bray-Curtis dissimilarity (B) of wine volatile profiles.

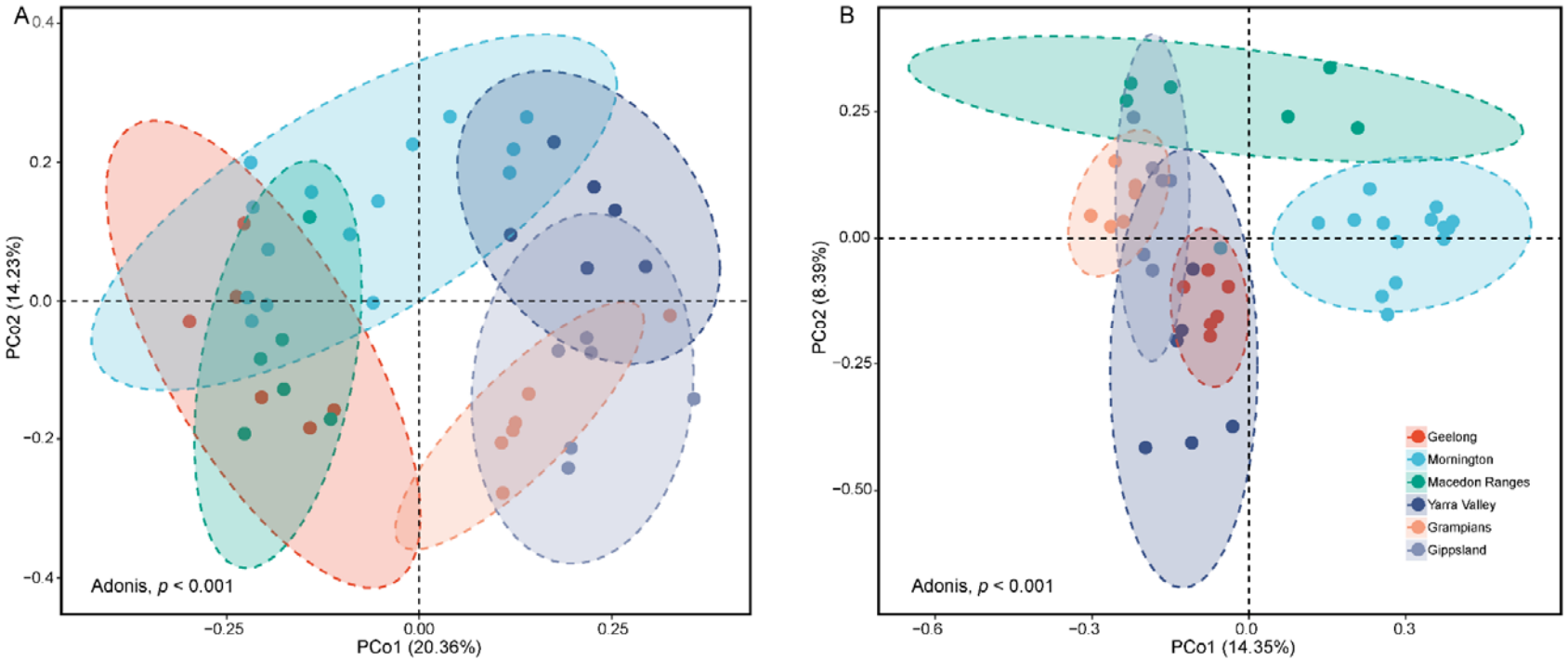

Figure 2 Vineyard soil microbial communities demonstrate distinct regional patterns. Bray-Curtis distance PCoA of bacterial communities (A) and fungal communities (B).

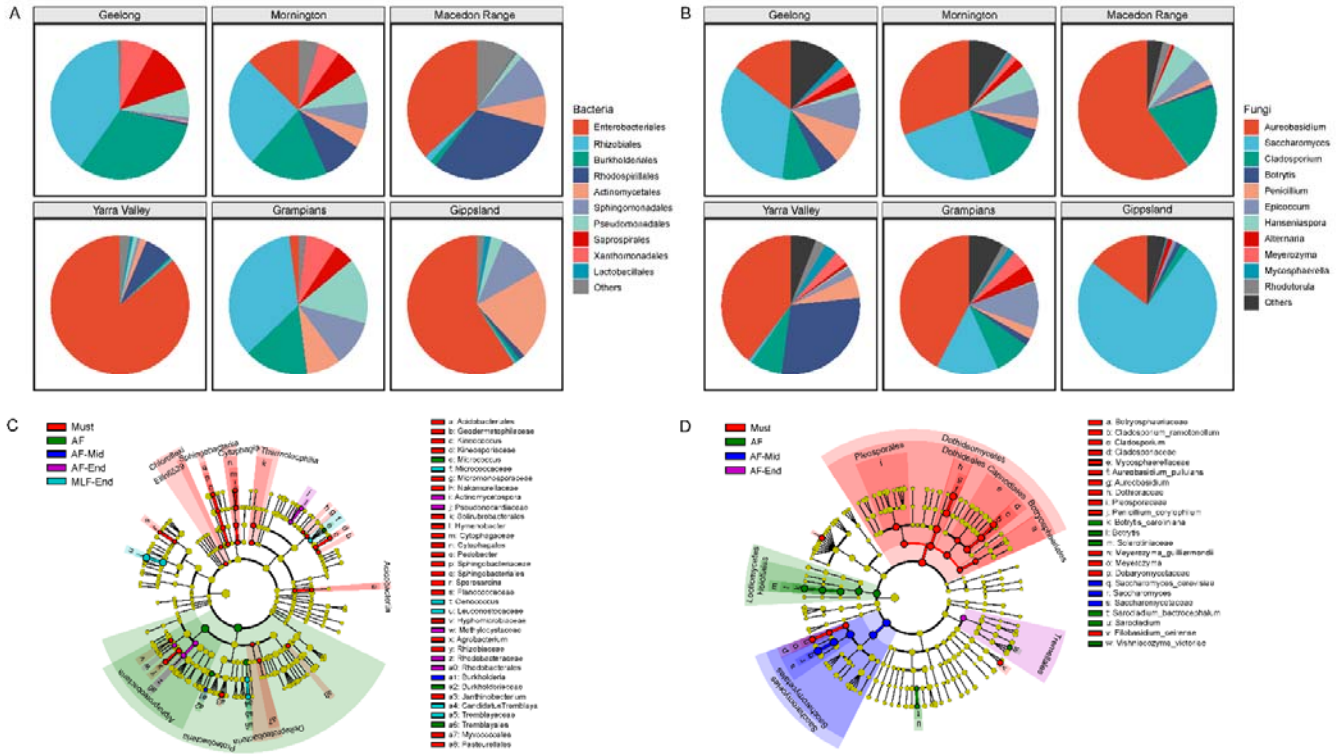

Figure 3 Microbiota exhibits regional differentiation in musts for both bacterial and fungal profiles. Stage of fermentation influences microbial composition of bacteria and fungi. (A) Must bacterial taxa in the order level with greater than 1.0% relative abundance, and *Lactobacillales.* (B) Must fungal taxa in the genus level with greater than 1.0% relative abundance. (C and D) LDA effect size taxonomic cladogram comparing all musts and wines categorised by fermentation stage. Significantly discriminant taxon nodes are coloured and branch areas are shaded according to the highest-ranked stage for that taxon. For each taxon detected, the corresponding node in the taxonomic cladogram is coloured according to the highest-ranked group for that taxon. If the taxon is not significantly differentially represented between sample groups, the corresponding node is coloured yellow.

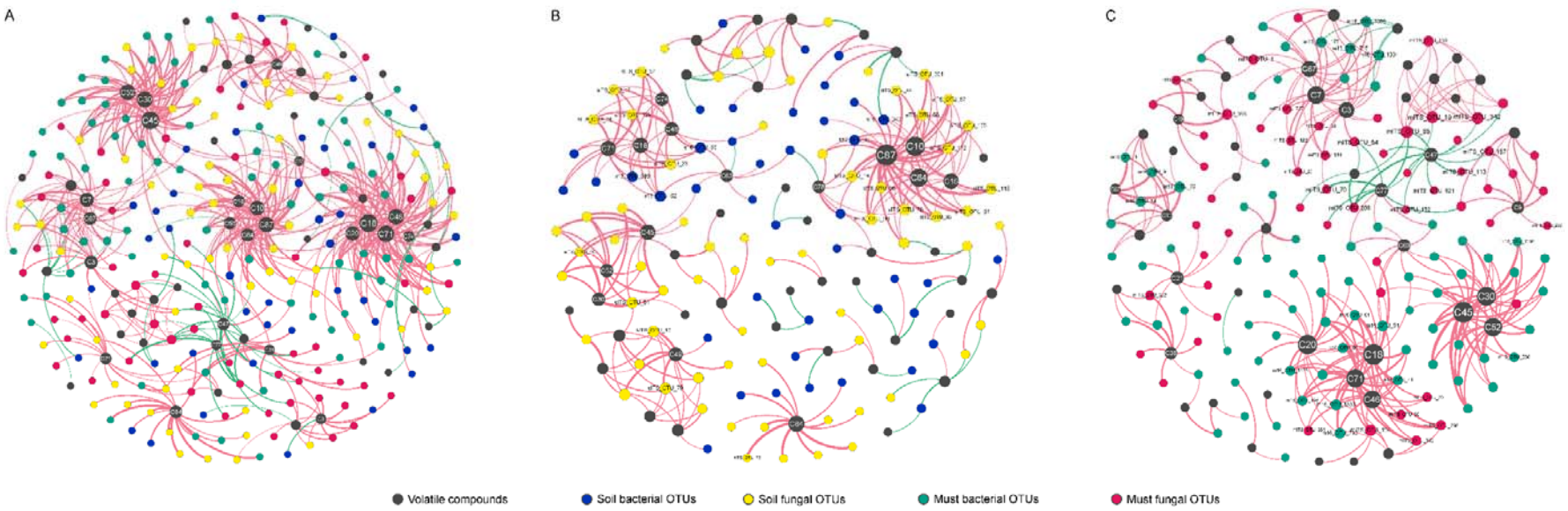

Figure 4 Statistically significant and strong co-occurrence relationships between wine volatile compounds and bacterial and fungal OTUs in vineyard soils and musts across six wine growing regions. Network plots for wine aroma with soil and must microbiota (A), and simplified network plots for wine aroma with soil microbiota (B) and with must microbiota (C), separately. Circle nodes represent assigned volatile compounds, bacterial and fungal OTUs, with different colours. Direct connections between compounds and OTUs indicate strong correlations (Spearman correlation coefficient, $\rho \geq 0.8$; $p < 0.01$). The colour of edges describes the positive correlation (pink) or the negative correlation (green). The size of nodes is proportioned to the inconnected degree.

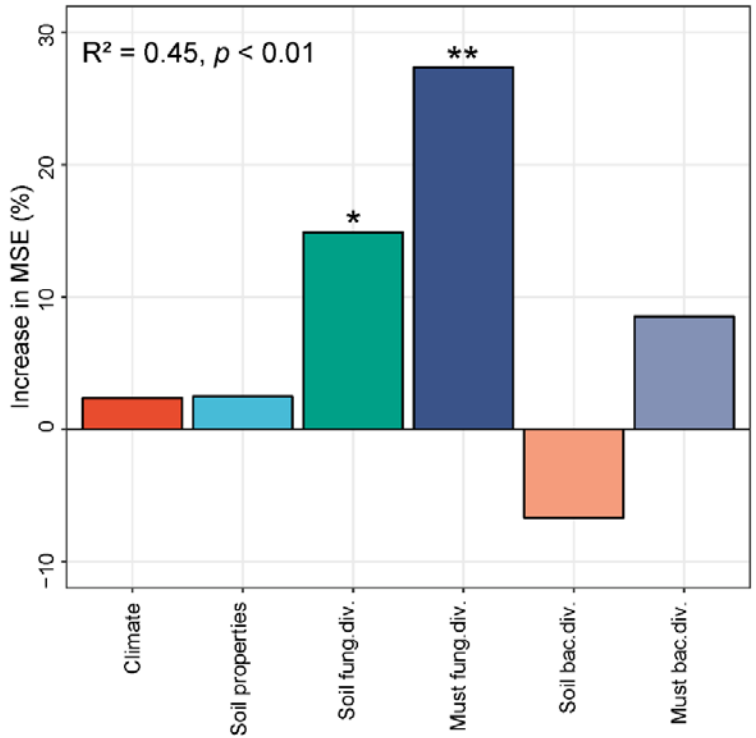

Figure 5 Main predictors of wine regionality. Shown is the Random Forest mean predictor importance (% of increase in MSE) of climate, soil properties and microbial diversity (Shannon index) on wine regionality. Soil bac. div., soil bacterial diversity; Soil fung. div., soil fungal diversity; Must bac. div., must bacterial diversity; Must fung. div., must fungal diversity. Significance levels: $*p < 0.05$, $**p < 0.01$.

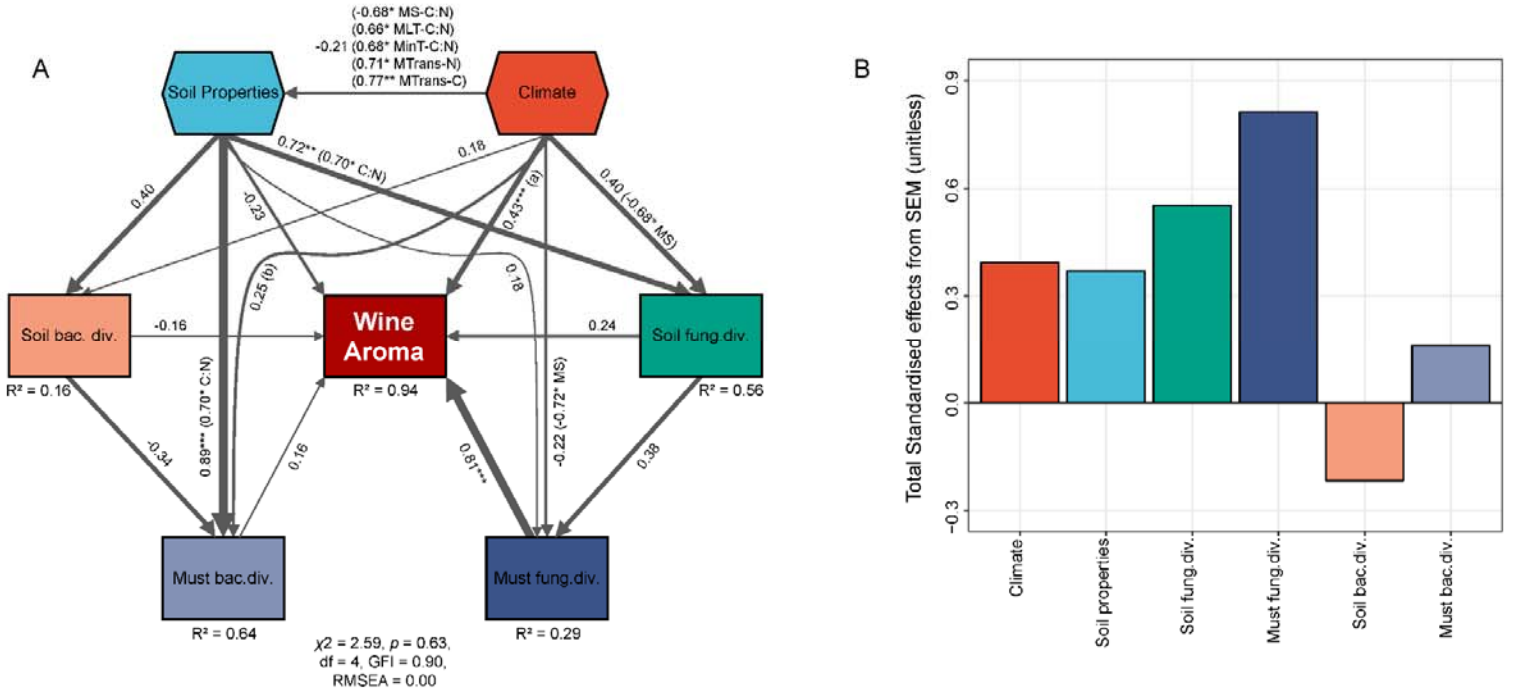

Figure 6 Direct and indirect effects of climate, soil properties and microbial diversity (Shannon index) on wine regionality. Structural equation model (SEM) fitted to the diversity of wine aroma profiles (A) and standardized total effects (direct plus indirect effects) derived from the model (B). Climate and soil properties are composite variables encompassing multiple observed parameters (see materials and methods for the complete list of factors used to generate this model). Numbers adjacent to arrows are path coefficients and indicative of the effect size of the relationship. The width of arrows is proportional to the strength of path coefficients. R^2 denotes the proportion of variance explained. (a) (0.75* MT) (0.67* MLT) (0.69* MinT) (-0.88** MS). (b) (0.75* MinT) (0.77* MLT) (-0.68* MS) (-0.74* MHT) (-0.84** MaxT). C, soil carbon; N, soil nitrogen; C:N, soil carbon nitrogen ratio; MS, mean solar radiation; MT, mean temperature; MLT, mean low temperature; MHT, mean high temperature; MinT, minimum temperature; MaxT, maximum temperature; MTrans, mean transpiration. Significance levels: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

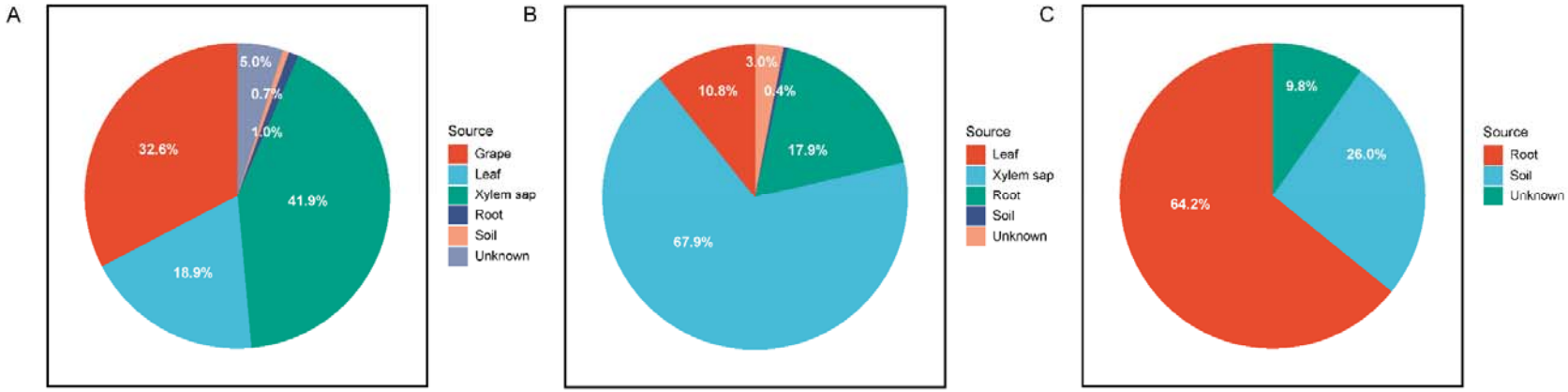

Figure 7 Fungal communities in musts emerge from multiple sources in the vineyard, but primarily from grape and xylem sap. Percent composition estimate for the contributions of possible sources in the vineyard to the wine-related fungal community for sourcetracking must (A), grape (B) and xylem sap (C).

Supplementary Table and Figures

Table S1 Permuted multivariate analysis of variance (PERMANOVA) of region and vintage effects on microbial patterns and wine profiles (beta-diversity)

Sample Group	Vintage	Factor	Soil				Must				Wine (MLF-End)				Wine Aroma	
			Bacteria		Fungi		Bacteria		Fungi		Bacteria		Fungi		MLF-End	
			R2	*p*	R2	*p*	R2	*p*	R2	*p*	R2	*p*	R2	*p*	R2	*p*
All	Both	Region	0.318	0.001	0.254	0.001	0.108	0.152	0.292	0.001	N/A	N/A	N/A	N/A	0.566	0.001
All	2017	Region	0.392	0.001	0.419	0.001	0.248	0.013	0.565	0.001	0.149	0.321	0.109	0.205	0.703	0.001
All	Both	Vintage	0.102	0.001	0.086	0.001	0.186	0.001	0.049	0.066	N/A	N/A	N/A	N/A	0.267	0.001
Mornington	Both	Vintage	0.155	0.001	0.151	0.001	0.305	0.001	0.143	0.001	N/A	N/A	N/A	N/A	0.355	0.001

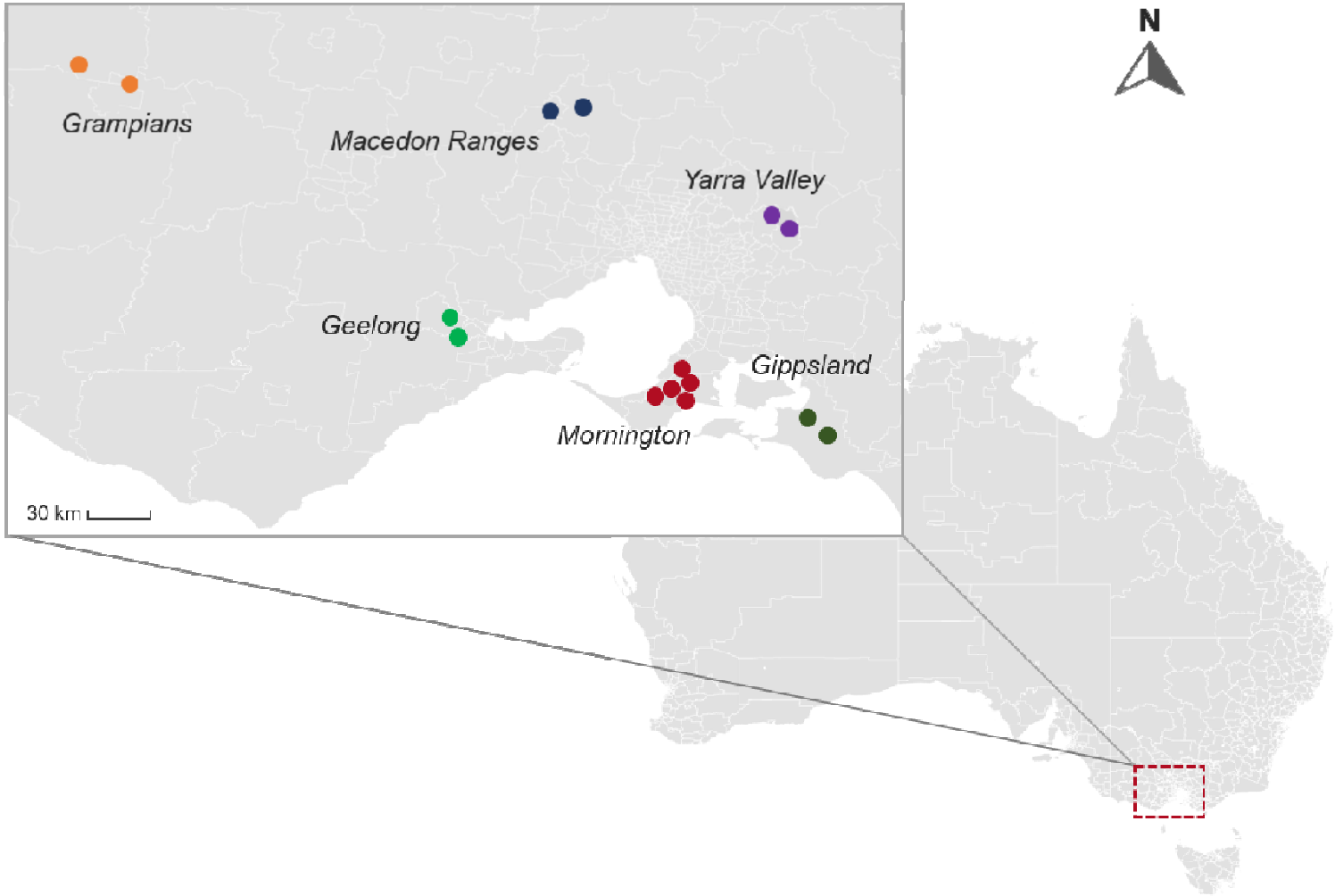

Figure S1 Map of 15 sampling vineyards from six Pinot Noir wine-producing regions in southern Australia, spanning 400 km (E-W).

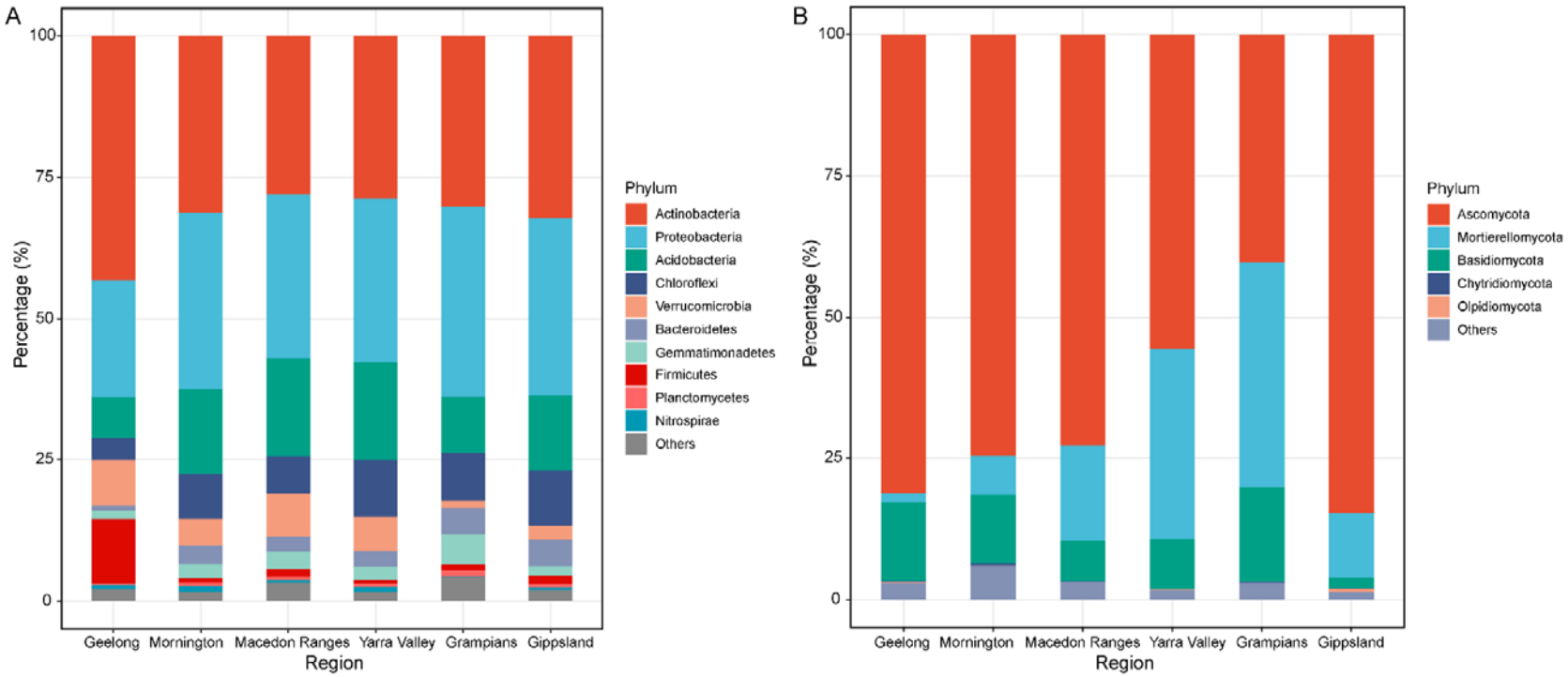

Figure S2 Vineyard soil microbial community structures across all samples from six grape growing regions. Microbial community compositions in soil samples are characterized to the phylum level: (A) dominant bacterial taxa with greater than 1.0% relative abundance (top 10 phyla); (B) fungal phyla.

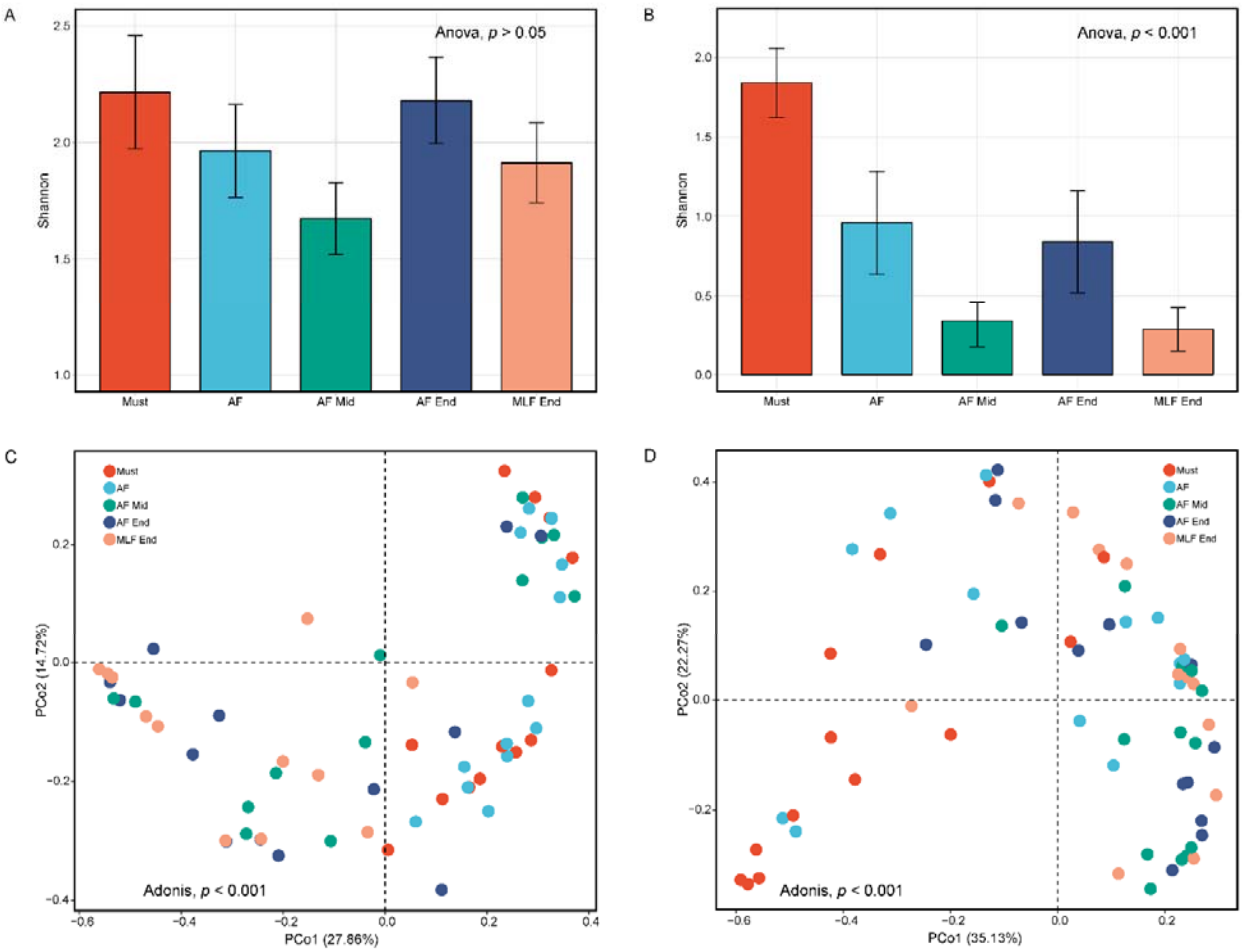

Figure S3 Stage of fermentation influences microbial diversities. Bacterial (A) and fungal (B) α-diversity (Shannon index) changes during wine fermentation. Bray-Curtis distance PCoA of bacterial communities (C) and fungal communities (D) according to the fermentation stage.

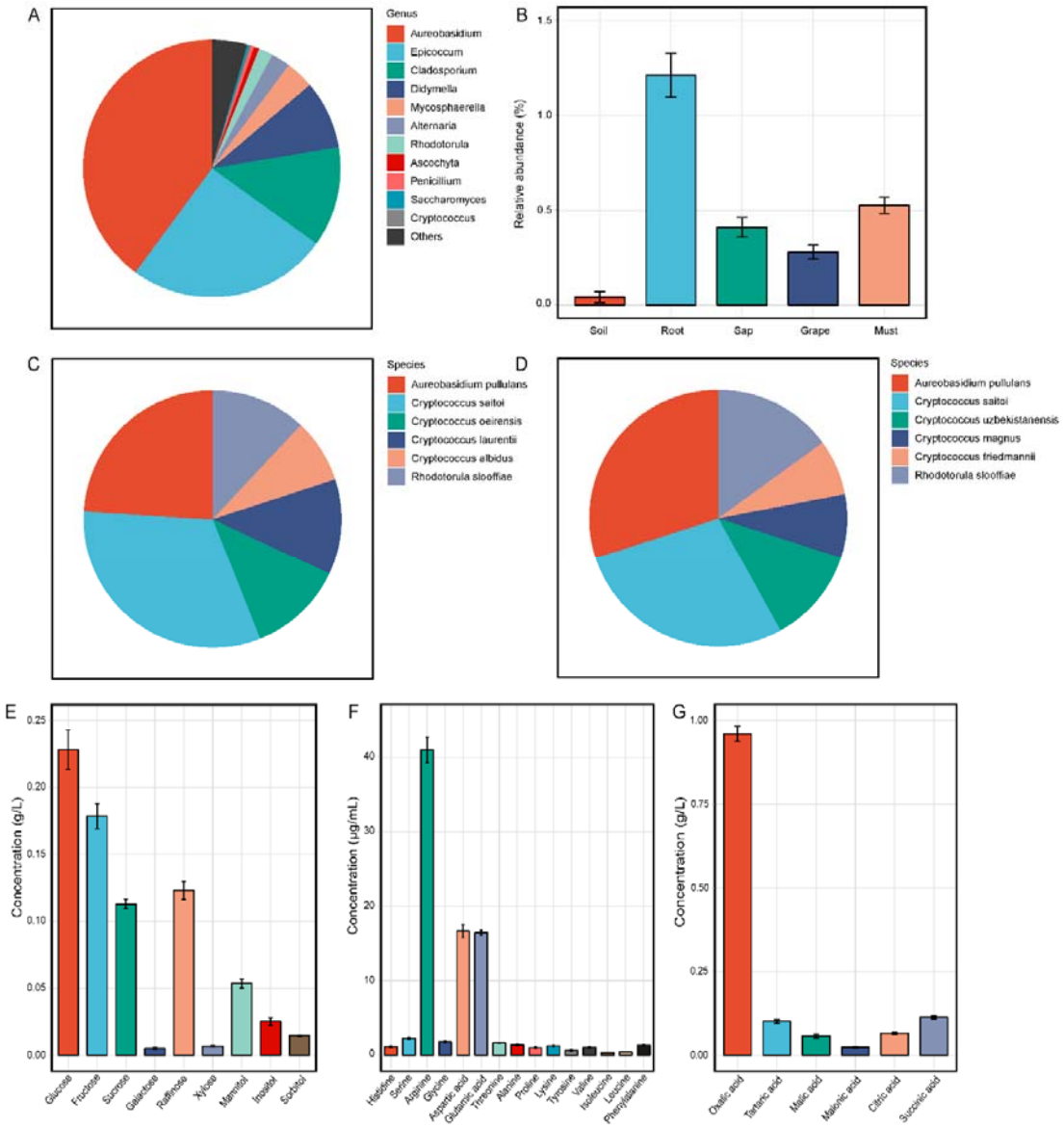

Figure S4 Xylem sap is a potential translocation medium of yeasts from the soil to the grapevine. (A) Fungal taxa in the genus level of xylem sap from the vineyard. (B) *S. cerevisiae* shared between niches of soil, root, xylem sap, grape, and must. Cultured yeast species from the xylem sap from vineyard (C) and glasshouse (D). Nutrient compositions of xylem sap: carbohydrates (E), total amino acids (protein and free) (F), and organic acids (G).

Supplementary Materials and Methods

Xylem sap collection

Xylem sap (n = 10) was collected from the vineyard using a centrifugation method in aseptic conditions (López-Millán et al 2000). Briefly, the shoots were peeled the outer bark and phloem layer, sterilised with 70% ethanol and cut to fit into 10-ml sterile centrifuge tubes that had 10 glass beads at the bottom. Xylem sap was centrifuged out the shoot pieces at 15,000 × *g* for 10 min at 4°C and collected at the bottom (~ 2.0 mL). Additional xylem sap (n = 5) were sampled from *Vitis vinifera* Shiraz grapevines grown in the glasshouse, the University of Melbourne. Xylem fluid was extracted with a pressure cylinder apparatus (similar with Scholander pressure chamber) (Schurr 1998). Grapevines were uprooted and carefully removed the soil from roots. In an aseptic bench, the main stems were cut 5-10 cm above roots with a sterile blade, girdled to remove phloem tissue, sterilised by 70% ethanol, and immediately inserted into the pressure cylinder. The cylinder was applied 60-70 kPa pressure for two hours to extract xylem sap (~ 4.0 mL). Xylem sap was divided into two subsamples, one for yeast culture experiment immediately, one for next-generation sequencing with flash frozen in liquid nitrogen and stored at -80°C until DNA extraction.

Chemical analysis of xylem sap composition

Carbohydrates of xylem sap were determined using enzymatic methods by Megazyme assay kits (Megazyme, Ireland) following the manufacturer's protocol. Amino acids (free and proteins) were determined using pre-column derivatisation with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate followed by separation and quantification with an ACQUITY Ultra Performance LC (UPLC; Waters, MA, USA) system by Australian Proteome Analysis Facility. The column employed was an ACQUITY UPLC BEH C18 column (1.7 µm × 2.1 mm × 5 mm) with detection at 260 nm (UV) and a flow rate of 0.7 mL/min at 57 – 60°C. Identification and quantitation were using a set of prepared standards, with DL-Norvaline as the internal standard (Cohen and Michaud 1993, Cohen 2001). Organic acids were determined using a Waters High-performance liquid chromatography (HPLC) (Waters, MA, USA) based on Andersen et al (1989) with modification. 20 µL xylem sap was injected

through a Synergi™ Hydro-RP LC Column (250 mm × 4.6 mm × 4 μm; Phenomenex Inc, CA, USA) at 60°C, with detection at 210 nm (UV). Mobile phases at a flow rate of 1.0 mL/min, with 20 mM potassium phosphate buffer (A, pH = 1.5) and 100% methanol (B) following a gradient programme: (0-2.5) min, 100% A; (2.5-2.9) min, linear ramp to 30% B; (2.9-8.0) min, 30% B; (8.0-8.5) min, linear ramp to 100% A; (8.5-10) min, 100% A. Identification and quantitation were using a set of prepared standards.

Isolation and identification of yeasts from xylem sap

Yeasts were isolated and identified from xylem sap to explore the potential translocation mechanism of yeasts in the vineyard ecosystem. Xylem sap was serially diluted and plated (0.1 mL) onto the yeast extract peptone dextrose (YPD) medium that was supplemented with 34 mg/mL chloramphenicol and 25 mg/mL ampicillin to inhibit bacterial growth. Plates were incubated at 28°C for 2-3 days in aerobic conditions. Single colonies with different morphological types were streaked onto Wallerstein Nutrient (WLN) agar media to obtain pure cultures, which were stored in 15% glycerol at -80°C. DNA was recovered from pure colonies using the MasterPure™ Yeast DNA Purification Kit (Epicentre, Madison, WI) following the manufacturer's instructions. The 26S rDNA D1/D2 domain was amplified using primers NL1/4 (Kurtzman and Robnett 1998) for sequencing by Australian Genome Research Facility (AGRF). Sequences were trimmed, aligned and analysed using BLAST at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

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