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Research Article

Grape-associated fungal community patterns persist from berry to wine on a fine geographical scale

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Abstract

Wine grape fungal community composition is influenced by abiotic factors including geography and vintage. Compositional differences may correlate with different wine metabolite composition and sensory profiles, suggesting a microbial role in the shaping of a wine's *terroir*, or regional character. While grape and wine-associated fungal community composition has been studied extensively at a regional and sub-regional scale, it has not been explored in detail on fine geographical scales over multiple harvests. Over two years, we examined the fungal communities on *Vitis Vinifera* cv. Pinot noir grape berry surfaces, in crushed grapes, and in lab spontaneous fermentations from three vineyards within a < 1 km radius in Canada's Okanagan Valley wine region. We also evaluated the effect of winery environment exposure on fungal community composition by sampling grapes crushed and fermented in the winery at commercial scale. Spatiotemporal community structure was evident among grape berry surface, crushed grape and fermentation samples, with each vineyard exhibiting a distinct fungal community signature. Crushed grape fungal populations were richer in fermentative yeast species compared to grape berry surface fungal populations. Our study suggests that, as on a regional level, fungal populations may contribute to fine-scale *-terroir,'* with significant implications for single-vineyard wines.

Keywords: wine yeast, microbial ecology, vineyard microbiome, wine fermentation, amplicon sequencing

Introduction

Wine is one of the world's most widespread and economically important beverages, and it is made by the fermentation of *Vitis vinifera* wine grape sugars to ethanol by yeast. While *Saccharomyces cerevisiae* is usually the dominant organism in later stages of fermentation, the fermentative process is characterized by a diverse succession of fungal taxa initially present on the grape berry or introduced into the fermentation via exposure to the winery environment. These fungi impact wine quality and flavour due to the production of a diverse range of flavour-active metabolites, particularly in spontaneous fermentation where no *Saccharomyces* inoculum is added (Fleet 2008, Jolly et al. 2014, Bokulich et al. 2016). Grape berry fungal taxa consist of hundreds of species spanning two phyla, Ascomycota and Basidiomycota, and some of these species may have profound influences on grapevine health and on wine quality. Filamentous fungal pathogens *Botrytis cinerea* and *Erysiphe necator* are ubiquitous and can have devastating impacts on grape harvest quality and yield, but these aerobic pathogens play little to no role in wine fermentation (Barata, Malfeito-Ferreira, and Loureiro 2012). Semi-fermentative yeast genera such as *Hanseniaspora*, *Metchnikowia*, *Pichia* and *Candida* among others are largely benign in the vineyard but can positively or negatively affect wine flavour and quality during fermentation depending on the species and strain (Fleet 2008). *S. cerevisiae* is found infrequently on the surface of intact grape berries but more frequently on damaged berries (Vaughan-Martini and Martini 1995, Goddard 2008). *S. cerevisiae* can also be introduced to fermentations through exposure of crushed grapes to winery equipment, vessels and environment (Martiniuk et al. 2016, Scholl et al. 2016) along with other wine-associated fungi harboured by winery surfaces during the harvest season and across vintages (Sabate et al. 2002, Santamaria et al. 2005, Bokulich et al. 2013, Grangeteau et al. 2016).

The environment of a wine region influences the sensory profiles of the wines that are produced in a region, such that the wine may have a region-specific character, or *terroir.* According to the International Organization of Wine and Vine, *terroir* is the 'collective knowledge of the interactions between the identifiable physical and biological environment and applied vitivinicultural practices' that create 'distinctive characteristics' for wines made in a particular region (Organisation Internationale du Vin 2010). *Terroir* effects are well-established on international and regional levels, but differences may also be identified at much smaller scales between and even within vineyard blocks (Van Leeuwen 2010). The concept of *terroir* is also more strongly associated with premium wines and can impact a wine's economic value. While abiotic environmental factors such as climate, topography and soil composition are traditionally associated with *terroir* and have a significant impact on the differentiation of wine organoleptic profiles, biotic factors such as soil, fruit and plant-associated microbiota may also contribute to differences in wine sensory attributes (Liu et al. 2019).Regional and temporal patterns have been identified in wine-associated microbial community composition (Bokulich et al. 2014, Taylor et al. 2014, Morrison-Whittle and Goddard 2015, 2018, Pinto et al. 2015, Jara et al. 2016, Liu et al. 2021) over scales of several to hundreds of kilometers. Additionally, regional variability in microbial community composition has been correlated

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with wine metabolite and sensory profiles, suggesting fungi and bacteria are significant contributors to wine differentiation and regional sensory character (Knight et al. 2015, Bokulich et al. 2016, Liu et al. 2020). Very little information exists about spatiotemporal patterns in vineyard grape fungal communities at smaller geographical scales and whether these patterns persist during wine fermentation. *S. cerevisiae* populations were found to be genetically similar in studies of vineyards within 1–2 km areas (Martiniuk et al. 2016, Knight et al. 2020), while vineyard soil fungal populations differed (Knight et al. 2020).

The Okanagan Valley is one of Canada's preeminent wine regions. The region is arid, cool-climate and is characterized by a wide range of microclimates and soil types that create many distinct *terroirs* for wine production over a relatively small area. The valley spans over 250 km but is planted with only ∼3500 hectares (ha) of vineyards, of which over 50% are in the southernmost 25 km (Bowen et al. 2005, Senese et al. 2012). In contrast to the vineyards of larger North American wine regions such as California and Washington State, the average Okanagan Valley vineyard size is only ∼4 ha (Bremmer 2014, Mertz et al. 2017). The Okanagan Valley's unique geography and the smaller scale of its wine industry has created a focus on premium wine production from a wide range of *Vitis vinifera* cultivars and an emphasis on 'place of origin' as an important marketing component (Senese et al. 2012). Herein, we used internal transcribed spacer (ITS) amplicon sequencing to evaluate fungal communities from two vintages on the berries, crushed grapes, and across four fermentation stages of grapes sourced from three Okanagan Valley vineyard blocks (*V. vinifera* cv Pinot Noir) within a 600 m radius. These blocks are farmed by a single winery and are used to make blended and single-vineyard wines. Our study reveals grape and wine fungal community structure across vintages on a fine geographical scale.

Materials and methods

Study site

The winery in this study farmed three Pinot Noir vineyard blocks (each < 1 ha in area) located in the south of the Okanagan Valley wine region—Home East (HE), Home West (HW) and Orchard Grove (OG). HE and HW are separated by approximately 100 m while OG is approximately 1 km to the north-east (Fig. S1a). OG is situated ∼200 m from the winery facility and is ∼15 m higher in elevation than HE and HW (GeoBC 2016). All three vineyards are planted with self-rooted vines on similar gravelly, moderately coarse soils (Agriculture Canada 1984). Sampling for this study occurred in late September and early October of 2013 and 2014; harvest dates and vineyard block characteristics are described in Table S1. All grapes were sampled within one week of the winery's scheduled harvest dates to ensure similar ripeness among vineyards.

Grape berry surface community sampling

Fourteen individual healthy Pinot Noir grape clusters were taken aseptically from each of HE, HW, and OG vineyard blocks. Clusters were sampled from middle vines of post panel sections distributed evenly throughout each block. Outer rows and panels were not sampled, and at least two panels were left between each sampled panel. An even number of clusters was taken from east and west-facing rows to account for differences in sun exposure. Grape clusters were transported on ice to the laboratory where 30 intact grape berries were aseptically removed from each cluster. Fungi and bacteria on the grape berry surface were removed according to (Setati et al. 2012). Thirty berries from each cluster were placed in a 250 mL Erlenmeyer flask with 50 mL rinsing buffer (0.9% NaCl w/v and 0.2% Tween 80) and shaken at 150 rpm, 30°C for 3 h. In total, 42 grape berry surface samples were processed per year. After collecting the rinsate, microorganisms were pelleted by centrifugation at 10 000 × *g* for 10 min. The supernatant was removed, and the pellet was frozen at −80°C until DNA extraction.

Lab and winery fermentation community sampling

Concurrent with grape berry surface sampling, grape sampling for fermentations was carried out according to Martiniuk et al. 2016 (Fig. S1b for experimental design). Briefly, triplicate bags of Pinot Noir grape clusters were collected from HE, HW, and OG vineyard blocks to be fermented in the lab. Six sections of three 6 m panels distributed throughout each vineyard block were selected for sampling (Fig. S2). One panel per sampling area was assigned to fermentation #1, 2, or 3. In each area, three healthy grape clusters were randomly selected from each side of the row laneway. Bags of 36 grape clusters were transported to the lab on the same day and processed immediately. Bags were aseptically crushed by hand and ∼3 L grape must (juice, skins, and seeds) were transferred from each bag to three sterile 3.8 L fermentation vessels. Fermentation samples were taken without grape skins after crushing and at early, mid, and late fermentation (0%, ∼25%, ∼50%, ∼90%, sugar depletion as determined by weight loss of CO_2). The winery harvested grapes from OG block and fermented them as described in Martiniuk et al. 2016, sampled at the same sugar depletion stages as described above, froze samples at −20°C and delivered them on dry ice to the lab. 2013 samples (5 mL) were diluted with sterile glycerol (final concentration 15%) and stored at −20° until DNA extraction. About 2014 samples (2 mL) were stored without glycerol at −80°C until DNA extraction.

DNA extraction and amplicon sequencing

DNA was extracted from grape berry rinsate pellets and fermentation samples in batches by year using a modified chemical and enzymatic method from Zott et al. 2010, Bokulich et al. 2012. Fermentation samples were thawed on ice and centrifuged to pellet microbes. 7.5 mL volumes of glycerol-spiked 2013 samples (5 mL undiluted volume) were pelleted, while 2 × 2 mL 2014 samples were pelleted. The pellets were rinsed 2–3 times with ice-cold phosphate-buffered saline. Pellets were incubated for 30 min with lysozyme in 50 mM EDTA followed by mechanical disruption with 0.5 mm ø glass beads on a vortex shaker. Disrupted samples were treated with Promega nuclei lysis buffer at 70°C then centrifuged to precipitate cell debris. About 600 μL of supernatant was removed to new tubes and 22.5 μL 20 mg/mL Proteinase K was added to denature soluble proteins. Promega protein precipitation buffer was added to remove residual protein before another centrifugation to precipitate debris. The supernatant was further treated with 10% polyvinylpyrrolidone to remove polyphenolic compounds, then DNA was precipitated with 2-propanol and cleaned with two 70% ethanol washes. DNA was resuspended in TE buffer and treated with RNase A prior to quantification and storage at −20°C.

The fungal ITS1 region was PCR amplified in 25 μL reactions using BITS/B58S3 primers from (Bokulich and Mills 2013) modified with Illumina overhang adapters. Reactions contained 1 unit of Phusion II polymerase (Thermo Fisher) and 25 ng of template DNA. The DNA extracted from 5 mL volumes of 2013 fermentation samples was amplified once, while DNA from duplicate 2 mL 2014

fermentation sample extracts were amplified and pooled. PCR was conducted using the following cycling conditions: an initial denaturation of 2 min 30 s at 98°C; 30 amplification cycles of 20 s at 98°C, 30 s at 55°C, and 30 s at 72°C; and a final elongation of 10 min at 72°C. PCR products were cleaned and indexed with Nextera indices according to Illumina's 16S Metagenomic Sequencing Library Preparation protocol (16S Metagenomic Sequencing Library Preparation 2013). Prior to pooling, sample library concentrations were normalized using SequalPrep (Invitrogen) plates. The pooled libraries were sequenced in three separate 2 × 250 bp MiSeq runs at the UBC Sequencing and Bioinformatics Consortium.

Data processing

Raw, unpaired FASTQ files were processed in cutadapt (Martin 2011) to remove primer and reverse complement sequences from read ends. Reads were joined, filtered and amplicon sequence variants (ASVs) inferred using the DADA2 plugin (Callahan et al. 2016) (e = 2, minimum length = 100 bp including primer lengths) in QIIME 2 version 2018.11 (Bolyen et al. 2019). One late-stage fermentation sample (2014 vintage, HE vineyard) was excluded due to a high proportion of low-quality reads and because 25% of ASV reads were < 5. Taxonomy was assigned using the naive Bayes feature-classifier plugin in QIIME 2 against the UNITE fungal ITS database version 7.2 (developer, dynamic classification) (Kõljalg et al. 2013). ASVs left unclassified below the phylum level and with total counts > 100 were queried against the NCBI nucleotide database using megablast (Morgulis et al. 2008); subject sequences from metagenomic datasets or uncultured organisms were excluded. ASV numbers, sequences and taxonomies can be found in Table S2. Sample FASTQ files and metadata were uploaded to the NCBI Short Read Archive under accession number PRJNA587381 and are available at https://www.ncbi.nlm.nih.gov/sra/PRJNA587381.

Data analysis

Diversity analyses and ordinations

Alpha- and beta-diversity analyses were performed in PRIMER version 7 (Anderson et al. 2008) and in R version 3.5.3 (R Core Team 2016) using the packages phyloseq (McMurdie and Holmes 2013) and vegan (Oksanen et al. 2015). Alpha diversity metrics ASV richness, Pielou's evenness and Shannon diversity were calculated on samples rarefied to even depth. Differences between vintages and among vineyards were evaluated by analysis of variance (ANOVA). Diversity metric normality was evaluated visually and Levene's tests were performed to confirm homoscedasticity prior to ANOVA. Beta diversity was evaluated using permutational multivariate analysis of variance (PERMANOVA) (Anderson 2001) performed in PRIMER on Bray–Curtis similarity matrices of square-root transformed ASV counts. Transformation was implemented to reduce the influence of highly dominant ASVs that may mask contributions of less abundant taxa (Clarke 1993), which can occur in later stages of wine fermentation. Because between-group heterogeneities in variance can affect interpretation of PERMANOVA results (Anderson and Walsh 2013), permutational multivariate analysis of dispersions (PERMDISP) (Anderson 2006) was also performed to assess differences in variance among vineyard, vintage and fermentation stage fungal community groups. To visualize differences in fungal community composition between vintages, among vineyard sites and/or fermentation stages, three-dimensional non-metric multi-dimensional scaling (NMDS) of Bray–Curtis similarity matrices (derived from square-root transformed ASV counts) was implemented using the 'metaMDS' function in vegan with square root transformation disabled. Canonical analysis of principal coordinates (CAP) (Anderson and Willis 2003) with cross-validation was used to test the hypothesis of significant between vineyard and vintage differences in fungal community composition. Statistical comparisons were performed only on fermentation samples within the same vintage because of differences in fermentation sample processing between vintages.

Indicator and network analyses

Indicator analysis (IA) was performed in the R package 'indicspecies' (De Cáceres and Legendre 2009) using the point biserial correlation coefficient function (corrected for unequal group sizes where necessary) with 99 999 permutations to identify ASVs significantly associated with a particular vineyard or combinations of blocks. IA was also performed on both grape berry surface and fermentation samples in vintage-vineyard or vineyard-stage combinations, where we defined samples isolated from a vineyard block and vintage or fermentation stage as belonging to distinct site groups. Indicator probabilities were corrected using the Benjamini-Hochberg method (Benjamini and Hochberg 1995). The number of vineyard/stage combinations tested was limited to 8 within a single vintage when evaluating fermentation data. Bipartite networks of indicator taxa were assembled in Cytoscape 3.7.1 (Shannon et al. 2003) with vineyard site, year and fermentation stage combinations used as source nodes and significantly associated indicator ASVs ($P < 0.05$) as target nodes. The networks were generated using an edge-weighted spring-embedded algorithm. Edge weight (i.e. thickness of lines connecting nodes) is proportional to each ASV's point biserial correlation coefficient generated in 'indicspecies' and ASV node size is proportional to its square-root transformed relative abundance.

Results

SBV farms three separate Pinot Noir vineyard blocks (HE, HW, OG) situated within a 600 m radius in the south of British Columbia's Okanagan Valley wine region (Fig. S1a). We investigated fungal populations on grape berry surfaces from each of the HE, HW, and OG vineyard blocks over two vintages (2013–2014), and sequenced ITS1 amplicons from a total of 84 rinsed grape berry surface samples (14 samples/vineyard/year, Fig. S1b). We also harvested triplicate bags of grape clusters from each vineyard over the same two vintages, then crushed and fermented the grapes aseptically in the lab to profile fungal population dynamics over time among vineyard blocks. In tandem, SBV harvested and fermented grapes from OG vineyard at commercial scale to evaluate the effect of the wine cellar environment on fungal community composition during fermentation. To evaluate fungal community composition, we obtained a total of 3332927 curated reads processed into 211 ASVs representing 99 genera from 84 grape berry surface samples and 4241394 reads (203 ASVs, 89 genera) from 95 fermentation samples.

Grape berry surface fungal communities

We began our study with the characterization of fungal communities on Pinot Noir grape berry surfaces. We calculated community alpha diversity metrics for each vineyard and vintage on the rarefied data set (11156 reads/sample, Fig. S3). Alpha diversity metrics varied widely across samples (richness 35–88 ASVs; evenness 0.28–0.71; Shannon diversity 1.10–2.89) indicating wide heterogeneity in ASV richness and proportion among individual grape

Table 1. Vineyard block and vintage effects on grape berry surface fungal α-diversity.

	Richness		Evenness		Shannon's H	
Main test[a]	F	P	F	P	F	P
Vineyard ($F_{2, 78}$)	**15.19**	**<0.001**	1.24	0.295	1.73	0.185
Vintage ($F_{1, 78}$)	**14.05**	**<0.001**	**6.76**	**0.011**	2.71	0.10
Vineyard × vintage ($F_{2, 78}$)	**4.81**	**0.011**	1.99	0.14	1.10	0.34

[a]Main factor and interaction effects were evaluated by one-way ANOVA with degrees of freedom and residual listed for each factor. Factors include the vintage sampled (2013 or 2014) and vineyard block (HE, HW, and OG). The F-value and probability are provided for main and interaction factors. Significant F-values (α<0.05) are in bold.

Table 2. Vintage and vineyard block effects on grape berry surface fungal β-diversity as assessed by permutational multivariate analysis of variance (PERMANOVA).

Main test[a]	F	VC	P
Vintage ($F_{1, 78}$)	**20.02**	**19.00**	**0.000 01**
Vineyard ($F_{2, 78}$)	**5.01**	**10.68**	**0.000 01**
Vineyard × vintage ($F_{2, 78}$)	**2.42**	**8.99**	**0.0037**
Pairwise tests[b]	t	$\overline{BC}$	P
HE vs HW	**1.73**	**54.43**	**0.0053**
HE vs OG	**2.00**	**54.85**	**0.003 83**
HW vs OG	**2.93**	**48.60**	**0.000 03**

[a]Main factor and interaction effects were evaluated by PERMANOVA (99 999 permutations) with degrees of freedom and the residual listed for each factor. F = test statistic, VC = estimated variance component. Exact p-values are listed. Significant p-values (α<0.05) are in bold.
[b]t = pairwise test statistic, $\overline{BC}$ = average between-group Bray-Curtis similarity in %. P-values are exact and corrected for multiple tests using the Benjamini-Hochberg method. Significant p-values (α<0.05) are in bold.

berry samples (Fig. S3). While there were significant vintage, vineyard, and vineyard x vintage interaction effects on ASV richness (Table 1) and vintage effects on ASV evenness, Shannon diversity, which accounts for both the richness and evenness of species, did not vary significantly across vintages or among vineyards (Table 1), indicating that while the number and relative proportions of ASVs vary, the overall diversity of fungal communities is relatively similar among vineyards and across vintages.

To examine factors driving community structure across vineyard blocks and vintages, a PERMANOVA was performed on the grape berry dataset. Both vintage and vineyard block were drivers of grape berry surface fungal community structure, with PERMANOVA revealing significant temporal and spatial effects (Table 2). Vintage was the largest driver, contributing nearly double the variance component of vineyard (VC = 19.00 vs 10.68, Table 2). To evaluate differences in fungal community composition between individual vineyards, pairwise PERMANOVA tests were conducted. Pairwise tests revealed significant differences between all vineyards (Table 2). HE and HW and HE and OG vineyards had significant between-group Bray–Curtis similarities of 54.43% ($P = 0.0053$) and 54.85% ($P = 0.00383$) respectively, while the two most distant vineyards, HW and OG, were significantly least similar (BC = 48.60, $P = 0.0003$). The PERMANOVA interaction between vintage and vineyard was also significant ($P = 0.0037$, VC = 8.99); however, when PERMANOVA was performed on vineyards partitioned by vintage, significant spatial effects were also observed (2013: VC = 12.34, $P = 0.0032$; 2014: VC = 12.52, $P = 0.00001$; Table S4). PERMDISP analysis revealed vintage dispersions were significantly different (Table S5), indicating some of the temporal effect identified by PERMANOVA may be attributable to dispersion effects. Grape berry surface fungal communities were separated by vintage and vineyard in unconstrained NMDS ordinations but other sources of variation are apparent (Fig. 1A**).** To better discriminate differences among vineyard berry surface fungal communities, we used a method of constrained ordination, canonical analysis of principal coordinates (CAP), which tests whether separation can be found among *a priori* groups of fungal communities, in this case, vineyard blocks (Anderson and Willis 2003). CAP revealed significant and distinct clustering of communities by vineyard block, with reclassification success rates of 89.3%–96.4%, indicating that vineyard fungal populations are distinct (Fig. 1B). When using both vintage and vineyard as grouping factors to examine possible interaction effects, 2013–2014 OG fungal communities clustered separately, while HE and HW fungal communities partially overlap in 2013 and between vintages, but populations remain largely separate (Fig. 1C).

The predominant fungi identified on grape berry surfaces with both vintages combined were *Aureobasidium pullulans* (21.5% average relative abundance in data set) and species of the filamentous fungal genera *Cladosporium* (22.9%), *Alternaria* (8.5%), and *Botrytis* (3.7%). Abundant yeast genera included *Vishniacozyma* (11.7%), *Filobasidium* (9.2%), *Starmerella* (6.2%), and *Pichia* (3.4%). Notably, *S. cerevisiae* abundance was very low (0.007%, Table S3). The average relative abundance of major taxa (>0.2% of reads in the rarefied dataset) in each vineyard in 2013 and 2014 are displayed in Fig. 2A. For several genera, multiple highly abundant ASVs representing different species or strains within those genera were identified **(**Fig. 2A**).** Over three quarters of ASVs were found in all vineyard blocks (160/211, or 75.8%) and in both vintages (170/211, or 80.6%), with 8.5% of ASVs unique to a particular block and 19.4% unique to a vintage (Fig. 2B, C), indicating the spatiotemporal distribution of fungal ASVs is relatively homogeneous when not accounting for abundance.

To identify which grape berry surface ASVs were significantly associated with a particular vineyard block (indicator ASVs), we conducted a multilevel indicator analysis, which assesses the association of an ASV with a particular vineyard or combination of vineyards using a correlation index (De Cáceres et al. 2010). A total of 41 out of 211 ASVs were significantly correlated with a vineyard block or combination of blocks ($P < 0.05$, Table S6). To incorporate vintage effects, we conducted the same analysis on communities from each vineyard partitioned by vintage, considering

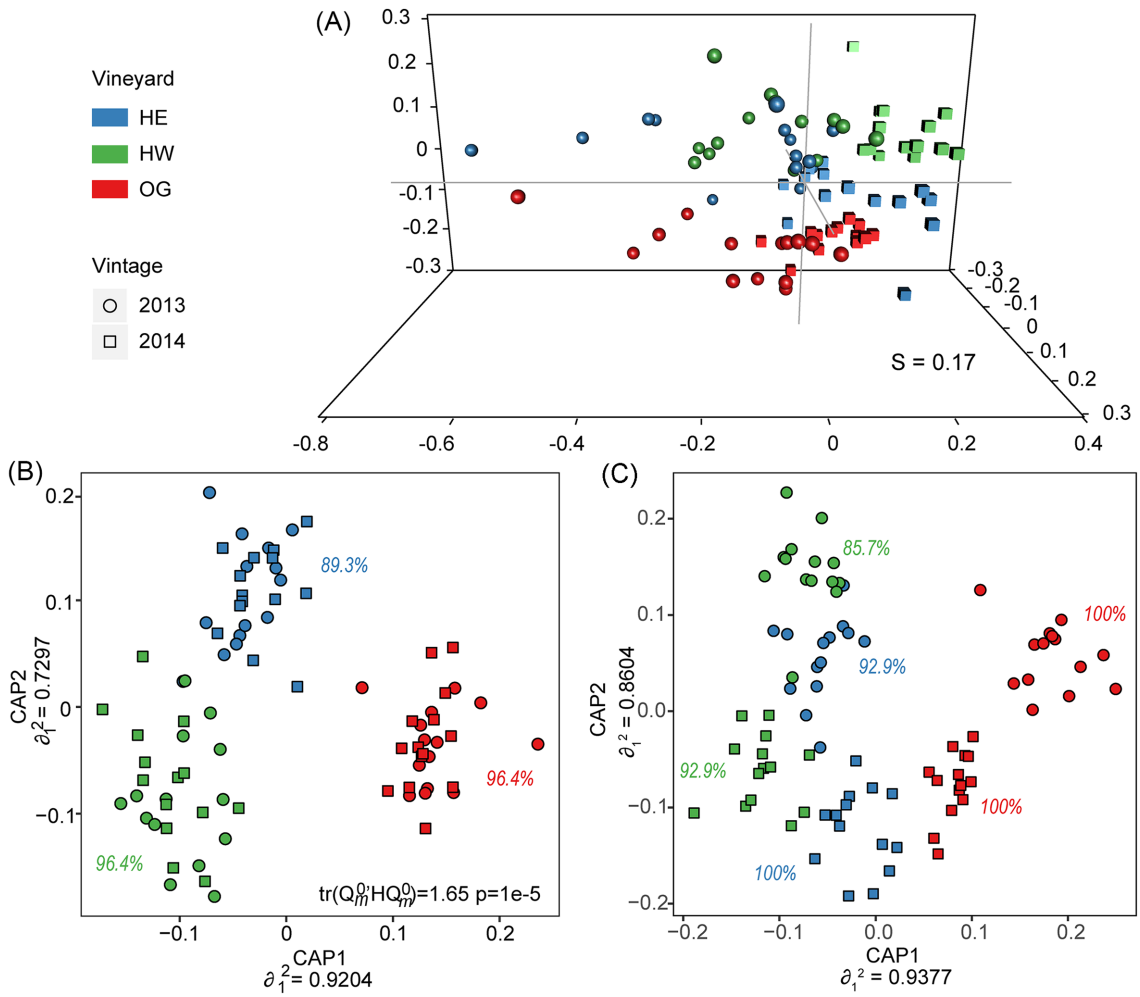

Figure 1. Vintage and geography drive grape berry surface fungal community structure. **(A)** Non-metric multidimensional scaling (NMDS) plot of grape berry surface fungal communities in three dimensions. The plot is based on a pairwise Bray-Curtis similarity matrix of grape berry surface ASV counts and is rotated to the first principal component. **(B)** CAP plot of grape berry surface fungal communities discriminated by vineyard. Canonical correlation values on each axis indicate the association strength between the plotted data and the sample groupings of vintage and vineyard. Percentages represent the reclassification accuracy of the CAP algorithm and indicate the magnitude of separation between groups. The trace statistic tr(*Qm*0'HQ*m*0) tests the hypothesis of significant differences among communities from different vineyards and vintages. **(C)** Canonical analysis of principal coordinates plot of 2013–2014 grape berry fungal communities grouped by vintage and vineyard.

each vintage-vineyard block pair as a separate site. This analysis identified 83 ASVs significantly associated with a vintage-vineyard site or combination of sites (Table S7). The most highly abundant indicator ASVs identified in this analysis (>0.2% of reads in the rarefied data set, highlighted in red in Fig. 2A) were significantly associated with samples of a particular vintage rather than a particular vineyard block. For example, *Aureobasidium*, *Cladosporium*, and *Pseudopithomyces* ASVs were significantly associated with all three vineyard blocks in 2014, but not 2013 (Fig. 2A). In contrast, certain *Starmerella, Hanseniaspora, Candida*, and *Pichia* ASVs were associated with all three vineyard blocks in 2013 but not 2014, indicating the strong role of vintage in driving fungal community compositional differences (Fig. 2A).

To further visualize the differences in fungal community composition among vineyards, vintages or combinations thereof, the relationships between indicator ASVs and their associated vineyard or vintage-vineyard block sites were modelled in bipartite networks, where ASVs are nodes and are connected to vineyard site nodes by edges (Fig. 3). The first network depicts ASVs associated with vineyard sites in both vintages (Fig. 3A). In this network, HW and OG have the highest number of unique ASVs (17 and 16, respectively) while HE has fewer (4). The second network depicts ASVs associated with vintage-vineyard site combinations, showing clear discrimination between vintages and vineyard blocks (Fig. 3B). Similar to the first network, the majority of indicator ASVs (44 or 53.0%) were associated with a single vineyard and only nine were associated with a particular vineyard in both vintages. In both the 2013 and 2014 vintage, six ASVs were associated with HW, three ASVs with OG whereas no ASVs were identified in HE in both years. Single-vineyard ASVs in this network were mostly low abundance (except for two *Vishniacozyma* ASVs associated with OG 2013) and comprised only 12.22% of reads in the rarefied data set (Table S7). The most highly abundant ASVs (*Aureobasidium*, *Cladosporium*, *Alternaria, Starmerella*, *Vishniacozyma*) were shared between multiple vintage-vineyard sites (Figs 2A and 3B), suggesting that differences in community composition are due to unique lowly abundant taxa, and to variations in abundance among highly abundant taxa in each vineyard.

Fermentation fungal communities

In addition to grape berry surface samples, grape samples from HW, HE, and OG vineyards were crushed and fermented spontaneously in the laboratory (HW, HE, and OG fermentations). SBV

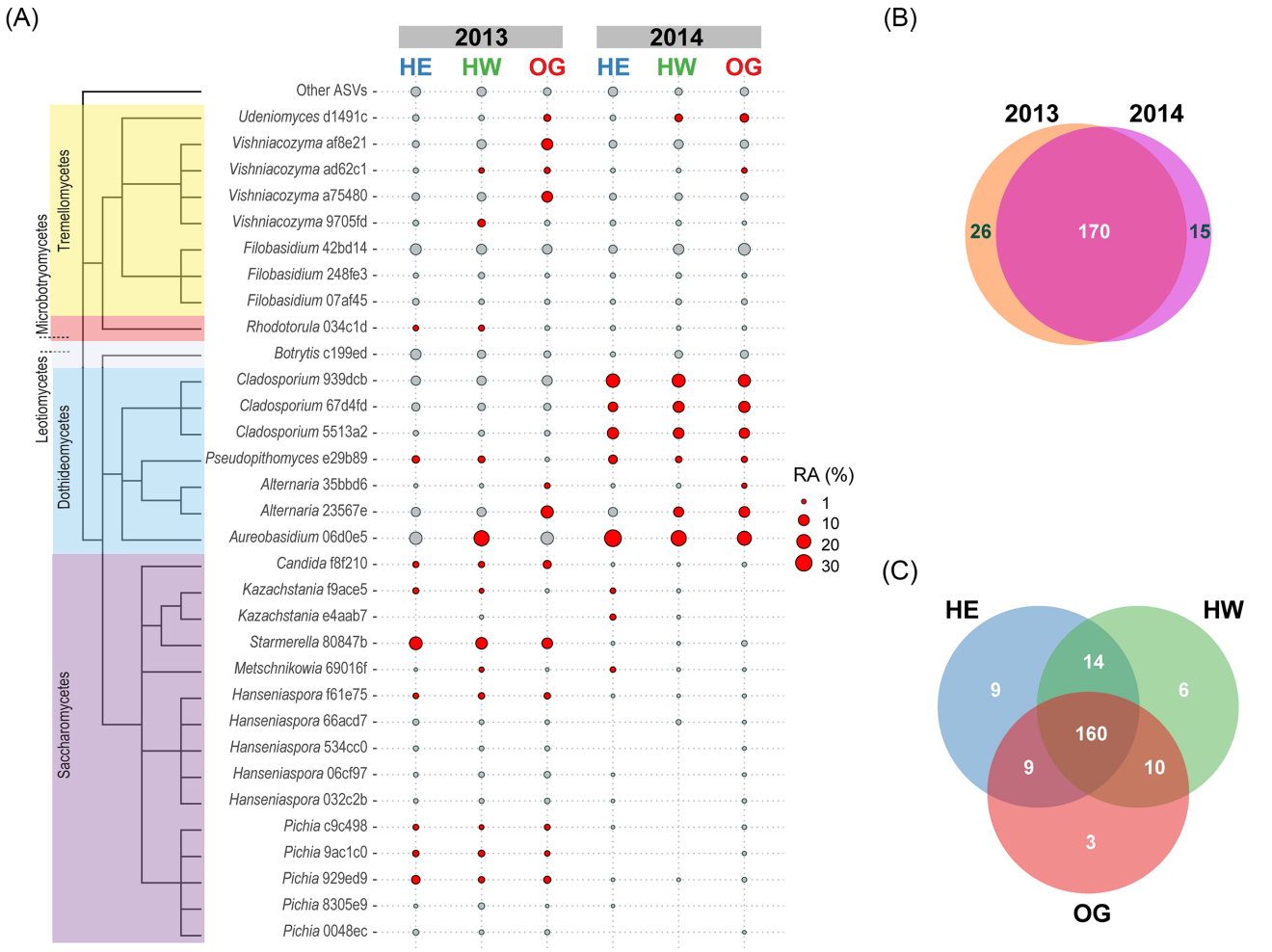

Figure 2. Relative abundance and distribution of grape berry surface fungal taxa across vintages and vineyard blocks. **(A)** Relative abundance of major taxa by vintage and vineyard. ASVs with abundances > 0.2% in the rarefied data set are represented by bubble scaled to their average relative abundance across samples from the same vintage and vineyard block. ASVs are ordered by taxonomy and names are shaded by taxonomic class. ASVs significantly associated with one or more vintage-vineyard site groups are highlighted in red. RA = relative abundance. Distribution of ASVs by **(B)** vintage and **(C)** vineyard.

winery also harvested, crushed and spontaneously fermented OG grapes in the winery facility (WIN fermentations). Samples were collected from fermentations in the lab and winery immediately after crushing and at early, mid and late fermentation stages. We found that crushed grape fungal populations were significantly different from grape berry surface populations when taking into account both abundance and occurrence of ASVs (PERMANOVA $F_{1,106} = 15.47$, $P = 0.00001$). Grape berry surface samples clustered separately from crushed grape samples in NMDS ordination (Fig. 4A). Most ASVs found on grape berry surfaces (174/216 or 80.6%) were also found in crushed grape samples, although a greater number (35/216 or 16.2%) were unique to grape berry surfaces compared to crushed grapes (7/216 or 3.2%, Fig. 4B). Most ASVs associated with grape berry surfaces were in very low abundance and include Basidiomycota such as *Trametes* and *Ganoderma* species (Table S8). *Botrytis* and Saccharomycetes yeasts *Pichia*, *Starmerella*, *Candida* were more abundant in and were significantly associated with crushed grape samples, whereas filamentous fungi (*Alternaria*, *Cladosporium*) and basidiomycetous yeasts (*Vishniacozyma*, *Filobasidium*) were more abundant in and associated with grape berry surface samples (Table S8).

We next calculated alpha diversity indices on the rarefied dataset for both the winery and lab spontaneous fermentation samples. HE vineyard crushed grape Shannon diversity (H) values were significantly lower than OG in both 2013 and 2014 (Fig. S4a). Grape processing in the winery environment had mixed effects on alpha diversity. OG grapes crushed in the lab versus those crushed in the winery had similar H values in both vintages (Fig. S4a). However, in 2013, OG crushed grape ASV richness was significantly higher in the winery compared to the lab fermentations (Fig. S4a). As expected, all three of the diversity metrics declined substantially from beginning to end of fermentation in all the lab and winery fermentations (Fig. S4b). A repeated measures ANOVA performed on ASV richness in winery and OG fermentations found that richness was significantly higher in the winery in 2013 (2013 $F_{1,19} = 49.20$, $P = 1.12 \times 10^{-6}$).

To evaluate differences in fungal community composition among vineyard blocks and fermentation stages, we performed a PERMANOVA on each of 2013 and 2014 fungal community samples from lab fermentations of grapes sourced from HE, HW and OG vineyard blocks and from winery fermentations of OG grapes. Both 2013 and 2014 PERMANOVAs revealed significant differences in community composition across fermentation stages and among vineyard blocks (Table 3). While the effect of fermentation stage was understandably strongest (2013 VC = 30.31, $P = 0.00001$; 2014 = 32.61, $p = 0.00001$), the magnitude of the

Table 3. Vineyard block and fermentation stage effects on fermentation fungal β-diversity determined by PERMANOVA.

Main test[a]	F	VC	P
2013 (all fermentations)[b]			
Stage ($F_{3, 32}$)	**43.66**	**30.31**	**0.000 01**
Ferm. group[c] ($F_{3, 32}$)	**5.96**	**13.27**	**0.000 01**
Ferm. group × stage ($F_{9, 32}$)	**1.91**	**11.38**	**0.0002**
2013 (without winery)			
Stage ($F_{3, 24}$)	**19.01**	**31.58**	**0.00 001**
Vineyard ($F_{2, 24}$)	**4.48**	**12.02**	**0.00 001**
Vineyard × stage ($F_{6, 24}$)	**1.64**	**10.32**	**0.0104**
2013 Winery effects			
Stage ($F_{3, 16}$)	**18.71**	**31.69**	**0.00 001**
Ferm. location (lab vs winery) ($F_{1, 16}$)	**4.79**	**10.37**	**0.00 001**
Location × stage ($F_{3, 16}$)	**2.28**	**12.04**	**0.0104**
2014 (all fermentations)			
Stage ($F_{3, 31}$)	**22.47**	**32.61**	**0.00 001**
Ferm. group ($F_{3, 31}$)	**10.96**	**22.22**	**0.00 001**
Ferm. group × vintage ($F_{9, 31}$) *2014 (without winery)*	0.82	– 5.99	0.8032
Stage ($F_{3, 23}$)	**13.75**	**32.86**	**0.00 001**
Vineyard ($F_{2, 23}$)	**7.09**	**19.69**	**0.00 001**
Vineyard × stage ($F_{6, 23}$) *2014 Winery effects*	0.48	– 11.48	0.9913
Stage ($F_{3, 16}$)	**29.67**	**33.44**	**0.00 001**
Ferm. location (lab vs winery) ($F_{1, 16}$)	**12.13**	**14.74**	**0.00 004**
Ferm. location × stage ($F_{3, 16}$)	**2.27**	**9.96**	**0.0073**

[a]Main factor and interaction effects were evaluated by PERMANOVA (99 999 permutations). Factor and residual degrees of freedom are in parentheses. F = PERMANOVA pseudo-F test statistic, VC = PERMANOVA estimated variance component. Exact *P*-values are listed. Significant *P*-values (α<0.05) are in bold.
[b]Analyses were performed on each vintage separately to account for differences in sample storage.
clab (HE, HW, OG) and winery (WIN) fermentations

vineyard block effect was pronounced in both vintages (2013 VC = 13.27, P = 0.00002; 2014 VC = 22.22, P = 0.00001; Table 3). Vineyard dispersion effects were not significant in both vintages (Table S10) and PERMANOVA conducted with WIN samples excluded (to remove variation between winery and lab fermentation processes) did not appreciably diminish the geographical effect, indicating that the vineyard block, and not other extraneous factors, drives differences in fermentation community composition. In examining winery environment effects, we found that WIN (OG) fermentations were significantly different from the lab (OG) fermentations (See winery effects, Table 3). We performed a separate pairwise PERMANOVA by fermentation stage which indicated that fungal communities became significantly and increasingly dissimilar from crushed grape populations as fermentation progressed (Table S9). For example, in 2013, early-stage fermentation fungal communities were 48.25% similar to crushed grape fungal communities whereas late-stage fermentation fungal communities were only 30.79% similar to crushed grape fungal communities (Table S9). Pairwise comparisons of fermentation communities by vineyard block revealed that all lab fermentation communities were significantly different from each other, with OG and HE fermentations being the least similar (BC = 46.88 in 2013 and 40.39 in 2014) and HW and HE fermentations most similar (BC = 50.78 in 2013 and 41.01 in 2014) in both vintages (Table S9).

NDMS ordination shows fermentation fungal communities were distinguishable by vineyard in both vintages, with the greatest separation between OG (lab and winery) and the other vineyard groups (HE and HW) in crush and early fermentation samples in both vintages (Fig. 5A and C). The OG and HE/HW vineyard fungal population separation decreased in the mid and late stages of 2013 fermentations, but in 2014, OG and the HE/HW vineyard groups remained separated throughout fermentation (Fig. 5A and C). WIN (OG) crush and fermentation samples generally clustered close to lab OG crush and fermentation samples in both vintages (Fig. 5A and C). CAP performed on fermentation samples using lab fermentation group (HE, HW, OG, and WIN) as a grouping factor classified 2013 samples with 50%–91.7% accuracy and 2014 samples with 75%–100% accuracy (Fig. 5B and D). While 2013 reclassification rates were lower (e.g. HW = 50% correct, OG = 58.3% correct, Fig. 5B), most sample misclassifications were OG samples classified as WIN (3/12) and HW samples misclassified as HE samples (5/12; Table S11). When expanding the groupings to OG + WIN and HE + HW these pairs, reclassification success rate improves to 87.5% and 95.8%, respectively (Fig. 5B). The lower 2013 reclassification success rates are also likely related to the spatiotemporal PERMANOVA interactions in 2013 (Ferm. group × stage $F_{9, 32}$ = 1.91, P = 0.0002; Table 3), which appear between later stages of lab fermentations (Fig. S5).

The most abundant ASVs (>0.2% relative abundance in dataset) identified in 2013 and 2014 fermentations include fungi in classes Dothideomycetes, Tremellomycetes and Saccharomycetes (Fig. 6). Indicator analyses were conducted on the 2013 and 2014 fermentations separately to identify ASVs significantly associated with a particular vineyard or fermentation stage. In 2013, 63 indicator ASVs were identified and in 2014, 73 indicator ASVs were identified (Table S12), the most abundant of which are highlighted in red in Fig. 6. Pronounced changes in the dominance of different fungal taxa are evident between crush and early fermentation. Filamentous fungi in the genera *Alternaria, Botrytis*, and *Cladosporium* and yeasts *Aureobasidium, Filobasidium* and *Vishniacozyma*, and were abundant at the crushing stage but declined by early fermentation in both vintages (Fig. 6). *Candida* and several *Pichia* ASVs were also present at crush and early stages of fermentation in 2013 and to a lesser extent in 2014. *Starmerella* ASVs were highly abundant in 2013 early fermentation samples and in mid to late fermentation samples across both vintages. *Saccharomyces* ASVs

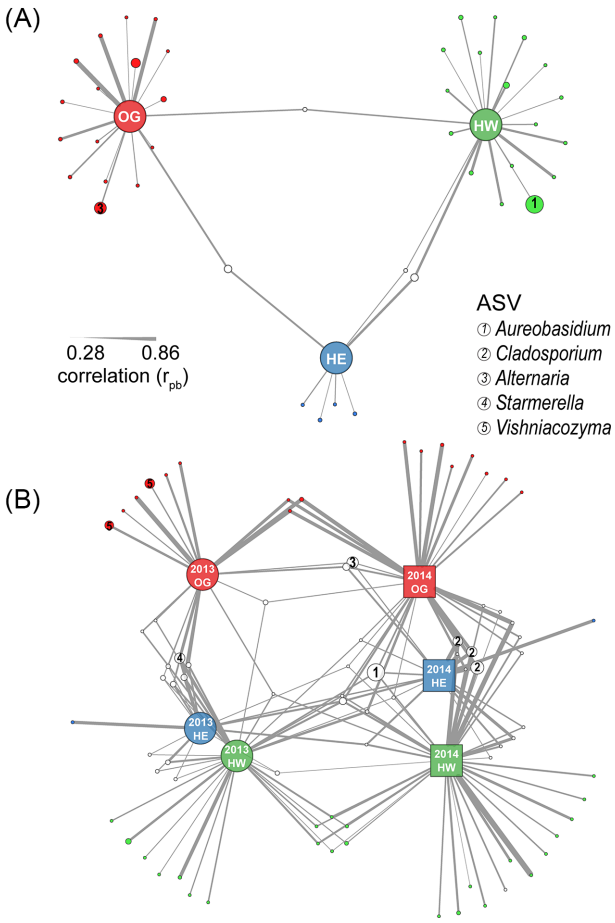

Figure 3. Bipartite networks of grape berry surface indicator ASVs significantly associated with **(A)** vineyard blocks and **(B)** vineyard blocks separated by vintage. Vineyard block nodes in (B) are represented by large circles (2013 vintage) and squares (2014). Smaller circular nodes represent significant indicator ASVs ($P < 0.05$). ASV circular nodes associated exclusively with OG vineyard are red, while nodes associated with HW vineyard are green and HE vineyard are blue. ASV white circular nodes are associated with more than one vineyard. ASV nodes are scaled to the average relative ASV abundance in the dataset and are arranged by the edge-weighted spring embedded algorithm, which weights node spacing by the ASV's strength of association (rpb) to site group(s). Edge thickness is scaled to the rpb value, with thicker edges representing closer associations. The five major ASV nodes (>5% abundance) are labelled numerically by genus.

were consistently abundant and significantly associated with all mid and late fermentations samples except for the 2014 winery late stage fermentation in which *Starmerella* was significant (Fig. 6). Two *Saccharomyces* ASVs (3c672c, 9dacaa) detected in later fermentation stages were significantly associated only with OG and winery fermentations (Fig. 6, Table S12). Other fermentative yeast ASVs in class Saccharomycetes such as *Hanseniaspora, Candida, Metschnikowia*, and *Pichia* were present in low abundance and most often significantly associated with early stage fermentations, except certain *Hanseniaspora* ASVs, which were associated with late stage fermentations, primarily in 2013 (Fig. 6, Table S12).

Visualizing associations between indicator ASVs and fermentation group, stage and their combinations in bipartite networks revealed division of communities by stage and fermentation group (Fig. S6). OG and WIN nodes are closely situated throughout the 2013 and 2014 fermentation stages relative to HE and HW fermentations, sharing many ASVs (Fig. S6, Table S12). As found in grape berry surface communities, highly abundant fermentation ASVs were associated with multiple fermentation groups and stages. An ASV in the genus *Starmerella* is one exception; while this ASV was significantly associated with grape berry surfaces (Fig. 2A) and fermentation groups in 2013, it was uniquely significantly associated only with winery fermentations in 2014 (Fig. 6; Fig. S6).

Discussion

SBV's three Pinot Noir vineyard blocks are geographically separate but are very closely situated, located within an approximate 600 m radius (Fig. S1). All the vineyards are conventionally farmed by the winery using the same equipment and are treated with identical spray regimes. The close vineyard proximity and the similarity of farming methods among blocks allowed an examination of the influence of small-scale geography on fungal community composition in isolation from other effects. The concept of *terroir* is traditionally associated with abiotic factors such as climate, soil and topography, and by these metrics, distinct *terroirs* that influence wine prices have been identified in closely-situated or adjacent vineyards at similar scales to this study such as the Côte de Nuits of France's Burgundy wine region (Haynes 1999). However, little research has been conducted on biotic factors that may also influence *terroir* on this small of a geographical scale. Previous studies of wine-associated microbial biogeography have focused primarily on evaluating community structure at larger scales or over single vintages (Bokulich et al. 2014, Morrison-Whittle and Goddard 2015, Liu et al. 2020, 2021). Given previous research linking microbial biogeography to wine metabolite profiles (Knight et al. 2015, Bokulich et al. 2016, Liu et al. 2021), our results suggest that fungal populations are also a defining contributor to very small-scale or 'micro' *terroir*, which is economically relevant to wine regions focused on producing premium single-vineyard wines.

Vintage and fine-scale geography influence grape-associated fungal community structure

Grape berry surface fungal community structure was driven by vintage and by geography (Table 2, Fig. 1). Herein, vintage differences were characterized by large shifts in abundance of *Starmerella* and *Pichia* ASVs (more abundant in 2013) and *Cladosporium* ASVs (more abundant in 2014) (Fig. 2). A multitude of factors may have contributed to the fungal community compositional differences between years. Climatic variation is a significant contributor (Bokulich et al. 2014). *Cladosporium,* a common grape berry pathogen, may proliferate in drier, sunnier conditions (Briceño and Latorre 2008, Latorre et al. 2011). *Cladosporium* was significantly associated with all vineyards in 2014, when rainfall was substantially lower than in 2013 in the month prior to our harvest dates: 9 mm and 42 mm respectively (Environment and Climate Change Canada 2021). In contrast, yeasts *Starmerella* and *Pichia* were associated with the rainier 2013 vintage. Fermentative yeasts can be more abundant on damaged grapes, which become more prevalent with increased rainfall (Barata et al. 2012). In crushed grapes, *Starmerella* and *Pichia* were more abundant in 2013 compared to *Cladosporium* which was more abundant in 2014 (Fig. 6). While vintage effects on fungal communities have not been studied as extensively as geographic effects, a study of Chardonnay grape musts from closely situated vineyards (<4 km) identified fungal population differences between two non-consecutive vintages; however, this effect did not extend to regional populations (Bokulich et al. 2014). Additionally, because grapes are harvested annually and are not a permanent microbial reservoir in the vine-

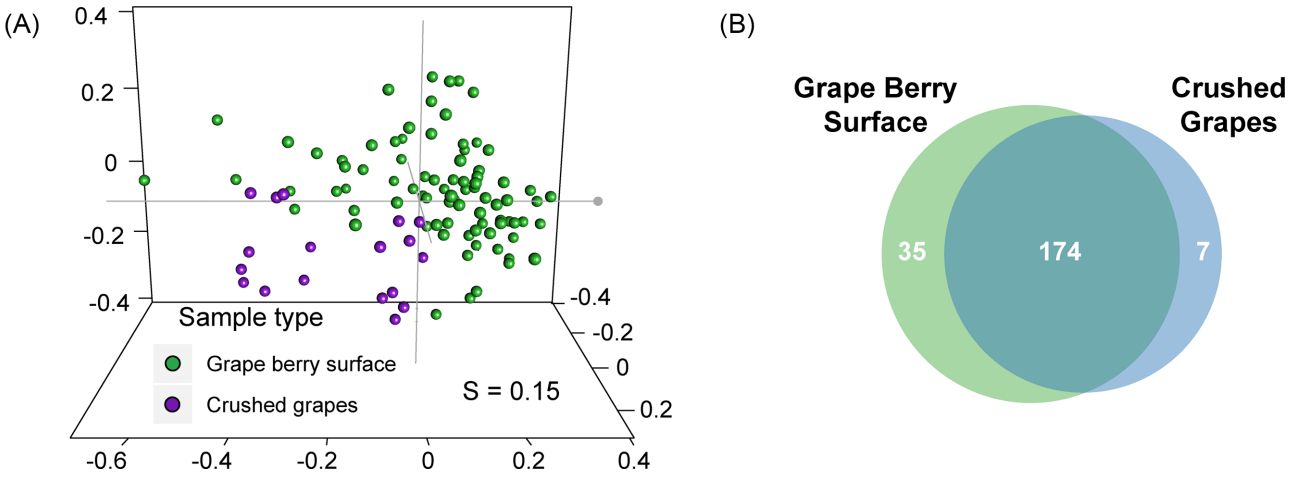

Figure 4. Grape berry surface and crushed grape fungal community structure. **(A)** NMDS plot of grape berry surface and crushed grape cluster fungal populations. **(B)** Venn diagram of grape berry surface and crushed grape ASVs.

yard, they may be susceptible to more pronounced changes in composition year-to-year within a microclimate.

Despite the proximity of the vineyards surveyed, our results indicate each vineyard block has a distinct fungal community pattern across vintages. While grape berry surface fungal communities are most strongly defined by vintage, PERMANOVA (Table 2), NMDS (Fig. 1A) and constrained CAP analyses (Fig. 1B and C) reveal community structure by vineyard, suggesting the berries from each vineyard have a fungal community "signature.' Most ASVs are shared by all vineyard blocks, and a small proportion of lowly abundant ASVs are significantly associated with a single vineyard block, indicating the distinction among vineyard fungal communities is largely defined by differences in abundance of taxa shared between sites (Figs 2 and 3). The distance between vineyard blocks may play a role in defining the degree of dissimilarity between fungal populations. Grape berry surface and fermentation communities from OG, which is situated approximately 1 km to the north-east of HE and HW, cluster most distantly from the other vineyards in CAP plots, whereas HW and HE, which are approximately 100 m apart, are more similar and have lower reclassification success rates (Figs 1B, C and 5B, D).

The distance-decay relationship of vineyard-associated fungal communities has been characterized at larger scales, with mixed findings. A study of Chilean vineyards within 35 km of each other found that grape berry and leaf fungal populations became more dissimilar with distance (Miura et al. 2017) whereas a New Zealand study covering vineyards across 1000 km identified the same trend at larger scales, but found this relationship deteriorated among vineyards less than 100 km apart (Morrison-Whittle and Goddard 2015). On a smaller scale, however, a study of soil fungi in four New Zealand Pinot Noir vineyards within a 2 km radius revealed community differentiation across sites, similar to our own results (Knight et al. 2020b). The appearance of differentiation at such small scales in contrast to larger scales may be attributable to a variety of factors. In both our study and Knight et al. 2020b, the vineyards were farmed similarly, potentially eliminating noise that may have obscured signals of population structure in more distant vineyards farmed with different techniques. The degree of sampling may also be a factor in elucidating population structure on finer geographical scales.

Crushed grape berry fungal communities also appear distinct among vineyards, however the distinguishing yeast populations differ from those found on the surface of grape berries. Basidiomycetous yeasts and ascomycetous filamentous fungi were significantly associated with grape berry surface samples, whereas semi-fermentative yeasts in the Saccharomycetales class were associated with crushed grapes (Table S8). Damaged or rotten berries may have been present inside the grape clusters gathered for crushing despite examination for damage, which could account for the increased number of Saccharomycetales. A similar finding was made in a culture-based study of grape berry and cluster isolates (Cadez et al. 2010). Yeast may also be present inside the grape berry, having translocated via xylem sap to other grapevine tissues (Liu et al. 2020). Sample handling may also account for these differences; for example, epiphytic filamentous fungi may not have been fully removed from the grape skins during crushing, and crush samples included only juice and not skins.

No *Saccharomyces* were found in crushed grape samples and were in very low abundance on grape berry surfaces, which is in line with our current understanding of *Saccharomyces* distribution (Martini 1993, Mortimer and Polsinelli 1999) and with similar vineyard studies (Taylor et al. 2014, Morrison-Whittle and Goddard 2015, Setati et al. 2015) (Tables S3 and S8). In contrast, Bokulich et al. 2014 collected must samples from wineries and found *Saccharomyces* at 4% average relative abundance, which may be due to harvesting method and to processing conditions in the winery (20).

The number of fungal ASVs identified in this study (211) is similar to those found on grape berry surfaces or in grape must samples from other studies covering much larger areas. Three amplicon sequencing surveys of hundreds of grape and wine samples spanning < 4 km to 1000 km identified 153 operational taxonomic units (OTUs) (Bokulich et al. 2014), 253 OTUs (Taylor et al. 2014) and 277 OTUs (Liu et al. 2021). The similar ASV counts found in this study, despite the smaller area surveyed, may be related to the robust sampling we conducted, analyzing 14 grape berry surface samples per vineyard and must from 36 grape clusters per fermentation replicate. Many of the most abundant grape berry surface ASVs (e.g. filamentous fungi *Aureobasidium pullulans*, *Cladosporium*, *Alternaria* and *Botrytis*; yeasts *Vishniacozyma*, *Filobasidium*, *Starmerella*, and *Pichia*) have been observed in high abundance in other studies (Bokulich et al. 2014, 2016, de Celis et al. 2022). The lack of significant variation in alpha diversity metrics

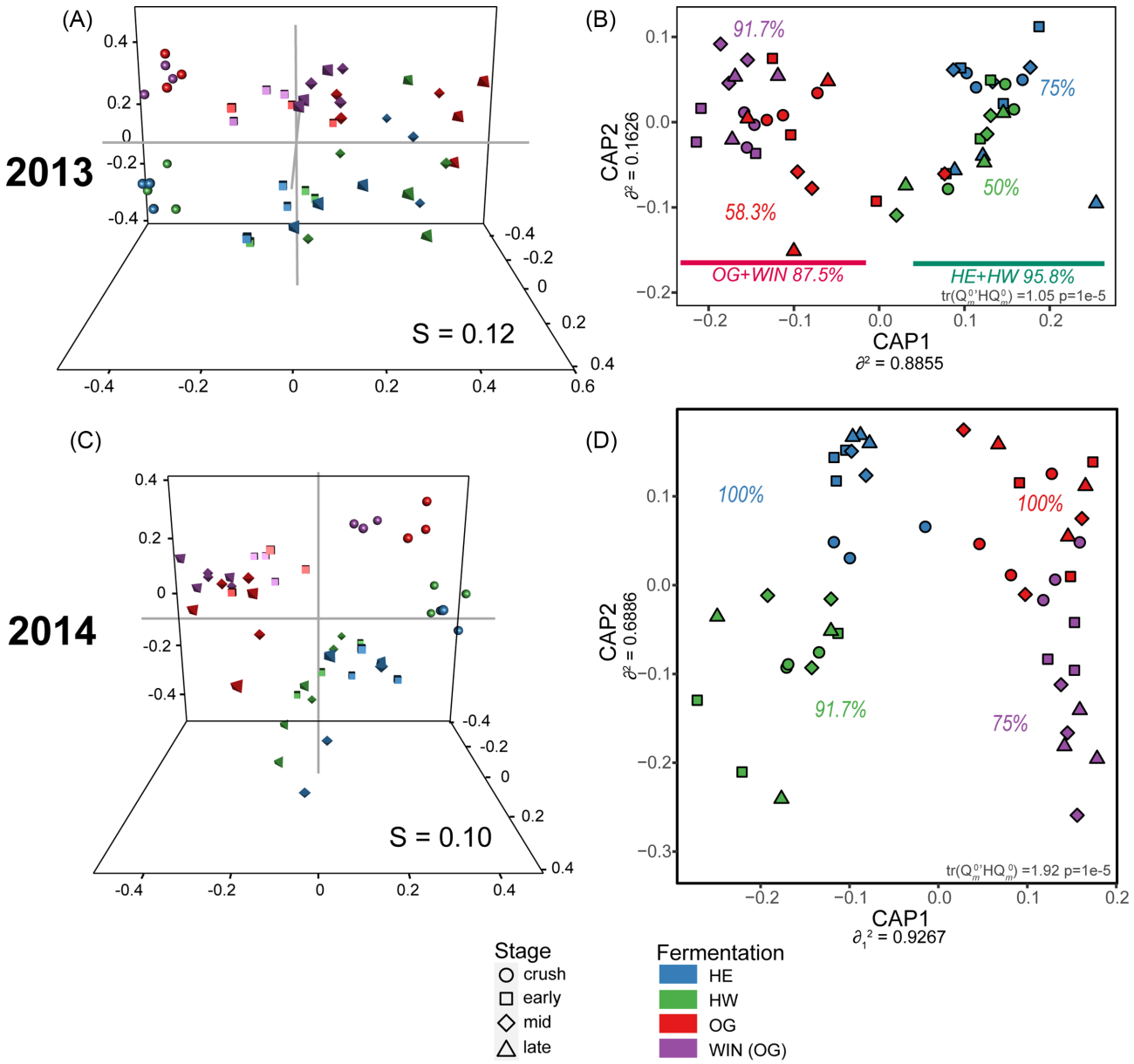

Figure 5. Location and fermentation stage effects on fermentation fungal community structure. **(A, C)** Three-dimensional NMDS and **(B, D)** CAP ordinations of fermentation samples from 2013 (A,B) and 2014 (C,D). HE, HW and OG fermentations were conducted in-lab with grapes sourced from these vineyards; WIN fermentations were conducted in the winery with OG grapes. NMDS plots are based on a pairwise Bray-Curtis similarity matrix of fermentation sample ASV counts and are rotated to the first principal component. CAP ordinations are discriminated by vineyard. Canonical correlation values (2) on each CAP axis indicate the association strength between the plotted data and the vineyard grouping. Percentages represent the reclassification accuracy of the CAP algorithm and indicate the magnitude of separation between groups. The trace statistic tr(Q*m*0'HQ*m*0) tests the hypothesis of significant differences among communities from different vineyards and vintages.

between vineyard sites in this study is reasonable to expect given that vineyard blocks are managed similarly.

Geographically-driven differences in fungal community structure persist during fermentation

We show that fungal populations in laboratory fermentations of grapes sourced from closely situated vineyards are significantly different at grape crush and that they retain this differentiation throughout fermentation, though the magnitude of this effect differs by vintage (Table 3, Fig. 5). Earlier stage fermentation populations from the lab and winery fermentation groups were significantly different in both vintages, but fermentation fungal communities became more similar as fermentation progressed (Table S9). Other studies have observed an increase in community similarity as fermentation progresses. For example, Bokulich et al. (2016) found that fungal communities in inoculated fermentations from different regions and vineyards became less distinguishable near the end of fermentation (Bokulich et al. 2016). In spontaneous fermentations, the diversity among fermentations has been shown to decline, but populations remain distinguishable by geographical origin (Stefanini et al. 2016, Mezzasalma et al. 2017, Morrison-Whittle and Goddard 2018).

In our study, spontaneous fermentation fungal populations changed dramatically over time, with a dramatic decline in ASV richness and increase in *Saccharomyces* abundance as fermentation progressed (Fig. S4, Fig. 6). This decline in richness is a well-

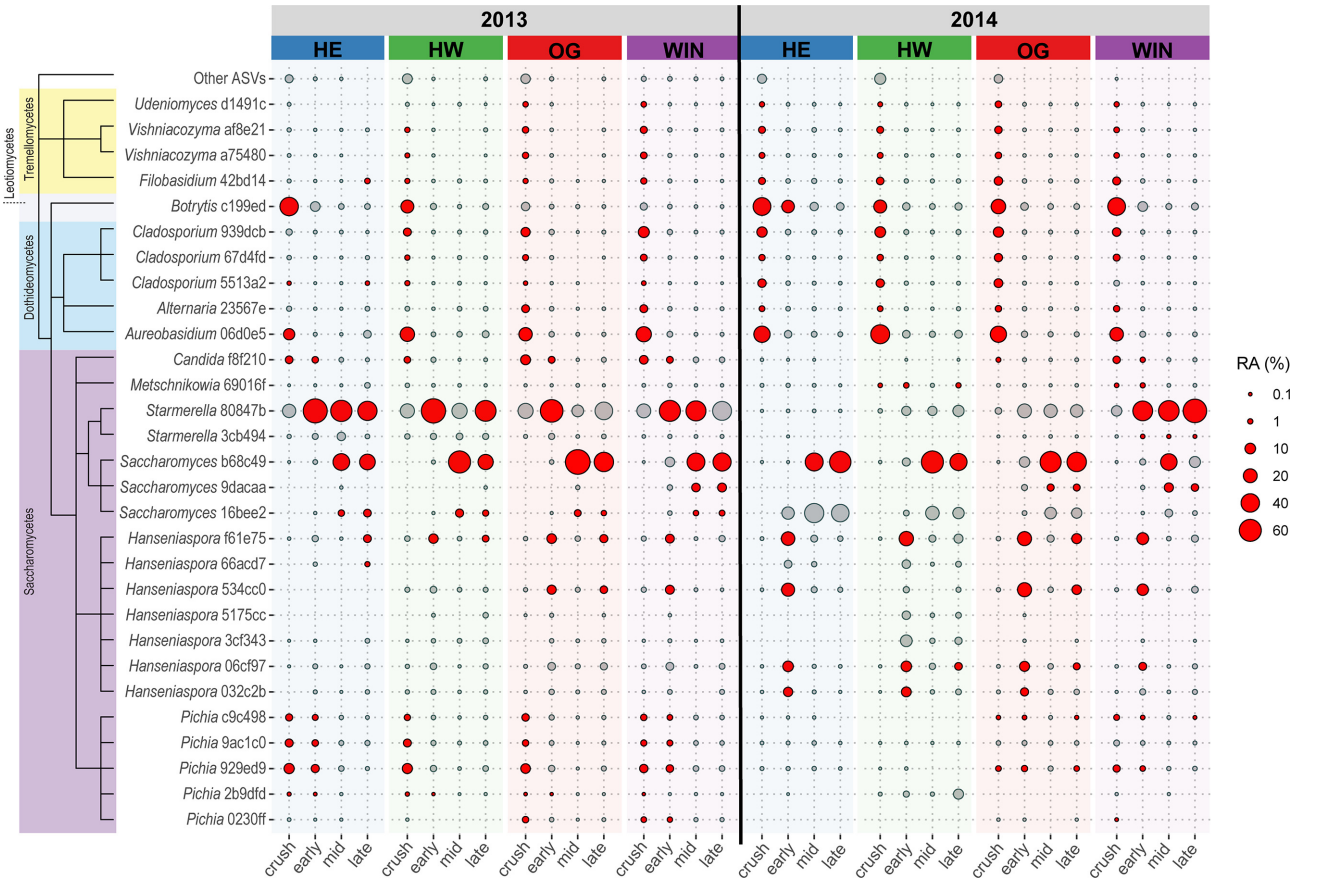

Figure 6. Relative abundance of major 2013 and 2014 fermentation fungal taxa arranged by fermentation stage and vineyard/winery. ASVs with abundances > 0.2% in the rarefied data set are represented by bubbles scaled to their average within-group relative abundance. ASVs are ordered by taxonomy and names are shaded by taxonomic class. ASVs significantly associated with one or more vintage-vineyard site groups are highlighted in red. RA = relative abundance.

documented phenomenon (Varela and Borneman 2017, Liu et al. 2021); *S. cerevisiae* is well-adapted to outcompete other organisms in high-sugar environments by using alcoholic fermentation as its preferred metabolism to render the environment less hospitable to other microorganisms (Goddard 2008). Interestingly, *Starmerella* relative abundance was quite high and remained constant or increased from crush to mid stages of fermentations, particularly in 2013 (Fig. 6). In 2014, *Starmerella* was significantly associated only with winery fermentations and was higher in abundance than *Saccharomyces* (Fig. 6). The species *Starmerella bacillaris* is commonly found in wine fermentations and can confer desirable sensory characteristics (Englezos et al. 2015). *S. bacillaris* may survive in ethanol concentrations up to 12% which may account for the high abundance of *Starmerella* at late fermentation stages (Masneuf-Pomarede et al. 2015). Interestingly, yeast belonging to the *Starmerella* clade are fructophilic (Gonçalves et al. 2018) and *S. bacillaris* could be useful in winemaking for resolving 'stuck fermentations' by removing residual fructose (Berthels et al. 2004).

Because of differences in fermentation sample storage between 2013 and 2014, we refrained from evaluating vintage effects on these. Were there storage effects, we would expect to see a substantial difference in ASV counts and/or a reduction in evenness due to DNA degradation between vintages, but trends in ASV richness and evenness across fermentation stages are relatively similar between vintages (Fig. S4). Additionally, the ratio of the number of ASVs detected in grape berry surface samples vs crushed grape samples is consistent between vintages (196:155 = 1.26 in 2013; 185 :154 = 1.20 in 2014); these ratios would likely be different if sample storage were to affect recovery of organisms.

Winery exposure and technique may influence fungal community structure in fermentation

We compared the effect of winery exposure to fungal populations in Pinot Noir grapes sourced from the OG vineyard as along with our lab fermentations, the winery spontaneously fermented OG grapes in 2013 and 2014. OG and WIN crushed grape populations were significantly richer only in 2013 (Fig. S4A). However, OG and WIN fermentation populations were significantly different in both vintages when taking all stages into account in pairwise PERMANOVA (Table S9; 2013: $t = 2.76$, $P = 0.00165$; 2014: $t = 3.48$, $P = 0.000024$). The winery environment can act as a reservoir of yeasts that are introduced to grapes through contact with winery surfaces and equipment, and the composition of the reservoir can consist of both seasonally transient and persistent yeasts (Sabate et al. 2002, Bokulich et al. 2013, Grangeteau et al. 2016). *S. cerevisiae* persistence in particular has been well documented in the winery environment (Constantí et al. 1997, Santamaria et al. 2005, Lange et al. 2014, Börlin et al. 2016, Martiniuk et al. 2016). Our results suggest two possibilities. Firstly, fermentative fungi may be introduced to crushed grapes in low numbers via exposure to the winery environment, which then increase in abundance during fermentation. For example, *Saccharomyces* ASV 9dacaa is significantly associated only with 2013 WIN fermentations and *Starmerella* ASV 3cb494 is significantly associated only with 2014 WIN fermenta-

tions (Fig. 6). The other possibility is that differences in fermentation conditions between the lab and the winery may have influenced population dynamics. WIN fermentations were conducted in wide, partially sealed vats with a higher surface area to volume ratio than for lab fermentations. The surface area to volume ratio can also affect phenolic extraction and heat release in red wines (Boulton et al. 1999), which could in turn impact microbial population dynamics. The surrounding vineyards may also be impacted by the winery environment and practices. We have documented the exchange of *S. cerevisiae* strains between the vineyard and the winery environment in a previous study (Martiniuk et al. 2016). In this study, winery and OG vineyard crushed grapes have higher Shannon diversity relative to HE and HW crushed grapes in both vintages (Fig. S4). This may be related to the winery's receipt of grapes from multiple vineyards and to the proximity of the OG vineyard to the winery environment, although further study is required to evaluate these impacts.

The discovery of persistent fungal community structure over two vintages at the 1.2 km scale evaluated in this study and the preservation of this structure throughout spontaneous fermentation have important implications for smaller-scale premium wine producers in regions like the Okanagan Valley, particularly for wineries that make single-vineyard wines. Given the differentiating influence of microbial community composition on wine organoleptic profiles at regional scales, our research demonstrates that the fungal ecology of vineyards at a smaller scale may be an important component of *terroir*, potentially contributing to the diversity of organoleptic characteristics in wines from different but closely-situated vineyards.

Author Contributions

JTM designed experiments, performed experimental work, analyzed data and wrote the manuscript. JH and TD performed experimental work. VM provided experimental direction and wrote the manuscript.

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Supplementary data

Supplementary data are available at FEMSYR online.

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. The funders played no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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