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Freeze-thaw Cycles Characterize Varietal Aroma of *Vidal Blanc* Grape during Late Harvest by Shaping Self-assembled Microeukaryotic Communities

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Abstract: To study the effects of microeukaryotic communities on development of varietal aroma of *Vidal blanc* grape during late harvest, metagenomics, gas chromatography–mass spectrometry, climate factors and scanning electron microscopy

were jointly used to discover how freeze-thaw stress influences grape skin and shapes the microeukaryotic consortia. Most free terpenes, monoterpenes and C13norisoprenoids developed during late harvest. Freeze-thaw cycles caused damage of the wax layer and microfissures on the cuticle of grape berries. This increased nutrients availability and supported the dominance of oxidative and fermentative ascomycetous populations. Microbial assemblages possessed certain ecosystem functions, which correlated to the development of characteristic volatile organic compounds, such as free terpenes and phenylalanine derivatives. Significantly upregulated genes of CAZy were identified as FENNEDOB12352, OJNJMGIN252777, EGHMEMAM161340, EGHMEMAM158447, KKFIDJDF144524, BEDCFLNK31943, LHALBBHN88115, BEDCFLNK41042. *Zymoseptoria tritici, Kluyveromyces lactis, Yarrowia lipolytica*, and *Pichia kudriavzevii* were selected as key factors in yeast assemblage.

KEYWORDS: Self-assembled microeukaryotic communities; Freeze-thaw cycles; Metagenomics; Climate factors; Ecosystem functions; Varietal aroma; Icewine

1. Introduction

Economic and cultural wine values can be defined by different geographical locations, which are commonly regarded as "*terroir*". Recent advances in high-throughput sequencing technology have improved our understanding of the correlation between microbial biogeography and wine terroir (R. Li et al., 2021). In particular, a suitable acclimatisation of indigenous yeast strains can not only construct good oenological conditions, but can largely improve the aroma profile complexity and exhibit regional wine characteristics (Kai Chen et al., 2021). For wine making, it is well known that good-quality grapes can be much more important than well-controlled fermentation processes. Wine grapes present a unique biogeography model, wherein microbial biodiversity simultaneously answers questions about the inherent components of the grape quality and grapevine health (Bokulich, Thorngate, Richardson, & Mills, 2014).

Icewine is a type of dessert wine produced from grape juice extracted from frozen grapes. It is generally characterised by a delicate aroma profile and an extremely smooth taste (Kai Chen, Han, Li, & Sheng, 2017). Icewine is widely produced in cold regions, such as Austria, Germany, Canada, and Northern China. In Canada, *Vidal Blanc* is the most common grape used for wine production. Fully ripe grapes hang on the vines for several months to be naturally frozen until the final harvest, which ensures the temperature ranges from -10 °C to -12 °C, so that the pressed juice attains a high

concentration of sugar (around 35 °Brix) prior to the fermentation process. This typical period for the harvest of frozen grapes is technically called late harvest. Grape hang time has been previously found to affect the organoleptic profile of icewine (Bowen & Reynolds, 2015). Particularly, grape berries in later harvest can substantially intensify several highly odour-active compounds, such as 1-octanol, *cis*-rose oxide, nerol oxide, ethyl benzoate, and ethyl phenylacetate (Bowen, Reynolds, & Lesschaeve, 2016).

Varietal aroma is composed of volatile organic compounds (VOCs) directly derived from grape fruit and determines the varietal typicality of wines (Silva Ferreira, Monteiro, Oliveira, & Guedes de Pinho, 2008). Terpenes are secondary metabolites synthesised by acetyl-CoA in plants and play an important role in the perception of varietal aroma (Fischer, 2007). These compounds exist in grape fruit in free forms or in tasteless glycosidic forms, and the highest concentration of terpenes accumulates in grape skin (Gil, Pontin, Berli, Bottini, & Piccoli, 2012). Linalool, geraniol, nerol, citronellol, and α -terpineol are the most common monoterpenes with the *Muscat* aroma, among which geraniol and linalool are regarded as the most fragrant in grapes and wines (Cabrita, Freitas, Laureano, & Stefano, 2006). According to our previous investigation, off-vine treatment for *Vidal Blanc* can positively influence the development of some glycosidic bound VOCs and free terpenes such as linalool oxide, α -ionone, 2-phenyl-1-ethanol, and α -terpineol during late harvest (Kai Chen, Wen, Ma, Wen, & Li, 2019). These reports suggest that the development of varietal aroma, especially for free terpenes in grapes, may be correlated to certain environmental factors during late harvest.

Predictably, icewine making is comparatively risky during late harvest because ripe grapes can be seriously affected by both abiotic and biotic factors. For instance, ripened grapes may rot, and crops will be lost if a freeze does not come quickly enough. Late harvest for icewine making involves natural freeze-thaw cycles and desiccation, which cause cellular degradation and compartmentalisation, and consequently influence hexose metabolism, cell wall composition, and secondary metabolism of grapes. Meanwhile, temperature fluctuation in freeze-thaw cycles results in a series of degradative effects in grape skin cells and the growth of epiphytic microbes. In particular, freeze-thaw stress induces a specific pattern of differential gene expression in Saccharomyces cerevisiae, indicating that the cellular response to this stress is regulated at least in part at the transcriptional level (Cabrera, Welch, Robinson, Sturgeon, Crow, & Segarra, 2020). Freezing is the main determinant of freeze-thaw damage. It directly induces high resistance during the lag phase in wild yeast strains (Park, Grant, Attfield, & Dawes, 1997). Besides S. cerevisiae, wild yeasts have been found to have a positive function in alcoholic fermentation by releasing extracellular hydrolytic enzymes, which catalyse the glycosidic bound aroma precursors and consequently increase aroma complexity in the wines (Benito, Palomero, Calderón, Palmero, & Suárez-Lepe, 2014). Although many wild yeast strains have been verified to enhance aroma intensity in different fermentation scenarios, and recent studies have focused on spontaneous fermentations (Kai Chen et al., 2018; Y. Chen et al., 2020; Lu et al., 2020; Shi, Chen, Xiao, Li, & Wang, 2020), the effects of grape-surface microeukaryotic communities on grape quality and aroma profile have been rarely reported. In particular, little work has been done to understand how a grape-surface microeukaryotic community is formed by natural freeze–thaw cycles and whether this microbial consortium actively interacts with wine grapes during late harvest.

This study combined metagenomics and scanning electron microscopy (SEM) to demonstrate that freeze-thaw stress and grape status shape the microeukaryotic consortia inhabiting the grape surface. Furthermore, we speculate that these microbial assemblages possess certain ecosystem functions, which would correlate with the development of varietal characteristics of the aroma profile, especially for free terpenes and phenylalanine derivatives. Meanwhile, to elucidate the scale and impact of freezethaw cycles on the microeukaryotic community during late harvest, the impact of major environmental factors, such as temperature, humidity, and precipitation on the grapesurface microeukaryotic community are also discussed in detail.

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2. Materials and Methods

2.1. Viticulture and sampling

Grape sampling was conducted in the core icewine production area of Northeast China. *Vidal blanc* vines were planted in a commercial vineyard in Sidaolingzi village adjacent to Huanlong Lake, Huanren County, Benxi City, Liaoning Province, P. R. China (latitude 41° 30' N, elevation 125° 23' E) on a 25° slope. The vines were 9 years old with a plant density of 2.5 m \times 1 m. A vertical trellis system was installed in the vineyard with east–west oriented rows. Vines were pruned to five to six shoots, and the overall crop yield was maintained at approximately 10000 kg/ha. Meteorological parameters (the average and lowest temperature, rainfall, atmospheric pressure, vapour pressure, precipitation, humidity, and sunshine hours) of the Huanren region for the 2020 vintage (Figure S1 in Supplementary Information (SI), A–E) were obtained from the China Meteorological Data Service Centre (http://data.cma.cn).

The specific process of grape sampling was based on our previous work (Kai Chen et al., 2019). Grape berries were randomly selected by hand from more than 50 different grapevines. Grape sampling started the 70th day after full bloom and continued every 10 days until the end of late harvest. In total, eleven grape berry stages were collected for the 2020 vintage (Figure S1, F). Grape berries were immediately frozen in liquid nitrogen and then transported to the laboratory for physicochemical analysis. Samples were preserved at -40 °C prior to subsequent experiments.

To collect epidermal microorganisms from grape berries, 200 g *of Vidal blanc* berries were soaked in 500 mL sterilised $10 \times$ PBS solution for 15 min and vortexed to elute epidermal microorganisms. Sediments were collected by filtering the eluate through a 0.45-µm cellulose nitrate membrane (Che, Xia, Liu, Li, Yang, & Zhang, 2019). All processed samples were stored at -40 °C prior to DNA extraction. Triplicate analyses were performed on each grape sample.

2.2. Determination of physicochemical parameters and antioxidant activity of Vidal blanc

After measuring the average berry weight, seeds were removed from 100 *Vidal blanc* berries, and grapes were pressed and centrifuged (10000 rpm at 4 °C for 10 min) for physicochemical analysis. Physicochemical parameters of grape berries, such as yeast assimilate nitrogen (YAN), total acidity, malic acid, lactic acid, iron, copper, tartaric acid, glucose/fructose, acetic acid, and glycerol were measured using a Y15 enzymatic autoanalyser (Biosystems S.A., Barcelona, Spain). pH values were determined using PB-10 pH Basic+ meter; Sartorius, Göttingen, Germany.

To determine the antioxidant activity of grape berries during late harvest, enzyme activities in the eleven stages were analysed using detection assay kits purchased from Solarbio Science & Technology Co., Ltd., Beijing. The determination included the

enzyme activities of total antioxidant capacity (T-AOC), polyphenol oxidase (PDC), superoxide dismutase (SOD), and the content of malondialdehyde (MDA). Detection measures were implemented according to the manufacturer's instructions.

2.3. Scanning electron microscopy (SEM)

The surface morphology of the grape skin was observed and photographed using a Hitachi UHR FE-SEM SU8010 (Hitachi, Tokyo, Japan) at an accelerating voltage of 10 kV after coating with a gold layer. The SEM operation was technically supported by the Institute of Electron Microscopy of the Chinese Academy of Agricultural Science, Beijing.

2.4. Extraction of free and glycosidically bound VOCs

Seed-removed grape berries (50 g) were blended with 1 g of polyvinylpolypyrrolidone (PVPP) and D-gluconic acid lactone (0.5 g) and subsequentially soaked in liquid nitrogen. Frozen berries were ground to powder, macerated at 4 °C for 4 h, and the supernatant was collected after centrifugation (10000 rpm at 4 °C for 10 min) and directly used for the determination of free VOCs (Kai Chen et al., 2019; Lan et al., 2016).

Isolation of glycosidically bound aromatic precursors was carried out using Cleanert PEP-SPE resins (Bonna-Angela Technologies, China), which were previously conditioned with 10 mL each of methanol and Milli-Q water. Five millilitres of the supernatant were gravitationally passed through the solid-phase extraction resin. The resin was then rinsed with 5 mL of Milli-Q water to remove sugars, acids, and other polar compounds, and washed with 5 mL of pentane/dichloromethane (2:1; v/v) to eliminate free fractions. The precursors were eluted using methanol (10 mL). The methanol extract was evaporated to dryness under a nitrogen stream and re-dissolved in 10 mL of citrate-phosphate buffer solution (0.2 M, pH 2.5) and 1 g of sodium chloride. Enzymatic hydrolysis was performed in an incubator with 100 mL of 100 g/L commercial glucosidase, Rapidase Revelation Aroma (Creative, Shirley, NY) at 40 °C for 24 h (Kai Chen et al., 2021; Kai Chen et al., 2019).

2.5. Headspace-solid phase microextraction (HS-SPME)

The grape supernatant (5 mL) was added to 10 μ L of internal standard 4-methyl-2pentanol (250 mg/L) and 1 g of sodium chloride in a 15-mL dark brown glass vial containing a magnetic stirrer. The mixture was tightly capped with a polytetrafluoroethylene (PTFE)–silicone septum. Subsequently, the sample was equilibrated at 40 °C for 30 min under continuous stirring. Pre-treated SPME fibre (PDMS/CAR/DVB; Supelco, Bellefonte, PA) was inserted into the headspace and



extracted at 40 °C for 30 min with continuous heating and agitation (Kai Chen et al., 2021; Kai Chen et al., 2019).

2.6. Gas chromatography-mass spectrometry (GC-MS)

An Agilent 7890B GC system equipped with an Agilent 5977B mass spectrometer and a 60 m × 0.25 mm × 0.25 μ m HP-INNOWAX capillary column (J&W Scientific, Folsom, CA) was used to separate and identify the aromatic volatiles. Pure helium (> 99.999%) was used as the carrier gas at a flow rate of 1 mL/min. The SPME extracts were injected in splitless mode with the following operating conditions: injector temperature, 250 °C; thermal analysis time, 8 min. The temperature program went from 60 °C (holding 1 min) to 220 °C by increasing the temperature at 3 °C/min and was held at 220 °C for 2 min. Mass spectrometry was performed using 70 eV electron ionisation (EI). The ion source and quadrupole were set at 230 °C and 150 °C, respectively. The mass detector was operated in full scan mode (*m/z* 30–350), and triplicate extractions were performed on each sample.

The retention index (RI) was calculated using *n*-alkanes C8–C40 (Sigma-Aldrich, St. Louis, MO) as an external reference under the same conditions. Data analysis was performed using ChemStation Software (Agilent Technologies, Inc., Santa Clara, CA). The VOCs were identified by comparing the mass spectra and RI with those of the

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standard substance. In addition, the VOCs without standards were tentatively identified by comparison with the mass spectra and RI values in the standard NIST14 library or compared with the RI values reported in the literature (Table S1) (Kai Chen et al., 2019; Lan et al., 2016). Standards for each level were extracted and analysed in the synthetic matrix. In addition, quantification of other compounds without establishing calibration curves was carried out from the total ion current peak areas according to the internal standard (Table S2).

2.7. Metagenomic sequencing and bioinformatic analysis

Raw metagenomic sequencing data were obtained from Wuhan Benagen Tech Solutions Co. Ltd. (Wuhan, China) for 500 bp library construction on the Illumina NovaSeq 5000 platform. The metagenomic data size of each sample was approximately 6 GB in clean reads. Host gene removal was performed using Bowtie2 to align the sequence with the reference genome, then SAMtools were used to extract the unmatched BAM file, and bam2fastx was used to convert the fq-sequence (H. Li et al., 2009). Contigs were obtained after gene assembly of short reads using MEGAHIT and quantified using QUAST (Gurevich, Saveliev, Vyahhi, & Tesler, 2013; D. Li, Liu, Luo, Sadakane, & Lam, 2015). The sum of the gene abundances corresponding to each species was used to calculate the species abundance. Gene functional annotation was mainly annotated against the Kyoto Encyclopedia of Genes and Genomes (KEGG), Gene Ontology (GO), and reference sequences (RefSeq), based on the gene quantification measured by Salmon (Patro, Duggal, Love, Irizarry, & Kingsford, 2017). To correlate the glycosidase gene and possible epidermal microorganisms, the predicted contigs were annotated against the carbohydrate-active enzyme database (CAZy) (http://www.cazy.org/) using the HMMER for sequence homologues and alignment.

2.8. Statistical analysis

Statistics for VOC evolution in *Vidal blanc* grapes, such as principal component analysis (PCA), partial least squares discriminant analysis (PLS-DA), and heatmap cluster with Euclidean distance, were calculated using the MetaboAnalyst 4.0 platform (www.metaboanalyst.ca). In addition, data visualisations of α -diversity, Spearman correlation ($p \le 0.05$) of free terpenes and epidermal fungi, volcano plots, redundancy analysis (RDA) of meteorological factors, and random forest algorithm of predicable correlation between epidermal yeasts and functional genes were realised using relative packages in R version 4.1.1.

3. Results and Discussion

3.1. Evolution of free terpenes and phenylalanine-derivatives in Vidal blanc during late harvest

Terpenes play an important role in varietal aroma development and characterise the floral and fruity properties of grapes and wines (Yuan & Qian, 2016). In particular, free terpenes in grapes and wines mainly include monoterpenes and C13-norisoprenoids. These groups of VOCs usually have strong aromatic characteristics and can be easily perceived by the nose because the sensorial thresholds of free terpenes are very low (K. Chen & Li, 2022). Heatmap cluster and PCA were jointly used to study the evolution of free terpenes in Vidal blanc during late harvest. Most free terpenes were highly developed during late harvest (November until harvest), while a few free terpenes were highly correlated with the period of grape ripeness (September to October), specifically, citronellol, geraniol, α -phellandrene, *cis*-carveol, and *trans-\beta*-ocimene. With increasing degree of freeze-thaw, grape berries gradually lost water, shrank, and browned during late harvest (Figure S1). Compared with the ripened grape berry, approximately 50% of the hundred-berry weight was lost by the end of late harvest (Figure S2, A). Previous studies suggested that late harvest grapes had increased total terpene concentration, which resulted in enhanced floral and fruity sensorial characteristics (Kai Chen et al., 2019; Lan et al., 2016). The development of monoterpene oxides was highly correlated with the late harvest. Nerol oxide and rose oxide are found in minor amounts in some essential oils and contribute to their unique odour. The formation of these monoterpene oxides was confirmed by bioconversion of citronellol in the co-culture of different fungi, such as Aspergillus sp. and P. roqueforti (Demyttenaere, Vanoverschelde, & De Kimpe,

2004). In contrast, 2-phenylethanal and 2-phenyl-1-ethanol were highly developed during late harvest. These groups of phenylalanine derivatives possess a strong flavour of rose and honey and can be synthesised in alcoholic fermentation by *S. cerevisiae* and other non-*Saccharomyces* yeasts, such as *Pichia kudriavzevii, Lachancea thermotolerans*, and *Metschnikowia pulcherrima* (Zhang et al., 2022). Three kinds of C13-norisoprenoids, ionone, geranyl acetone, and β -damascenone, were highly correlated with late harvest grapes. In plants, carotenoids are catalysed into C13-norisoprenoids by members of the carotenoid cleavage dioxygenase family and commonly exist in the glycosidic bound form (Lashbrooke, Young, Dockrall, Vasanth, & Vivier, 2013). These terpenoids are responsible for the "varietal character", with a complex floral and fruity aroma at very low perception thresholds. For instance, the sensory thresholds in a model wine solution of β -damascenone and ionone were 0.8 $\mu g/L$ and 0.05 $\mu g/L$, respectively.

To understand the total VOC dimensions, PCA was performed to analyse the correlations in different grape samples. The score plot showed that 73.5% of the total variance could be explained by the first two principal components. The first group consisted of the ripening period samples (70 to 100 days after full bloom) and the second group comprised the late harvest period samples (120 to 176 days after full bloom). The results of PCA indicated that the varietal aroma of grapes in the late harvest was different from that of grape maturation. Grape samples collected on the 110th day

and the 176th day can be regarded as biomarkers of berry maturation and late harvest, respectively. Moreover, many indexes, such as glycose/fructose, total sugar, lactic acid, malic acid, polyphenols, and tartaric acid, showed significant changes on the 110th day. This indicated that the 110th day after full bloom in October was the dividing line between the end of grape maturation and the beginning of late harvest (Figure S3).

Characteristic free terpenes and phenylalanine derivatives were identified and rated by PLS-DA. The importance of VOCs was measured using the variable input in projection (VIP) score of component 1. The top twenty characteristic free terpenes and phenylalanine derivatives were rated, and correlations between the important VOCs and different harvest periods were jointly shown using a heatmap cluster. VIP scores of VOCs higher than 1 suggest the key contributors to PLS-DA. Therefore, β -cymene, 2-phenyl-1-ethanol, hotrienol, 2-phenylethanal, geraniol, nerol oxide, *cis*-rose oxide, and terpinen-4-ol were selected as the characteristic VOCs of *Vidal blanc*. The present study suggests that *Saccharomyces* species and hybrids are responsible for the bioconversion of geraniol into linalool and *a*-terpineol during fermentation (Gamero, Manzanares, Querol, & Belloch, 2011). Apart from geraniol, the characteristic VOCs showed high correlations with late harvest, which was the critical period for the development of varietal aroma in *Vidal blanc*.

3.2. Comparison of free and glycosidically bound terpenes and phenylalanine

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derivatives in Vidal blanc during late harvest

Terpenes and phenylalanine derivatives were found in either free or glycosidically bound forms. Glycosidically bound forms predominate in the berry ripening stage and continue to increase after over ripeness, whereas the free forms may stop or decrease (Bhat, Desai, & Suleiman, 2010). Monoterpenyl disaccharide glycosides originating from plants and microbes, such as α -arabinofuranosidase, β -glycosidase, and β galactosidase, hydrolysed glycosidically-bound terpenes into free forms, which largely enhance the aroma profiles of grape juice and wine (Bhat et al., 2010; K. Chen et al., 2022). Considerable concentration loss was found in some important monoterpenes during late harvest, such as α -terpineol, β -cymene, β -terpineol, geraniol, linalool, and nerol (Figure 2). These monoterpenes were largely synthesised during the berry ripening stage and gradually decreased until almost complete loss during late harvest. Either free forms or glycosidic forms are in agreement with this similar change, which is comprehensively affected by biological and abiotic factors, such as berry water loss, exposure to sunshine, enzymatic hydrolysis, or bioconversion by microbes. Obviously, geraniol, linalool, linalool oxide, nerol, β -ionone, and 2-tridecyne were released during the ripening period, and the majority remained in the glycosidically bound form in the grape skin. In contrast, hotrienol, β -cymene, and 2-phenylethanal were continuously released in free form throughout the berry ripening period and late harvest. The glycosidically bound form of geranyl acetone was synthesised during the berry ripening

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stage. Afterwards, free geranyl acetone significantly increased, whereas the glycosidic bound form was completely hydrolysed until the end of late harvest. α -Ionene, β damascenone, and 1,1,6-trimethy1-1,2-dihydronaphthalene (TDN) were mainly synthesised and released as free forms during the veraison period (Luo, Brotchie, Pang, Marriott, Howell, & Zhang, 2019). Apart from slight decreases during berry maturation, the concentration of these norisoprenoids barely changed during late harvest. Notable concentration increases in nerol oxide, rose oxide, 2-phenylethanal, and 2-phenyl-1ethanol possibly resulted from fungal metabolism. Although accumulation of plantderived 2-phenylethanal and 2-phenyl-1-ethanol in berries could be part of the reason for the increase in concentration, this sharp rise was highly correlated to the bioconversion of both VOCs through the Ehrlich pathway by yeasts. Moreover, 2phenyl-1-ethanol has been confirmed as a quorum-sensing molecule, which plays an important role in nitrogen metabolism and nicotinate/nicotinamide metabolism among yeasts (Han, Turnanov, Cannon, & Villas-Boas, 2013).

3.3. Freeze-thaw induced self-assembled microeukaryotic communities on the grape surface during late harvest

A complex microbial ecology on grapes is formed of filamentous fungi, yeasts, and bacteria, which jointly determine grape quality with regional characteristics and further influence wine properties. SEM was used to observe the microeukaryotic community availability during the late harvest (Figure S4). As the number of freeze-thaw cycles increased, the damage to the wax layer became increasingly serious (Figure S4, A–E). Freeze-thaw cycles caused damage to the wax layer and microfissures on the cuticle of visually intact berries. It increased nutrient availability and supported the dominance of oxidative and fermentative ascomycetous populations (e.g., *Candida spp.*, *Hanseniaspora spp.*, and *Pichia spp.*) during late harvest. As expected, a group of yeastlike fungi with smooth surfaces could be observed in the wax layer (Figure S4, F), whereas the other group of fungi had a "plush" surface (Figure S4, G). Interestingly, we also clearly observed a group of yeasts in agamocytog, which indicated the growth of yeasts under freeze-thaw stress during late harvest (Figure S4, H).

Recent studies have reported that basidiomycetous yeasts and the yeastlike fungus *Aureobasidium pullulans* are commonly found on different grape berries after veraison, and the proportion of these microorganisms changes with the grape ripening stages (Barata, Malfeito-Ferreira, & Loureiro, 2012; Shi et al., 2020). However, the relative importance of environmental factors in shaping grape surface microeukaryotic communities is still poorly understood. The neutral community model (NCM), proposed by Sloan et al. (Sloan, Lunn, Woodcock, Head, Nee, & Curtis, 2006), was used to understand the deep mechanisms (deterministic or stochastic processes) underlying self-assembled microeukaryotic communities and to quantify the importance of neutral processes. The NCM predicted a large fraction of the relationship

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between the occurrence frequency of OTUs and their relative abundance variations (Figure 3, A-C), with 90.6%, 90%, and 87.6% of the explained community variance for the entire season (from grape maturation to late harvest), late harvest, and grape maturation, respectively. The NCM suggested that stochastic processes always played an important role in shaping the microeukaryotic community assembly throughout the season. Moreover, the Nm value was slightly lower for microeukaryotic taxa in the late harvest (Nm = 32482) than for grape maturation (Nm = 33055). This suggests that species dispersal of microeukaryotes could be similar throughout the season. To provide more details for microeukaryote dispersal, the relative importance of determinism and stochasticity was assessed using a modified stochasticity ratio (MST), which was developed with 50% as the boundary line between a more deterministic (< 50%) assembly and a more stochastic (> 50%) assembly (Ning, Deng, Tiedje, & Zhou, 2019). The results showed that MST during harvest (grape maturation) and late harvest was higher than 50%, which indicated stochasticity dominated in the microeukaryotic community assembly for both seasons (Figure 3, D). Although the MST of microeukaryotic species in late harvest (65.7%) was higher than that in grape maturation (64.3%), the significance (p < 0.05) between the MSTs in both seasons was not identified. In other words, the eukaryotic communities and grape qualities could dynamically change and were simulated by considering abiotic filtering, competition, environmental noise, and spatial scales. Our results first quantified the effect of ecological stochasticity on self-assembled microeukaryotic communities of wine

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grapes and further elucidated the importance of stochastic processes in shaping microeukaryotic communities for both grape maturation and late harvest.

To understand the effects of climatic factors on self-assembled microeukaryotic communities, the coefficients between climatic factors and OTUs of microeukaryotic species were calculated. The dynamic changes in climatic factors during late harvest are shown in Figure S5. Herein, atmospheric pressure, temperature (average temperature), vapour pressure, atmospheric humidity, sunshine hours, lowest temperature, and hundred berry weight showed significant correlations ($p \le 0.05$) with OTU abundance of microeukaryotic species (Figure 3, E). In particular, three freeze-thaw related climatic factors, temperature, lowest temperature, and vapour pressure, negatively affected the self-assembly process of microeukaryotic communities during late harvest. Thus, the species richness and diversity of microeukaryotes on grape surfaces could be inhibited by freeze-thaw cycles. Moreover, RDA further confirmed that vapour pressure, lowest temperature, temperature, and hundred berry weight (water loss related factor) were key climatic factors that jointly affected the self-assembled microeukaryotic communities during late harvest (Figure S6, D).

3.4. Species diversity of self-assembled microeukaryotic communities and the correlations to the development of varietal aroma

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Calculating a-diversity is useful for comparing the differences between microeukaryotic species from harvest (grape maturation) to late harvest (Kai Chen et al., 2021). The within-habitat diversity (α -diversity) of self-assembled microeukaryotic communities was jointly evaluated using the Chao1, Simpson, Shannon, and Pielou indices (Figure 4A). The richness of the microeukaryotic communities was similar to that of the Chao1 index. Herein, grape samples in the harvest stage showed the highest richness and Chao1 index, which indicated more prolific microeukaryotes than those in late harvest. In addition,, the Simpson, Pielou, and Shannon indices were used to evaluate the evenness of microeukaryotic species. Similarly, the grape samples at the end of late harvest reached the lowest value, which was statistically different from the samples during maturation and the beginning of late harvest. In general, harvest grape samples showed the highest richness and evenness of the microeukaryotic communities. The ability of microeukaryotes to survive under freeze-thaw cycles varies between different strains and species of microeukaryotes. A minority of microeukaryotic species have adapted freeze-thaw stress and have evolved a larger genetic network of survival responses during late harvest. For icewine fermentation, it is predictable that the different species diversity of self-assembled microeukaryotic communities would be much more determinable for varietal aroma profiles rather than the selection of a practical commercial yeast.

The most abundant top twenty microeukaryotes were selected, and the correlation levels

between OTU abundance of microeukaryotes and harvest stages were quantified and shown with cricoid strips (Figure 4B). Most of these microeukaryotes were classified as Ascomycota. Botrytis cinerea and Zymoseptoria tritici were widely distributed in different harvest stages. B. cinerea is described as a 'Jekyll and Hyde' fungus. An epidemic of B. cinerea results in devastating grey mould disease, whereas wellcontrolled B. cinerea infection promotes berry dehydration, increased sugar concentration, and improved grape quality (termed 'noble rot'), which can then be used for the production of sweet, smooth, full-bodied 'botrytised wines' (Negri et al., 2017). Apart from freeze-thaw cycles, the penetration of B. cinerea through stomata, wounds, or microfissures on the Vidal blanc surface, and the permeabilisation of the grape skin exacerbated water loss and sugar concentration during late harvest. B. cinerea infection developed and reached a high level during grape maturation, and B. cinerea growth was greatly inhibited by the accumulation of high sugar concentrations and the development of other fungi genera, such as Zymoseptoria, Fusarium, and Cryptococcus. However, the second development of B. cinerea was found at the end of the late harvest (Figure S6, B). The results indicated that biotic factors on grape skin are the main inhibitors of B. cinerea development, instead of the previously reported high sugar concentration.

To further explore the influence of self-assembled microeukaryotic communities on the evolution of varietal aroma of *Vidal blanc*, the correlations between the top forty abundant microeukaryotic species and the determined free terpenes and phenylalanine-

derivatives were studied via the calculation of Spearman coefficients; significant differences were labelled with $p \le 0.05$ and $p \le 0.01$ (Figure 4C). B. cinerea showed negative correlations with many terpenes, while most of the Ascomycota species showed positive and significant correlations with terpenes and phenylalanine derivatives. In particular, non-Saccharomyces strains, Zymoseptoria tritici, Kluyveromyces lactis, Saccharomyces eubayanus, Lachancea thermotolerans, Torulaspora delbrueckii, Pichia kudriavzevii, and Schizosaccharomyces pombe have been reported to positively influence the aroma profile in different winemaking scenarios (Kai Chen et al., 2018; Kai Chen et al., 2021; Zhang et al., 2022). Although this is the first study to show a positive correlation between Aspergillus spp., Eremothecium spp., and Thermothelomyces spp. on the development of varietal aroma, further verification needs to be carried out to evaluate the safety and effects of fermentation. Interestingly, the evolution of terpinen-4-ol was highly correlated with almost all microeukaryotes. α -Terpineol and its isomer terpinen-4-ol are the most common terpenes found in natural plants, and terpinen-4-ol has been shown to have effective antifungal activity, which can significantly reduce the fungal biofilm mass (de Fátima Souto Maior et al., 2019).

3.5. Enrichment analysis and biomarkers selection of OTUs of epiphytic microbes, proteins and genotypes in different harvest compartments

Commented [j7]: Really? What about limonene, pinene, etc.?

Because the bacterial and microeukaryotic assemblages on grape surfaces result in combined interactions, current analytical methods are limited to defining specific functions according to their classification. We performed analyses using combined samples from all genotypes across all harvest batches. Metagenomic data of genotypes and relatively expressed proteins were analysed in three compartments (Figure 5). Spatial dispersal of relatively expressed proteins was mainly enriched during the beginning of late harvest, whereas protein enrichment at the end of late harvest was lower and relatively expressed proteins in grape maturation had the lowest enrichment (Figure 5, A). The results indicated that there were two stages of protein expression during the late harvest. We speculated that the relative proteins, namely, enzymes, were widely expressed in the dark green area (the end of October until the beginning of November, late harvest 1), and a similar trend was observed in the dark purple area (the beginning of December, late harvest 2). However, epiphytic OTUs were enriched at the fully ripened stage (orange area) and then decreased in the two late harvest compartments (Figure S6, C). However, the majority of genotypes from epiphytic microbes were enriched in the dark green area, wherein the freeze-thaw cycles caused microfissures in grape skin, high release of free terpenes, α -diversity decrease in microeukaryotes, and growth inhibition of B. cinerea (Figure 5, B).

To compare the differences in epiphytic microbes between harvest and late harvest, volcano plots were constructed for the selection of differentially expressed and CAZy

involved genes. The top twenty characteristic microbial species, eighteen species of bacteria, and two species of yeast were identified (Figure 5, C). Some of these compounds have antifungal activity and have been used as biological control agents. For instance, a highly developed species, Pantoea vagans, is effective in controlling postharvest fungal Penicillium expansum (Bi et al., 2021), whereas Pseudomonas is known for its antifungal properties, such as P. extremorientalis against B. cinerea (Liu et al., 2021). P. oryzihabitans exhibits chemotaxis toward Fusarium and Rhizoctonia mycelia. Moreover, a fermentable yeast, Kluyveromyces lactis, is well known for the production of renin and β -galactosidase in cheese processing (Hollá, Karkeszová, Antošová, & Polakovič, 2021). Comparison of relative proteins suggested that multispecies interactions of epiphytes were severe during late harvest (Figure 5, D). It is understandable that grape skin was clearly damaged by freeze-thaw cycles and B. cinerea infection during late harvest, and the availability of high sugar concentrations and other nutrients on the berry surface favours an increase in polymicrobial communities. These microbial species rarely exist in isolation and interact with each other. Interplay is a major driver of cooperative behaviour, which is similar to animal societies (Khare, 2021). According to the KEGG results, pathways involved in metabolism integrated the majority of microbial activities; herein, the number of genes involved in carbohydrate metabolism was the highest among the five main classes of KEGG pathways (Figure S7). In this case, we analysed all genes and identified the top twenty significantly upregulated genes involved in microbial CAZy (Figure 5, E).

CAZy are a family of enzymes that work on carbohydrates for their biotransformation, that is, synthesis, metabolism, modification, and transport (André, Potocki-Véronèse, Barbe, Moulis, & Remaud-Siméon, 2014). The significantly upregulated CAZy genes participated in the synthesis of different types of glycosidases, such as FENNEDOB12352, OJNJMGIN252777, EGHMEMAM161340, EGHMEMAM158447, KKFIDJDF144524, BEDCFLNK31943, LHALBBHN88115, and BEDCFLNK41042, which were involved in the synthesis of β -galactosidase, β glucosidase, bacterial α -L-rhamnosidase, trehalase, oligogalacturonate lyase, glycoside hydrolase (GH), α -galactosidase, and cellulase family glycosylhydrolase, respectively.

3.6. Important CAZy pathways in epiphytic microbes and the correlation analysis for selection of high CAZy expressed yeasts assemblage

CAZy plays an important role in the diversity of carbohydrate-based products, which have been classified into several classes according to their catalytic activities. In this study, the classes included 21.3% glycoside hydrolases (GHs), 17.82% carbohydrate esterases (CEs), 0.62% polysaccharide lyases (PLs), 44.98% glycosyltransferases (GTs), 0.36% carbohydrate-binding modules (CBMs), and 14.92% auxiliary activities (AA), respectively. The GHs and GT families were the principal enzymes that dominated 66.28% of CAZy in total and were important to the development of free varietal aroma, as most terpenes in grapes are present in glycosidically bound forms. The results indicated that these odourless glycosidic bound terpenes and phenylalanine derivatives were greatly hydrolysed into free forms, which could be the main reason for the significant differences in varietal aroma between grape samples during maturation and late harvest (Kai Chen et al., 2019). Therefore, we extracted the top twenty GO differential pathways, and the enrichment scores of GTs and GHs were calculated (Figure 6, B).

In terms of harvest and late harvest 1 (beginning of late harvest), the differential pathways of GTs and GH metabolism were mainly involved in maltose α -glucosidase activity, maltose metabolic process, mannose metabolic process, cellobiose glucosidase process, sucrose metabolic process, and maltose catabolic process. In addition, major stress-resistant pathways were selected as cellular responses to glucose starvation, trehalose transport, maltose transport, trehalose transmembrane transport activity, and invasive growth in response to glucose limitation. Furthermore, enrichment of triosephosphate transmembrane transporter activity indicated active microeukaryote metabolism, as yeasts have been found in some functionally uncharacterised triose phosphate translocator (TPT) paralogs that are homologues in plants (Saier, 1999). Comparing harvest to late harvest 2 (end of late harvest), the important pathways related to glycosidically bound aroma release included maltose α -glucosidase activity, raffinose α -galactosidase activity as well as the cellulose synthase UDP-glucose metabolic process. In particular, glycosidically bound terpenes and phenylalanine derivatives are potential precursors of the VOCs with sensorial properties of flowers and fruits, whereas UDP-GTs can effectively catalyse plant-originated glycosides and mediate the transfer of an activated nucleotide sugar to acceptor aglycones (Yauk et al., 2014). During late harvest 2, UDP activity and UDP-glucose metabolic process were also identified as important factors for hydrolysis of glycosidically-bound VOCs.

Yeasts have been widely used in the food industry, especially in wine production. The co-effects in yeast assemblages characterised by effective expression of different glucosidases will greatly enhance the complexity of varietal aroma in vinification of icewine. To select potential yeasts with high expression of GTs and GHs, a random forest model was used to establish the correlation matrix for the top 25 highly expressed GT/GH-related genes and the top 25 active yeast strains, and the expressed gene abundance was monitored from grape maturation to late harvest. Accordingly, *Zygosaccharomyces rouxii, Lachancea thermotolerans, Saccharomyces eubayanus, Eremothecium cymbalariae, Cercospora beticola, Zymoseptoria tritici, Kazachstania naganishii, Kluyveromyces lactis, Yarrowia lipolytica, and Pichia kudriavzevii were selected as important factors in the yeast assemblage. In particular, <i>Zymoseptoria tritici, Kluyveromyces lactis, Yarrowia lipolytica,* and *Pichia kudriavzevii* have strong correlations with highly expressed GT/GHs-related genes. However, the specific gene functions were mainly involved in the synthesis of β -galactosidase (FENNEDOB12352,

HINDIEJE218024), β -glucosidase (OJNJMGIN252777), α-L-rhamnosidase (EGHMEMAM161340, OJNJMGIN71722), GH (BEDCFLNK31943, KKFIDJDF107323, LHALBBHN213309, LHALBBHN168497), α-galactosidase (LHALBBHN88115), and GT (BEDCFLNK47353, BEDCFLNK70396, KEHBHALF35750). Nevertheless, the expression of GT/GH-related genes increased on the 110th day (red line) and reached the highest level around the 125th day after bloom.

4. Conclusion

In this study, we observed that freeze-thaw cycles caused damage to the wax layer and microfissures on the cuticle of visually intact berries. This increased nutrient availability and supported the dominance of oxidative and fermentative ascomycetous populations. Quantification of the effect of ecological stochasticity on self-assembled microeukaryotic communities elucidated the mechanism of stochastic processes in shaping microeukaryotic communities for the grape maturation and late harvest periods.

In addition, the evolution of characteristic VOCs in *Vidal blanc* was studied. Most of the free terpenes, monoterpenes, and C13-norisoprenoids were highly developed during late harvest and differed from the aroma profile of grape ripeness. In this study, β -cymene, 2-phenyl-1-ethanol, hotrienol, 2-phenylethanal, geraniol, nerol oxide, *cis*-rose oxide, and terpinene-4-ol were identified as the characteristic VOCs of *Vidal blanc*. By

comparing free and glycosidically-bound terpenes and phenylalanine derivatives, considerable concentration loss in some important monoterpenes was mainly due to enzymatic hydrolysis or bioconversion by microbes.

Related enzymes were widely expressed at the end of October until November. Based on the KEGG and GO results, pathways involved in carbohydrate metabolism were the highest among the five main classes. By calculating the importance of CAZy genes and potential yeast strains, *Zymoseptoria tritici, Kluyveromyces lactis, Yarrowia lipolytica,* and *Pichia kudriavzevii* were identified as key factors in the yeast assemblage. Accordingly, further studies will be performed to verify the design of artificial yeast assemblages on terpenes and phenylalanine-derivative evolutions in alcoholic fermentation of icewine.

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Declaration of Competing Interest

Author acclaimed there was no conflict of interests in this article.

References

- André, I., Potocki-Véronèse, G., Barbe, S., Moulis, C., & Remaud-Siméon, M. (2014). CAZyme discovery and design for sweet dreams. *Current Opinion in Chemical Biology*, 19, 17-24. https://doi.org/https://doi.org/10.1016/j.cbpa.2013.11.014.
- Barata, A., Malfeito-Ferreira, M., & Loureiro, V. (2012). The microbial ecology of wine grape berries. *International Journal of Food Microbiology*, 153(3), 243-259. https://doi.org/https://doi.org/10.1016/j.ijfoodmicro.2011.11.025.
- Benito, S., Palomero, F., Calderón, F., Palmero, D., & Suárez-Lepe, J. A. (2014). Selection of appropriate *Schizosaccharomyces* strains for winemaking. *Food Microbiology*, 42, 218-224. https://doi.org/https://doi.org/10.1016/j.fm.2014.03.014.
- Bhat, N. R., Desai, B. B., & Suleiman, M. K. (2010). Flavor in Grapes: Its Characterization and Commercial Applications. In *Handbook of Fruit and Vegetable Flavors* (pp. 279-302).
- Bi, W., Wang, R., Yang, Y., Wang, Y., Ma, Z., Wang, Q., & Zhang, D. (2021). Pantoea vagans strain BWL1 controls blue mold in mandarin fruit by inhibiting ergosterol biosynthesis in *Penicillium expansum*. *Biological Control*, 161, 104639. https://doi.org/https://doi.org/10.1016/j.biocontrol.2021.104639.
- Bokulich, N. A., Thorngate, J. H., Richardson, P. M., & Mills, D. A. (2014). Microbial biogeography of wine grapes is conditioned by cultivar, vintage, and climate. *Proceedings of the National Academy of Sciences*, 111(1), E139-E148. https://doi.org/10.1073/pnas.1317377110.
- Bowen, A. J., & Reynolds, A. G. (2015). Aroma compounds in Ontario Vidal and Riesling icewines. I. Effects of harvest date. Food Research International, 76, 540-549. https://doi.org/https://doi.org/10.1016/j.foodres.2015.06.046.
- Bowen, A. J., Reynolds, A. G., & Lesschaeve, I. (2016). Harvest date and crop level influence sensory and chemical profiles of Ontario Vidal blanc and Riesling icewines. Food Research International, 89, 591-603. https://doi.org/https://doi.org/10.1016/j.foodres.2016.09.005.
- Cabrera, E., Welch, L. C., Robinson, M. R., Sturgeon, C. M., Crow, M. M., & Segarra, V. A. (2020). Cryopreservation and the Freeze–Thaw Stress Response in Yeast. *Genes*, 11(8), 835. https://www.mdpi.com/2073-4425/11/8/835.
- Cabrita, M. J., Freitas, A. M. C., Laureano, O., & Stefano, R. D. (2006). Glycosidic aroma compounds of some Portuguese grape cultivars. *Journal of the Science of Food and Agriculture*, 86(6), 922-931. https://doi.org/https://doi.org/10.1002/jsfa.2439.
- Che, Y., Xia, Y., Liu, L., Li, A.-D., Yang, Y., & Zhang, T. (2019). Mobile antibiotic resistome in wastewater treatment plants revealed by Nanopore metagenomic sequencing. *Microbiome*, 7(1), 44. https://doi.org/10.1186/s40168-019-0663-0.
- Chen, K., Escott, C., Loira, I., del Fresno, J. M., Morata, A., Tesfaye, W., ... Benito, S. (2018).

Use of non-*Saccharomyces* yeasts and oenological tannin in red winemaking: Influence on colour, aroma and sensorial properties of young wines. *Food Microbiology*, 69, 51-63. https://doi.org/https://doi.org/10.1016/j.fm.2017.07.018.

- Chen, K., Han, S.-Y., Li, M., & Sheng, W.-J. (2017). Use of Lysozyme and Oligomeric Proanthocyanidin to Reduce Sulfur Dioxide and the Evolution of Volatile Compounds in *Italian Riesling* Ice Wine During Aging Process. *Journal of Food Processing and Preservation*, 41(1), e12755. https://doi.org/https://doi.org/10.1111/jfpp.12755.
- Chen, K., & Li, J. (2022). Chapter 24 A glance into the aroma of white wine. In A. Morata (Ed.), White Wine Technology (pp. 313-326): Academic Press.
- Chen, K., Liu, C., Wang, Y., Wang, Z., Li, F., Ma, L., & Li, J. (2021). Predominance of indigenous non-Saccharomyces yeasts in the traditional fermentation of greengage wine and their significant contribution to the evolution of terpenes and ethyl esters. Food Research International, 143, 110253. https://doi.org/https://doi.org/10.1016/j.foodres.2021.110253.
- Chen, K., Wen, J., Ma, L., Wen, H., & Li, J. (2019). Dynamic changes in norisoprenoids and phenylalanine-derived volatiles in off-vine *Vidal blanc* grape during late harvest. *Food Chemistry*, 289, 645-656. https://doi.org/https://doi.org/10.1016/j.foodchem.2019.03.101.
- Chen, Y., Zhang, W., Yi, H., Wang, B., Xiao, J., Zhou, X., . . . Shi, X. (2020). Microbial community composition and its role in volatile compound formation during the spontaneous fermentation of ice wine made from *Vidal* grapes. *Process Biochemistry*, 92, 365-377. https://doi.org/https://doi.org/10.1016/j.procbio.2020.01.027.
- de Fátima Souto Maior, L., Maciel, P. P., Ferreira, V. Y. N., de Lima Gouveia Dantas, C., de Lima, J. M., Castellano, L. R. C., . . Bonan, P. R. F. (2019). Antifungal activity and Shore A hardness of a tissue conditioner incorporated with terpinen-4-ol and cinnamaldehyde. *Clinical Oral Investigations*, 23(7), 2837-2848. https://doi.org/10.1007/s00784-019-02925-w.
- Demyttenaere, J. C. R., Vanoverschelde, J., & De Kimpe, N. (2004). Biotransformation of (R)-(+)- and (S)-(-)-citronellol by *Aspergillus sp.* and *Penicillium sp.*, and the use of solidphase microextraction for screening. *Journal of Chromatography A*, 1027(1), 137-146. https://doi.org/https://doi.org/10.1016/j.chroma.2003.08.090.
- Fischer, U. (2007). Wine Aroma. In R. G. Berger (Ed.), Flavours and Fragrances: Chemistry, Bioprocessing and Sustainability (pp. 241-267). Berlin, Heidelberg: Springer Berlin Heidelberg.
- Gamero, A., Manzanares, P., Querol, A., & Belloch, C. (2011). Monoterpene alcohols release and bioconversion by Saccharomyces species and hybrids. International Journal of Food Microbiology, 145(1), 92-97.

https://doi.org/https://doi.org/10.1016/j.ijfoodmicro.2010.11.034.

- Gil, M., Pontin, M., Berli, F., Bottini, R., & Piccoli, P. (2012). Metabolism of terpenes in the response of grape (*Vitis vinifera L.*) leaf tissues to UV-B radiation. *Phytochemistry*, 77, 89-98. https://doi.org/https://doi.org/10.1016/j.phytochem.2011.12.011.
- Gurevich, A., Saveliev, V., Vyahhi, N., & Tesler, G. (2013). QUAST: quality assessment tool

for genome assemblies. *Bioinformatics*, 29(8), 1072-1075. https://doi.org/10.1093/bioinformatics/btt086.

- Han, T. L., Tumanov, S., Cannon, R. D., & Villas-Boas, S. G. (2013). Metabolic Response of *Candida albicans* to 2-phenylethanol under Hyphae-Inducing Conditions. *PloS One*, 8(8). https://doi.org/10.1371/journal.pone.0071364.
- Hollá, V., Karkeszová, K., Antošová, M., & Polakovič, M. (2021). Transglycosylation properties of a *Khuyveromyces lactis* enzyme preparation: Production of tyrosol βfructoside using free and immobilized enzyme. *Process Biochemistry*, 110, 168-175. https://doi.org/https://doi.org/10.1016/j.procbio.2021.08.016.
- Khare, A. (2021). Experimental systems biology approaches reveal interaction mechanisms in model multispecies communities. *Trends in Microbiology*. https://doi.org/https://doi.org/10.1016/j.tim.2021.03.012.
- Lan, Y. B., Qian, X., Yang, Z. J., Xiang, X. F., Yang, W. X., Liu, T., . . . Duan, C. Q. (2016). Striking changes in volatile profiles at sub-zero temperatures during over-ripening of 'Beibinghong' grapes in Northeastern China. Food Chemistry, 212, 172-182. https://doi.org/10.1016/j.foodchem.2016.05.143.
- Lashbrooke, J. G., Young, P. R., Dockrall, S. J., Vasanth, K., & Vivier, M. A. (2013). Functional characterisation of three members of the *Vitis vinifera L*. carotenoid cleavage dioxygenase gene family. *BMC Plant Biology*, 13(1), 156. https://doi.org/10.1186/1471-2229-13-156.
- Li, D., Liu, C.-M., Luo, R., Sadakane, K., & Lam, T.-W. (2015). MEGAHIT: an ultra-fast single-node solution for large and complex metagenomics assembly via succinct de Bruijn graph. *Bioinformatics*, 31(10), 1674-1676. https://doi.org/10.1093/bioinformatics/btv033.
- Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., . . . Subgroup, G. P. D. P. (2009). The Sequence Alignment/Map format and SAMtools. *Bioinformatics*, 25(16), 2078-2079. https://doi.org/10.1093/bioinformatics/btp352.
- Li, R., Lin, M., Guo, S., Yang, S., Han, X., Ren, M., . . . Huang, W. (2021). A fundamental landscape of fungal biogeographical patterns across the main Chinese wine-producing regions and the dominating shaping factors. *Food Research International*, 150, 110736. https://doi.org/https://doi.org/10.1016/j.foodres.2021.110736.
- Liu, Y., Song, Z., Zeng, H., Lu, M., Zhu, W., Wang, X., . . . Zhang, Q. (2021). Pseudomonas eucalypticola sp. nov., a producer of antifungal agents isolated from Eucalyptus dunnii leaves. Scientific Reports, 11(1), 3006. https://doi.org/10.1038/s41598-021-82682-7.
- Lu, Y., Sun, F., Wang, W., Liu, Y., Wang, J., Sun, J., ... Gao, Z. (2020). Effects of spontaneous fermentation on the microorganisms diversity and volatile compounds during *Marselan*^{*} from grape to wine. *LWT*, *134*, 110193. https://doi.org/https://doi.org/10.1016/j.lwt.2020.110193.
- Luo, J., Brotchie, J., Pang, M., Marriott, P. J., Howell, K., & Zhang, P. (2019). Free terpene evolution during the berry maturation of five *Vitis vinifera L.* cultivars. *Food Chemistry*, 299, 125101. https://doi.org/https://doi.org/10.1016/j.foodchem.2019.125101.
- Negri, S., Lovato, A., Boscaini, F., Salvetti, E., Torriani, S., Commisso, M., . . . Guzzo, F.

(2017). The Induction of Noble Rot (*Botrytis cinerea*) Infection during Postharvest Withering Changes the Metabolome of Grapevine Berries (*Vitis vinifera L., cv. Garganega*). *Frontiers in Plant Science, 8*(1002). https://doi.org/10.3389/fpls.2017.01002.

- Ning, D., Deng, Y., Tiedje, J. M., & Zhou, J. (2019). A general framework for quantitatively assessing ecological stochasticity. *Proceedings of the National Academy of Sciences*, *116*(34), 16892-16898. https://doi.org/10.1073/pnas.1904623116.
- Park, J. I., Grant, C. M., Attfield, P. V., & Dawes, I. W. (1997). The freeze-thaw stress response of the yeast *Saccharomyces cerevisiae* is growth phase specific and is controlled by nutritional state via the RAS-cyclic AMP signal transduction pathway. *Applied and Environment Microbiology*, 63(10), 3818-3824. https://doi.org/10.1128/aem.63.10.3818-3824.1997.
- Patro, R., Duggal, G., Love, M. I., Irizarry, R. A., & Kingsford, C. (2017). Salmon provides fast and bias-aware quantification of transcript expression. *Nature Methods*, 14(4), 417-419. https://doi.org/10.1038/nmeth.4197.
- Saier, M. H. (1999). Eukaryotic Transmembrane Solute Transport Systems. In K. W. Jeon (Ed.), International Review of Cytology (pp. 61-136): Academic Press.
- Shi, X., Chen, Y., Xiao, J., Li, D., & Wang, B. (2020). Effects of harvest dates on microbial communities of ice grape skins from Xinjiang of China. *Process Biochemistry*, 98, 202-210. https://doi.org/https://doi.org/10.1016/j.procbio.2020.08.002.
- Silva Ferreira, A. C., Monteiro, J., Oliveira, C., & Guedes de Pinho, P. (2008). Study of major aromatic compounds in port wines from carotenoid degradation. *Food Chemistry*, 110(1), 83-87. https://doi.org/10.1016/j.foodchem.2008.01.069.
- Sloan, W. T., Lunn, M., Woodcock, S., Head, I. M., Nee, S., & Curtis, T. P. (2006). Quantifying the roles of immigration and chance in shaping prokaryote community structure. *Environmental Microbiology*, 8(4), 732-740. https://doi.org/https://doi.org/10.1111/j.1462-2920.2005.00956.x.
- Yauk, Y.-K., Ged, C., Wang, M. Y., Matich, A. J., Tessarotto, L., Cooney, J. M., . . . Atkinson, R. G. (2014). Manipulation of flavour and aroma compound sequestration and release using a glycosyltransferase with specificity for terpene alcohols. *The Plant Journal*, 80(2), 317-330. https://doi.org/https://doi.org/10.1111/tpj.12634.
- Yuan, F., & Qian, M. C. (2016). Development of C13-norisoprenoids, carotenoids and other volatile compounds in *Vitis vinifera L. Cv. Pinot noir* grapes. *Food Chemistry*, 192, 633-641. https://doi.org/10.1016/j.foodchem.2015.07.050.
- Zhang, B., Tang, C., Yang, D., Liu, H., Xue, J., Duan, C., & Yan, G. (2022). Effects of three indigenous non-Saccharomyces yeasts and their pairwise combinations in cofermentation with Saccharomyces cerevisiae on volatile compounds of Petit Manseng wines. Food Chemistry, 368, 130807. https://doi.org/https://doi.org/10.1016/j.foodchem.2021.130807.

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- Kai Chen: Conceptualization, Methodology, Formal analysis, Visualization, Writing - Original Draft, Writing - Review & Editing, Data Curation, Software;
- 2. Lei Zhang: Review;
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- 4. Xugao Wu: Resources;
- 5. Jingming Li: Validation, Review, Supervision, Investigation;
- 6. Liyan Ma*: Funding acquisition, Project administration, Supervision.

Declaration of interests

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

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Figure 1. Evolution of free terpenes and phenylalanine-derivates of *Vidal blanc* grape during late harvest. (A) Heatmap cluster of free terpenes and phenylalanine-derivates in the different months of late harvest, herein, methodology of distance measure was selected as Euclidean, clustering algorithm was Ward. (B) Score plot of PLS-DA for analysis of free terpenes and phenylalanine-derivates by observation of sampling points. (C) Ranking of characteristic terpenes and phenylalanine-derivates by weighting sum of absolute regression coefficients in PLS-DA. The colored boxes on the right indicate the correlations between free terpenes and phenylalanine-derivates and different months during late harvest.

Figure 2. Dynamic changes of free and glycosidic bound free terpenes and phenylalanine-derivates during late harvest. (A) Concentration changes of characteristic monoterpenes in free and glycosidic bound forms. (B) Concentration changes of characteristic C13-norisoprenoids in free and glycosidic bound forms. (C) Concentration changes of characteristic phenylalanine-derivates in free and glycosidic bound forms.

Figure 3. Fit of the neutral community model of community assembly. The predicted occurrence frequencies for the periods of (A) entire season, (B) late harvest, and (C) grape maturation representing microeukaryotic communities from entire season, late harvest, and maturation, respectively. The solid blue lines indicate the best fit to the NCM, and the dashed blue lines represent 95% confidence intervals around the model prediction. OTUs occurred more or less frequently than prediction by the NCM are shown in different colors (blue dots represent above prediction, and red dots represent below prediction). Nm indicates the metacommunity size times immigration, R² indicates the fit to this model. Meanwhile, modified stochasticity ratio was used to assess the relative importance of stochasticity (D), which was developed with 50% as the boundary line. (E) coefficients between climatic factors and OTUs of microeukaryote species were calculated to evaluate the correlative fitting curves between climatic factors and self-assembled microeukaryote communities.

Figure 4. (A) Chao1, Simpson and Shannon, Pielou indices jointly evaluated the α diversity of self-assembled microeukaryote communities. (B) The most abundant top 20 microeukaryotes were listed. The correlation levels between OTU abundance of microeukaryotes and harvest stages were quantified and showed with cricoid strips. (C) Correlations between the top 40 abundant microeukaryote species and the determined free terpenes and phenylalanine-derivates were studied via the calculation of Spearman coefficients, the significant differences were labeled with $p \le 0.05$ and $p \le 0.01$,

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respectively.

Figure 5. (A) Spatial dispersal of relatively expressed proteins (based on NCBI Reference Sequence Database) during late harvest. (B) Spatial dispersal of the genotypes from epiphytic microbes during late harvest. (C) Volcano plot with padj < 0.01 and log2FoldChange ≥ 2 use to select the characteristic microbial species during late harvest. (D) Volcano plot with padj < 0.01 and log2FoldChange ≥ 2 use to select the upregulated relative proteins (based on NCBI Reference Sequence Database) during late harvest. (E) Volcano plot with padj < 0.01 and log2FoldChange ≥ 2 use to select the upregulated relative proteins (based on NCBI Reference Sequence Database) during late harvest. (E) Volcano plot with padj < 0.01 and log2FoldChange ≥ 2 use to select the upregulated CAZy related genes in microbial species during late harvest.

Figure 6. (A) Pie plot showed the percentage of CAZy classifications. (B) Top 20 differential pathways of gene ontology and the enrichment scores of GTs and GHs were ranked. (C) Correlation matrix for top 25 high expressed GTs / GHs related genes and top 25 active yeast strains was established using random forest, significances of coefficients ($p \le 0.05$) was shown with blue color intensity. Meanwhile, dynamic changes of the expressed genes abundances were represented as log10 (gene count) from grape maturation to late harvest.











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Highlights

- Varietal aroma of *Vidal blanc* in late harvest was different from that in maturation.
- Characteristic VOCs and the diversity of microeukaryotes were correlated.
- Quantification of stochastic process on shaping microeukaryotic communities.
- Important CAZy pathways of epiphytic microbes were identified.
- Correlation matrix for high expressed GTs / GHs and active yeasts was established.