

Article

Alterations in Yeast Species Composition of Uninoculated Wine Ferments by the Addition of Sulphur Dioxide

Kathleen Cuijvers ^{1,†}, Steven Van Den Heuvel ^{1,†}, Cristian Varela ^{1,2}, Mark Rullo ¹, Mark Solomon ¹ , Simon Schmidt ¹  and Anthony Borneman ^{1,3,*} 

¹ The Australian Wine Research Institute, PO Box 197, Glen Osmond, SA 5064, Australia; kate.cuijvers@awri.com.au (K.C.); steven.vandenheuvel@awri.com.au (S.V.D.H.); cristian.varela@awri.com.au (C.V.); mark.rullo@adelaide.edu.au (M.R.); mark.solomon@awri.com.au (M.S.); simon.schmidt@awri.com.au (S.S.)

² School of Agriculture, Food & Wine, Faculty of Sciences, University of Adelaide, Adelaide, SA 5005, Australia

³ School of Biological Sciences, University of Adelaide, Adelaide, SA 5005, Australia

* Correspondence: anthony.borneman@awri.com.au; Tel.: +618-8313-6600; Fax: +618-8313-6601

† Contributed equally.

Received: 3 June 2020; Accepted: 19 June 2020; Published: 23 June 2020



Abstract: Uninoculated wine fermentations are conducted by a consortium of wine yeast and bacteria that establish themselves either from the grape surface or from the winery environment. Of the additives that are commonly used by winemakers, sulphur dioxide (SO₂) represents the main antimicrobial preservative and its use can have drastic effects on the microbial composition of the fermentation. To investigate the effect of SO₂ on the resident yeast community of uninoculated ferments, Chardonnay grape juice from 2018 and 2019 was treated with a variety of SO₂ concentrations ranging up to 100 mg/L and was then allowed to undergo fermentation, with the yeast community structure being assessed via high-throughput meta-barcoding (phylotyping). While the addition of SO₂ was shown to select against the presence of many species of non-*Saccharomyces* yeasts, there was a clear and increasing selection for the species *Hanseniaspora osmophila* as concentrations of SO₂ rose above 40 mg/L in fermentations from both vintages. Chemical analysis of the wines resulting from these treatments showed significant increases in acetate esters, and specifically the desirable aroma compound 2-phenylethyl acetate, that accompanied the increase in abundance of *H. osmophila*. The ability to modulate the yeast community structure of an uninoculated ferment and the resulting chemical composition of the final wine, as demonstrated in this study, represents an important tool for winemakers to begin to be able to influence the organoleptic profile of uninoculated wines.

Keywords: wine; uninoculated fermentation; yeast; sulphur dioxide

1. Introduction

Wine is a complex beverage, produced through the interplay of grape and microbial metabolomes during the process of fermentation. While the majority of modern commercial wine fermentation is performed using inoculated commercial strains of the major wine yeast *Saccharomyces cerevisiae*, a significant proportion of commercial wine fermentations are now being performed using uninoculated grape must. In these situations, the fermentation is conducted by a consortium of wine yeast and bacteria that establish themselves either from the grape surface or from the winery via shared equipment or other vectors such as insects [1].

In the very early stages of fermentation, apiculate yeasts, and yeast-like fungi which reside on the surface of intact grape berries or winery equipment and include the genera *Aureobasidium*, *Rhodotorula*,

Pichia, *Candida*, *Hanseniaspora* and *Metschnikowia*, represent the majority of the microbiota [2]. However, the majority of these yeasts succumb very early after fermentation commences. Mildly fermentative yeasts, such as *Hanseniaspora uvarum*, *Candida stellata*, *Metschnikowia pulcherrima*, *Torulaspora delbrueckii* and *Lachancea thermotolerans* have been shown to proliferate and survive well into the fermentation, but reduce in numbers as ethanol levels increase above 6% [3–6].

Vineyard geography, environment and management practices, and harvest, juice/must processing and fermentation conditions can all affect yeast population dynamics during wine fermentation [7–13]. Of those fermentation conditions that are readily modulated by winemakers, the addition of the antimicrobial sulphur dioxide (SO₂) represents the most broadly available intervention practice. Previous microbiological studies have shown that species (and strains) of the major wine yeasts can respond differently to the application of SO₂. Typically, commercial strains of *S. cerevisiae* display high tolerance to SO₂, while “wild” yeasts display lower tolerances and are therefore thought to be broadly selected against through the application of moderate amounts of SO₂ prior to the start of fermentation [14–16].

In order to explore the effect of SO₂ addition on the yeast microbiota during uninoculated Chardonnay wine, meta-barcoding (phylotyping) analysis was used to assess the population dynamics of wine produced across two successive vintages using a range of pre-ferment SO₂ levels.

2. Results

In order to evaluate the effect of SO₂ addition on the yeast population structure, triplicate uninoculated fermentations were established in Chardonnay grape juice across two consecutive vintages. The effect of these different SO₂ concentrations on wine volatile composition was also evaluated.

2.1. SO₂ Addition Affects Yeast Population Structure

In vintage 2018, the grape juice was treated with one of five different concentrations of total SO₂ (0, 40, 60, 80 and 100 mg/L). In addition to its antimicrobial effect, SO₂ is also a powerful antioxidant [17]. To differentiate between the antimicrobial and antioxidant effects of the SO₂ addition, an alternate antioxidant, glutathione (GSH, 250 mg/L), was also assessed for its effects on the yeast community structure.

The progress of each ferment was tracked via sugar consumption (Figure S1), with samples taken immediately after SO₂ or GSH addition (T1), at 90% of sugar remaining (T2), 50% sugar remaining (T3) and 10% sugar remaining (T4), for meta-barcoding analysis using the fungal Internal Transcribed Spacer (ITS) region [18,19]. The addition of GSH did not affect the duration of fermentation, however, SO₂ had a significant impact on the length of time required for the fermentation to reach completion, with two of the 100 mg/mL treatments requiring five to seven days longer than the control ferments (26 day fermentation) and one of the 100 mg/mL treatments becoming stuck with 13 g/L of residual sugar.

Across the 18 samples from 2018 (6 treatments in triplicate), Operational Taxonomic Units (OTUs) that could be assigned to a total of 26 fungal genera were detected that exceeded 0.01% of the total abundance in at least one sample (Figure 1; full results in Table S1). Triplicate samples were shown to be highly concordant for each combination of SO₂ concentration and timepoint (Figure S2). The highest level of fungal diversity was observed at the T1 timepoint, while *Hanseniaspora*, *Metschnikowia*, *Saccharomyces* and *Torulaspora* dominated the fermentations from T2 through T4, accounting over 95% of the total ITS reads (Figure 1).

As seen for the fermentation kinetics, the GSH addition did not affect the overall population structure relative to the control samples, however, the addition of SO₂ had a significant, but differential effect on the four main genera observed across the samples (Figure 1). *Metschnikowia* displayed the highest sensitivity to SO₂, with 40 mg/L completely inhibiting the detection of this genus by the T2 timepoint. *Torulaspora* was shown to have a higher abundance at 40 mg/L relative to 0 mg/L, however, this genus was progressively inhibited by higher concentrations of SO₂ in a gradient from 60 through to 100 mg/L, at which point it was completely inhibited at timepoint T2. *Hanseniaspora* and *Saccharomyces*

were both shown to be tolerant across all the tested SO₂ concentrations, with *Hanseniaspora* increasing in its total proportion relative to the other genera as the concentration of SO₂ was increased.

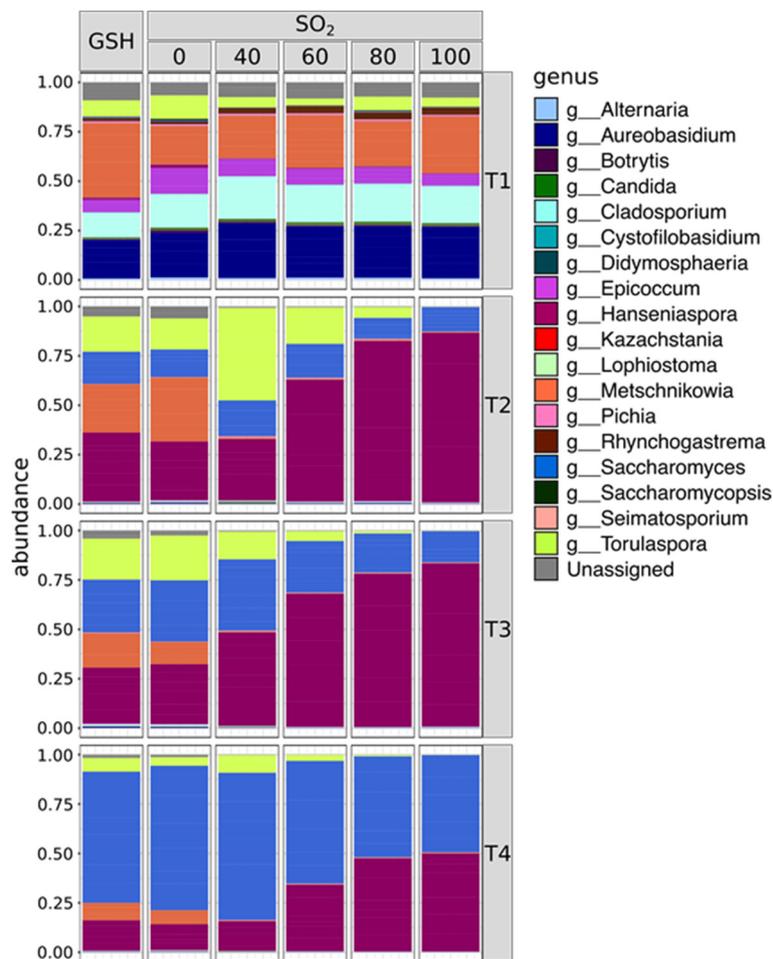


Figure 1. Genus-level metabarcoding analysis of community response to SO₂ addition. Vintage 2018 Chardonnay juice was treated with increasing concentrations of total SO₂ (mg/L) or glutathione (GSH, 250 mg/L) as an alternate antioxidant. Ferment samples were taken at four timepoints (T1, at crush; T2, 10% sugar utilization; T3, 50% sugar utilization; T4, 90% sugar utilization) and subjected to ITS metabarcoding. Only genera that exceeded 0.1% abundance in at least one sample are shown.

As fungal ITS sequencing generally affords the ability to define OTUs to the species level, the genus level counts were partitioned into species-level units to determine the effect of SO₂ concentration on the abundance of individual species. There were 29 species that exceeded 0.1% of the total abundance in any sample, with the genus *Hanseniaspora* displaying the highest number of individual species ($n = 4$). While the addition of SO₂ was shown to increase the overall abundance of *Hanseniaspora* at the genus level, there was a far more complex response profile when species designations were taken into account (Figure 2). Rather than a general increase in all species of *Hanseniaspora*, two species, *H. uvarum* and *H. opuntiae*, were the dominant species when SO₂ was absent (GSH and SO₂ 0 mg/L treatments). However, the addition of 40 mg/L of SO₂ resulted in a drastic shift in the species composition such that *H. osmophila* was the sole representative of this genus at 40 mg/L of SO₂. The relative abundance of this species increased substantially as SO₂ levels were raised, producing the overall increase in *Hanseniaspora* that was observed at the genus level.

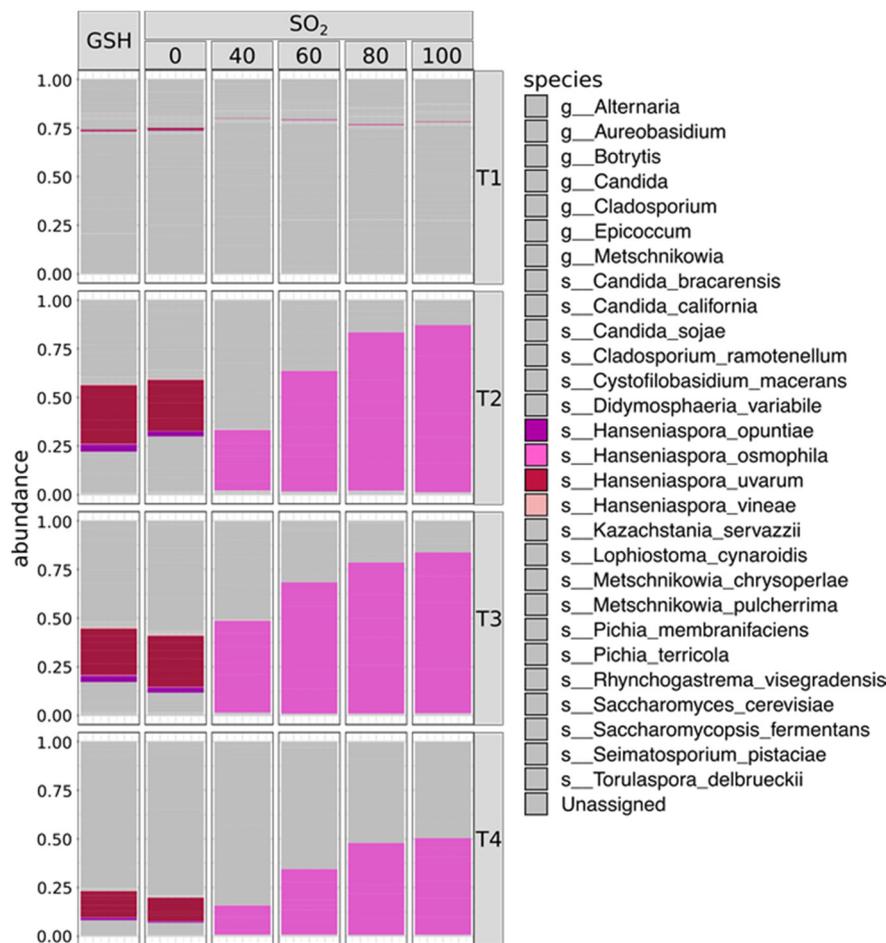


Figure 2. Species-level meta-barcoding analysis of community response to the SO₂ addition. Vintage 2018 Chardonnay juice was treated with increasing concentrations of total SO₂ (mg/L) or glutathione (GSH, 250 mg/L) as an alternate antioxidant. Ferment samples were taken at four timepoints (T1, at crush; T2, 10% sugar utilization; T3, 50% sugar utilization; T4, 90% sugar utilization) and subjected to ITS metabarcoding. Only species that exceeded 0.1% abundance in at least one sample are shown. For those OTU where a species-level designation was not possible, the genus-level taxonomic classification of the OTU was used. Only members of the genus *Hanseniaspora* are colored.

A second set of fermentations were established in the subsequent year (2019) using a finer set of SO₂ treatment intervals (0, 10, 20 and 40 mg/L). Consistent with the observations from the 2018 vintage, the SO₂ addition affected fermentation kinetics, particularly for the 40 mg/L treatments (Figure S1). The 2019 ferments displayed a different overall yeast diversity compared with the 2018 samples, with a lack of OTUs that could be assigned to *Metschnikowia* and prominent contributions from OTUs assigned to *Candida* spp., which increased over the range of SO₂ concentration used, and *Kazachstania* spp. that were present at up to 20 mg/L of SO₂ (Figure 3). However, when the species level contributions were investigated, there were clear similarities between the two vintages in the dynamics of the OTUs assigned to the genus *Hanseniaspora* (Figure 4). As also seen in 2018, *Hanseniaspora* was represented by the greatest number of species designations in 2019. The 2019 ferments also displayed a clear species shift that was associated with the use of SO₂, with the proliferation of *H. uvarum*, *H. opuntiae* and *H. vineae* all being inhibited by SO₂ in a concentration-dependent manner, while the proportion of *H. osmophila* was shown to be enhanced by the addition of 40 mg/L of SO₂.

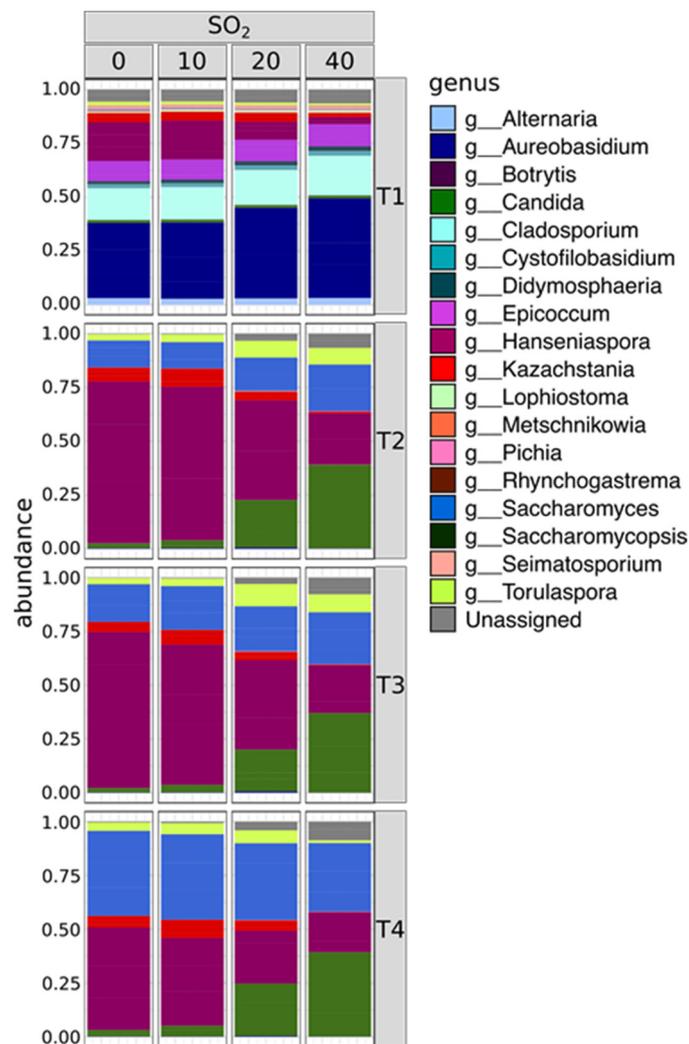


Figure 3. Genus-level meta-barcoding analysis of community response to the SO₂ addition. Vintage 2019 Chardonnay juice was treated with increasing concentrations of total SO₂ (mg/L). Ferment samples were taken at four timepoints (T1, at crush; T2, 10% sugar utilization; T3, 50% sugar utilization; T4, 90% sugar utilization) and subjected to ITS metabarcoding. Only genera that exceeded 0.1% abundance in at least one sample are shown.

In order to compare the 2018 and 2019 data, the metabarcoding time course results were analyzed by Bray–Curtis dissimilarity analysis (Figure 5). The T1 samples from across both vintages were broadly similar, with the ferments characterized by the presence of non-fermentative genera such as *Aureobasidium*, *Cladosporium* and *Epicoccum*. As observed in the abundance plots, ferments progressed towards being dominated by *S. cerevisiae* (Axis 1), however, there was a clear division between samples with 40 mg/L or more of added SO₂, which deviated along Axis 2 towards *H. osmophila*, while the samples with less than 40 mg/L were dominated by the signal from *H. uvarum*. Thus, despite differences in the overall microbial populations of the wild fermentations performed across the two vintages, both displayed consistent alterations in the microbial community due to the amount of SO₂ addition.

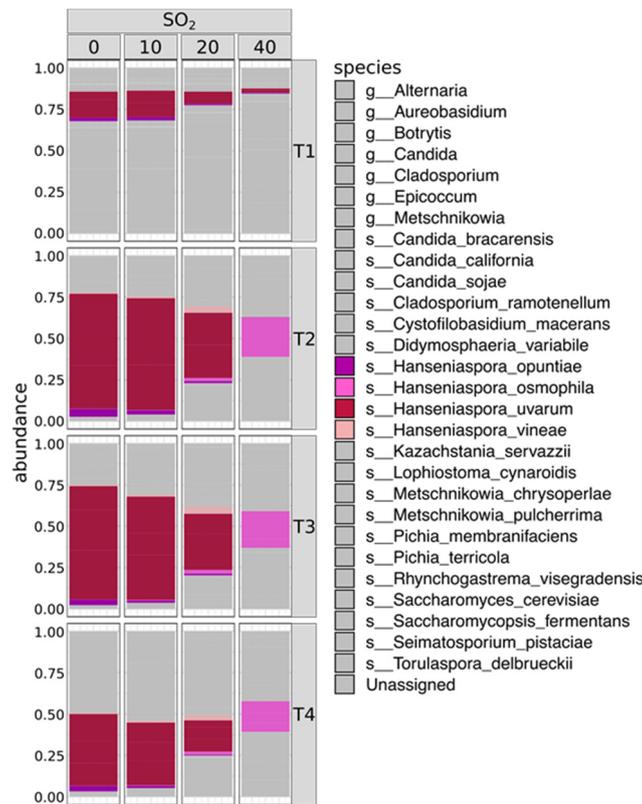


Figure 4. Species-level metabarcoding analysis of community response to the SO₂ addition. Vintage 2019 Chardonnay juice was treated with increasing concentrations of total SO₂ (mg/L). Ferment samples were taken at four timepoints (T1, at crush; T2, 10% sugar utilization; T3, 50% sugar utilization; T4, 90% sugar utilization) and subjected to ITS metabarcoding. Only species that exceeded 0.1% abundance in at least one sample are shown. For those OTU where a species-level designation was not possible, the genus-level taxonomic classification of the OTU was used. Only members of the genus *Hanseniaspora* are colored.

2.2. SO₂ Addition Influences Wine Volatile Composition

Given the significant effect of SO₂ addition on the microbial community structure, it was of interest to understand whether these different SO₂ treatments were also associated with changes to the chemical composition of the wine. This was assessed through an analysis of the volatile yeast metabolites known to contribute to the aromatic profile of wine. Of the 39 aroma compounds analyzed, 18 displayed a significant difference (ANOVA, $p \leq 0.001$) in concentration across one of the SO₂ regimes in either 2018 or 2019 (Table S2). Of these, ten analytes displayed more than a 1.5-fold decrease in at least one of the SO₂ treatments, while seven displayed an increase of the same magnitude (Figure 6). Three analytes, 2-methylpropanol (decreasing in response to SO₂) and 2-phenylethyl acetate and hexyl acetate (increasing), displayed the same effect across the 2018 and 2019 vintages. In all cases, there was a significant effect at 40 mg/L SO₂. Furthermore, in most situations in which a significant difference in analyte concentration was observed across multiple SO₂ regimes, there was a correlation between SO₂ concentration and the magnitude of change. The largest change in analyte concentration was observed for the desirable aroma compound 2-phenylethyl-acetate, with the 100 mg/L SO₂ treatment in 2018 displaying over two orders of magnitude more of this metabolite than the control, and the 2019 40 mg/L SO₂ treatment having over nine times as much 2-phenylethyl-acetate as the control (Table S2). More generally, higher SO₂ concentrations resulted in decreases in short chain acetates and higher alcohols and increases in 6-carbon and 8-carbon esters and acids. There was no effect of SO₂ concentration on low molecular weight volatile sulphur compound production.

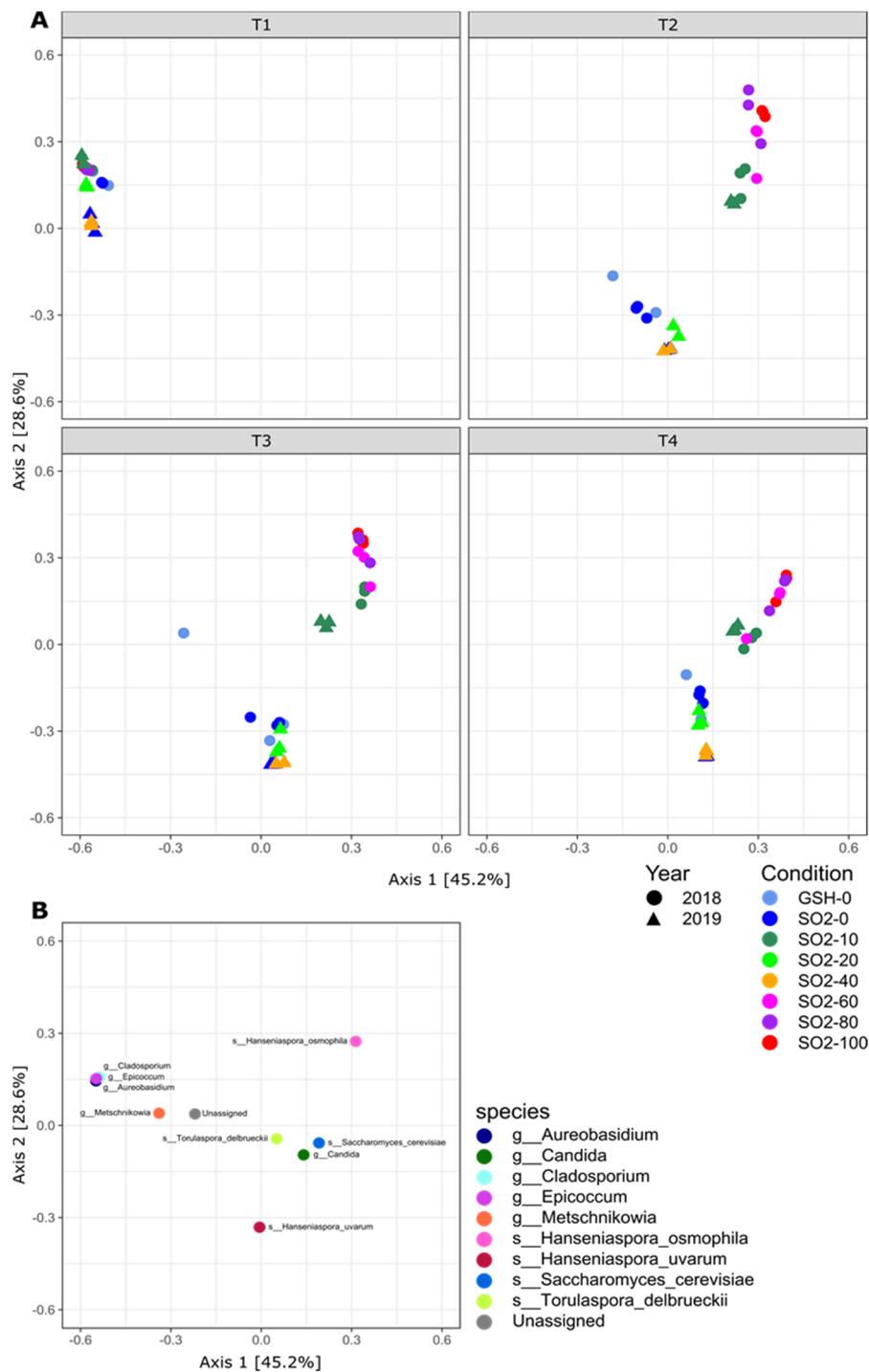


Figure 5. Dissimilarity analysis of ITS-amplicon abundance from vintage 2018 and 2019 fermentations. (A) Triplicate samples from each time point were subjected to Bray–Curtis dissimilarity analysis (clustered by PCoA) based upon the top 10 most abundant species and are shaded by treatment condition. (B) The weightings of the top 10 most abundant species relative to the plots in part (A). Points are shaded by species. For those OTU where a species-level designation was not possible, the genus-level taxonomic classification of the OTU was used.

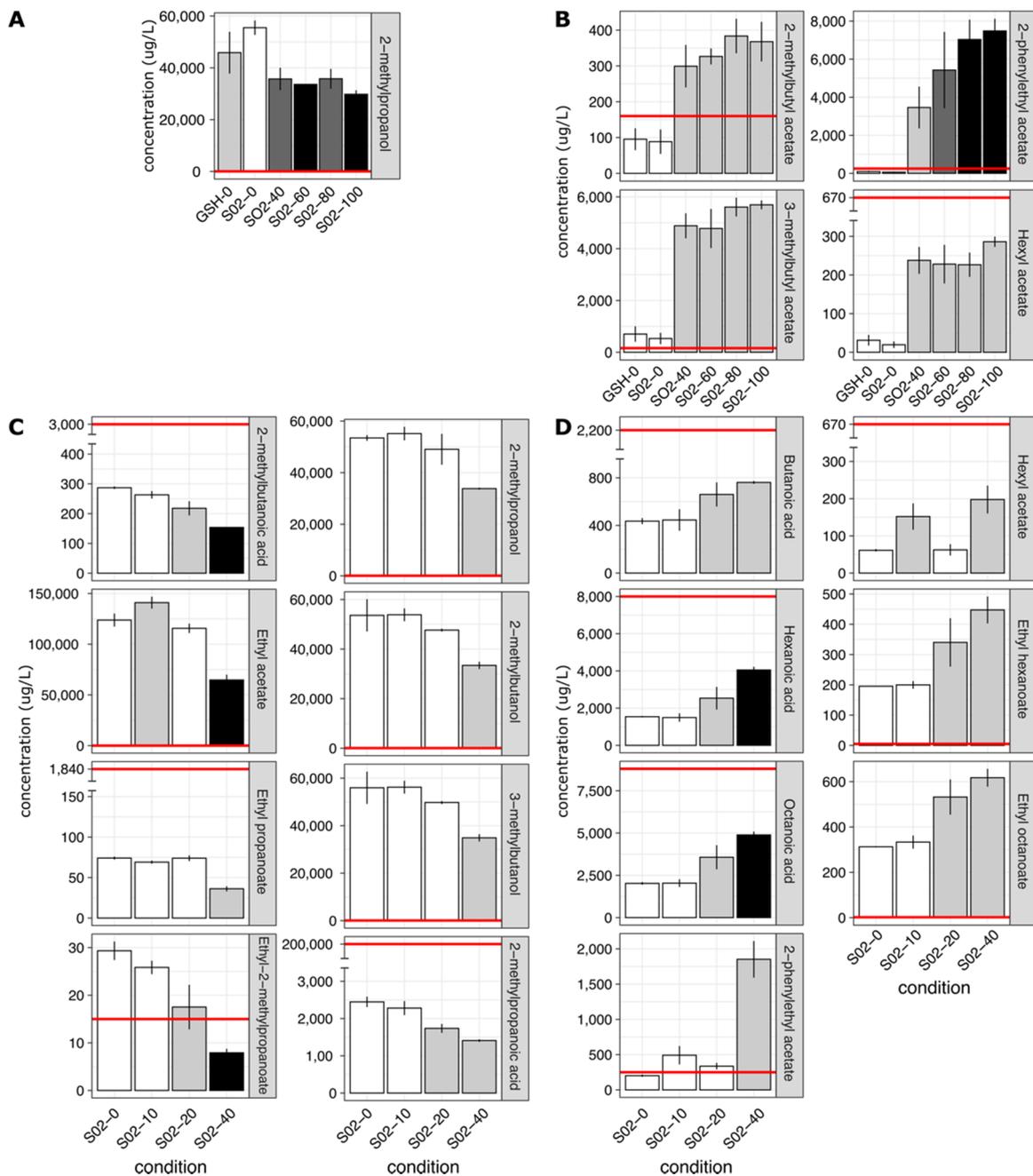


Figure 6. Concentration differences in aroma compounds due to the addition of SO₂. (A) Analytes with significantly reduced (ANOVA, $p \leq 0.001$) concentrations in 2018. (B) Analytes with significantly increased (ANOVA, $p \leq 0.001$) concentrations in 2018. (C) Analytes with significantly decreased (ANOVA, $p \leq 0.001$) concentrations in 2019. (D) Analytes with significantly increased (ANOVA, $p \leq 0.001$) concentrations in 2019. Individual bars are shaded according to their significance group and the estimated aroma thresholds (see Materials and Methods) are indicated in red.

3. Discussion

Winemakers are limited in their ability to influence the native microbial population of grape juice, with SO₂ addition representing the main available intervention. Previous microbiological studies have shown that species (and strains) of the major wine yeasts can respond differently to the application of SO₂, with commercial strains of *S. cerevisiae* displaying diverse but higher tolerance to SO₂ [20], while “wild” yeasts display lower tolerances and are therefore thought to be broadly selected against through the

application of moderate amounts of SO₂ prior to the start of fermentation [14–16,21]. Through the application of ITS meta-barcoding, this study demonstrated that the addition of over 40 mg/L of total SO₂ favored the presence of the non-*Saccharomyces* species *H. osmophila* at the expense of other genera such as *Metschnikowia*, *Torulasporea* and *Kazachstania*. *H. osmophila* has previously been shown to be resistant to SO₂ concentrations of over 40 mg/L [22,23]. While previous research into the effects of SO₂ on grape juice consortia did observe the antagonistic effect of SO₂ against non-*Saccharomyces* yeasts, the presence of *H. osmophila* was not specifically reported, although *Hanseniaspora* yeasts were observed at levels of SO₂ above 40 mg/L [16,24,25].

Much is known regarding the molecular basis of SO₂ tolerance in *S. cerevisiae*, where the sulfite efflux pump *SSU1* provides the main mode of resistance [26,27], but very little information is available on the main genetic determinants of SO₂ resistance in non-*Saccharomyces* species, although *SSU1* has been suggested to have a major role in *Brettanomyces bruxellensis* [28]. However, recent comparative genomic studies of *Hanseniaspora* spp. have shown that there is a clear differentiation of this genus into two well-defined phylogenetic clades, in which one of the differentiating factors is a homolog of *SSU1*, which is absent in the large clade containing *H. uvarum*, but present in the clade containing *H. osmophila* and *H. vineae* [29–31]. While this may explain the different response to SO₂ of *H. osmophila* versus *H. uvarum*, there are likely many other factors that impact the response of a specific species, as *T. delbrueckii*, which also possesses an *SSU1* homolog and displayed increased abundance at up to 40 mg/L SO₂, and is less tolerant at higher SO₂ concentrations than *H. osmophila*. Likewise, *H. vineae*, which was present at levels similar to *H. osmophila* in the control ferments and contains an *SSU1* homolog, did not increase in abundance in response to increases in the concentrations of SO₂.

Detailed chemical analysis showed that the addition of SO₂ resulted in a significant increase in the concentration of key esters and particularly the aroma compound 2-phenylethyl-acetate, which increased over 9-fold, to levels well above the sensory threshold for this compound, even under modest SO₂ additions (40 mg/L). Given the microbiological shift that was observed, it is likely that this change in ester production is due to the increasing prevalence of *H. osmophila* in these ferments with higher SO₂ levels. This is supported by published data from fermentations established with purified non-*Saccharomyces* strains, in which mixtures of *H. osmophila* and *S. cerevisiae* (90:10 ratio) were shown to produce higher concentrations of acetate esters (with the exception of isoamyl acetate) and concentrations of 2-phenylethyl-acetate almost 10-fold greater than those observed using *S. cerevisiae* alone [32,33].

In summary, uninoculated fermentations can provide desirable complexity, however, the process lacks the ability to introduce specific fermentation characteristics through the use of commercial starter strains with distinct fermentation aroma and/or flavor profiles. The ability to modulate the yeast community structure of an uninoculated ferment, and the resulting chemical composition of the final wine, demonstrated in this study represents an important tool for winemakers to begin to be able to influence the organoleptic profile of uninoculated wines.

4. Materials and Methods

4.1. Fermentation

Commercial, high-solids Chardonnay juice (not pre-treated with SO₂ during harvesting or processing) was obtained directly after destemming and crushing from Yalumba wineries during the 2018 and 2019 vintages (Table 1). The juice was transferred to 2 L Schott bottles and then treated with either potassium metabisulfite (ACE Chemical Company, Camden Park, Australia) to the appropriate final total SO₂ concentration or 250 mg of reduced glutathione (GSH, Sigma-Aldrich Sydney, Australia) as indicated. Each experiment was performed in triplicate. Bottles were sealed with airlocks and incubated at 18 °C. Ferments were assessed at least every 24 h by refractometry and sugar analysis (see below), with samples taken for meta-barcoding at four approximate sugar levels (T1, directly after

treatment; T2, 90% sugar remaining; T3, 50% sugar remaining; T4, 10% sugar remaining) from an in-built sampling port.

Table 1. Juice composition.

2018	
pH	3.32
Total soluble solids	22.3° Brix
Yeast assimilable nitrogen	249 mg/L
Ammonia	87 mg/L
Alpha amino nitrogen	177 mg/L
Titrateable acidity pH 7.0	5.7 g/L
Titrateable acidity pH 8.2	6.0 g/L
2019	
pH	3.41
[Glucose + Fructose]	229.6 g/L
Yeast assimilable nitrogen	412 mg/L
Ammonia	147 mg/L
Alpha amino nitrogen	291 mg/L
Titrateable acidity pH 7.0	5.7 g/L
Titrateable acidity pH 8.2	6.0 g/L

4.2. Meta-Barcoding

DNA was isolated using the DNeasy PowerFood Microbial DNA Isolation Kit (Qiagen Hilden, Germany) following the manufacturer's instructions. Bead-beating was carried out using a combination of 0.1 mm and 0.5 mm zirconia/silica beads (BioSpec Products, Butlersville, Oklahoma) in a Precellys Evolution homogenizer (Bertin Instruments, Montigny-le-Bretonneux, France) at 8000 RPM for 4 × 60 s. In order to prepare amplicons for sequencing, a two-step PCR was performed using sequences designed to amplify the fungal ITS region, while adding experiment-specific inline barcodes and appropriate adaptors for the Illumina sequencing platform. Briefly, first-round amplification of the ITS region was performed using the fungal-specific primers BITS (ACCTGCGGARGGATCA) and B58S3 (GAGATCCRTTGYTRAAAGTT) [18] which were modified to include both an inline barcode and Illumina adaptor sequences [19]. Second-round amplification added sequences required for Illumina dual-indexed sequencing via overhang PCR. Sequencing was performed using 2 × 300 bp chemistry (Ramaciotti Centre for Functional Genomics, Sydney, Australia). Paired-end reads were quality trimmed (Trimmomatic v0.38 [34]), adaptor trimmed (cutadapt v1.16 [35]) and merged into single synthetic reads (FLASH2 v2.2.00 [36]). Merged reads were de-replicated (USEARCH v10.0.240 [37]) and clustered (Swarm v2.2.2 [38]) into operational taxonomic units (OTUs) as presented previously [19]. Taxonomic annotation was performed against the UNITE database (qiime_ver8_dynamic_02.02.2019) using a 98% similarity cut off (assign_taxonomy.py module of QIIME v1.9.1 [39]). All sequence reads have been lodged in the NCBI database under the Bioproject accession PRJNA634973.

4.3. Chemical Analysis

Titrateable acidity and pH were determined using a TitraLab 840 (Radiometer) and the yeast assimilable nitrogen concentration was estimated by the NOPA + enzymatic ammonia method [40] on a Gallery Discrete Analyser (ThermoFischer, Waltham, USA) by AWRI Commercial Services (Australia).

During fermentation, [Glucose + Fructose] concentrations were determined spectrophotometrically using a Randox kit (Randox Laboratories Ltd., Crumlin, Antrim, UK) with adaptations for use in a 96-well microplate format [41]. Volatile acetates, esters and higher alcohol concentrations in the finished wines were determined using large-volume, stable-isotope dilution headspace-GC/MS analysis (Metabolomics Australia, Adelaide Australia) as adapted from [42] and as described by [43]. Volatile sulphur compounds contributing sulfidic off-aromas, were determined by gas chromatography with

sulphur chemiluminescence detection (GC/SCD) [42,44]. Aroma thresholds were in wine estimated using data from Siebert et al., [42]

Free SO₂ was measured in grape juice supplemented with freshly prepared PMS using the aspiration/titration method [45].

4.4. Statistical Analysis

An analysis of variance (ANOVA) was conducted using the formula aov (analyte ~ treatment) in R (version 3.6.3) to determine whether mean aroma-active compound concentrations ($n = 3$ for all treatments) differed with regard to SO₂ treatment. If ANOVA p values were less than 0.05, a multiple comparison of the analyte concentration with respect to treatment was undertaken using the function HSD.test (agricolae) to determine the grouping of the treatments at $\alpha = 0.05$. ANOVA $F_{3,8}$ values, p values, treatment means, standard deviations and treatment group are reported in Table S2.

Supplementary Materials: The following are available online at <http://www.mdpi.com/2311-5637/6/2/62/s1>, Figure S1: Sugar-consumption kinetics, Figure S2: Replicate fermentation comparisons, Table S1: OTU abundance measurements, Table S2: Chemical analysis results.

Author Contributions: Conceptualization, A.B.; methodology, K.C., S.V.D.H., M.S., C.V.; investigation, K.C.; S.V.D.H.; M.R., M.S., C.V.; formal analysis, S.S., A.B.; writing—original draft preparation, A.B., S.S.; writing—review and editing, all authors. All authors have read and agreed to the published version of the manuscript.

Funding: The AWRI is supported by Australia's grape growers and winemakers through their investment body Wine Australia with matching funds from the Australian Government.

Acknowledgments: Thank you to Caroline Bartel for performing SO₂ analysis and Ross Sanders for performing the volatile sulphur compound analysis and Markus Herderich for critical review of the manuscript. The AWRI is a member of the Wine Innovation Cluster in Adelaide.

Conflicts of Interest: The authors declare that they have no conflict of interest.

References

- Varela, C.; Borneman, A.R. Yeasts found in vineyards and wineries. *Yeast Chichester Engl.* **2017**, *34*, 111–128. [CrossRef]
- Fleet, G.H. Wine yeasts for the future. *FEMS Yeast Res.* **2008**, *8*, 979–995. [CrossRef] [PubMed]
- Fleet, G.H.; Lafon-Lafourcade, S.; Ribéreau-Gayon, P. Evolution of yeasts and Lactic acid bacteria during fermentation and storage of Bordeaux wines. *Appl. Environ. Microbiol.* **1984**, *48*, 1034–1038. [CrossRef]
- Fleet, G.H. Growth of yeasts during wine fermentations. *J. Wine Res.* **1990**, *1*, 211–223. [CrossRef]
- Beltran, G.; Torija, M.J.; Novo, M.; Ferrer, N.; Poblet, M.; Guillamón, J.M.; Rozès, N.; Mas, A. Analysis of yeast populations during alcoholic fermentation: A six year follow-up study. *Syst. Appl. Microbiol.* **2002**, *25*, 287–293. [CrossRef] [PubMed]
- Combina, M.; Elía, A.; Mercado, L.; Catania, C.; Ganga, A.; Martinez, C. Dynamics of indigenous yeast populations during spontaneous fermentation of wines from Mendoza, Argentina. *Int. J. Food Microbiol.* **2005**, *99*, 237–243. [CrossRef] [PubMed]
- Epifanio, S.I.; Gutierrez, A.R.; Santamaría, M.P.; López, R. The influence of enological practices on the selection of wild Yeast strains in spontaneous fermentation. *Am. J. Enol. Vitic.* **1999**, *50*, 219–224.
- Torija, M.J.; Rozès, N.; Poblet, M.; Guillamón, J.M.; Mas, A. Yeast population dynamics in spontaneous fermentations: Comparison between two different wine-producing areas over a period of three years. *Antonie Van Leeuwenhoek* **2001**, *79*, 345–352. [CrossRef]
- Jemec, K.P.; Raspor, P. Initial *Saccharomyces cerevisiae* concentration in single or composite cultures dictates bioprocess kinetics. *Food Microbiol.* **2005**, *22*, 293–300. [CrossRef]
- Sturm, J.; Grossmann, M.; Schnell, S. Influence of grape treatment on the wine yeast populations isolated from spontaneous fermentations. *J. Appl. Microbiol.* **2006**, *101*, 1241–1248. [CrossRef]
- Albertin, W.; Miot-Sertier, C.; Bely, M.; Marullo, P.; Coulon, J.; Moine, V.; Colonna-Ceccaldi, B.; Masneuf-Pomarede, I. Oenological pre-fermentation practices strongly impact yeast population dynamics and alcoholic fermentation kinetics in Chardonnay grape must. *Int. J. Food Microbiol.* **2014**, *178*, 87–97. [CrossRef] [PubMed]

12. Bokulich, N.A.; Thorngate, J.H.; Richardson, P.M.; Mills, D.A. Microbial biogeography of wine grapes is conditioned by cultivar, vintage, and climate. *Proc. Natl. Acad. Sci. USA* **2014**, *111*, E139–E148. [[CrossRef](#)] [[PubMed](#)]
13. Maturano, Y.P.; Mestre, M.V.; Combina, M.; Toro, M.E.; Vazquez, F.; Esteve-Zarzoso, B. Culture-dependent and independent techniques to monitor yeast species during cold soak carried out at different temperatures in winemaking. *Int. J. Food Microbiol.* **2016**, *237*, 142–149. [[CrossRef](#)] [[PubMed](#)]
14. Andorrà, I.; Landi, S.; Mas, A.; Guillamón, J.M.; Esteve-Zarzoso, B. Effect of oenological practices on microbial populations using culture-independent techniques. *Food Microbiol.* **2008**, *25*, 849–856. [[CrossRef](#)]
15. Morgan, S.C.; Scholl, C.M.; Benson, N.L.; Stone, M.L.; Durall, D.M. Sulfur dioxide addition at crush alters *Saccharomyces cerevisiae* strain composition in spontaneous fermentations at two Canadian wineries. *Int. J. Food Microbiol.* **2017**, *244*, 96–102. [[CrossRef](#)]
16. Morgan, S.C.; Tantikachornkiat, M.; Scholl, C.M.; Benson, N.L.; Cliff, M.A.; Durall, D.M. The effect of sulfur dioxide addition at crush on the fungal and bacterial communities and the sensory attributes of Pinot gris wines. *Int. J. Food Microbiol.* **2019**, *290*, 1–14. [[CrossRef](#)]
17. Danilewicz, J.C.; Secombe, J.T.; Whelan, J. Mechanism of interaction of polyphenols, oxygen, and sulfur dioxide in model wine and wine. *Am. J. Enol. Vitic.* **2008**, *59*, 128–136.
18. Bokulich, N.A.; Mills, D.A. Improved selection of internal transcribed spacer-specific primers enables quantitative, ultra-high-throughput profiling of fungal communities. *Appl. Environ. Microbiol.* **2013**, *79*, 2519–2526. [[CrossRef](#)]
19. Sternes, P.R.; Lee, D.; Kutyna, D.R.; Borneman, A.R. A combined meta-barcoding and shotgun metagenomic analysis of spontaneous wine fermentation. *GigaScience* **2017**, *6*, gix040. [[CrossRef](#)]
20. Nardi, T.; Corich, V.; Giacomini, A.; Blondin, B. A sulphite-inducible form of the sulphite efflux gene *SSU1* in a *Saccharomyces cerevisiae* wine yeast. *Microbiol. Read. Engl.* **2010**, *156*, 1686–1696. [[CrossRef](#)]
21. Henick-Kling, T.; Edinger, W.; Daniel, P.; Monk, P. Selective effects of sulfur dioxide and yeast starter culture addition on indigenous yeast populations and sensory characteristics of wine. *J. Appl. Microbiol.* **1998**, *84*, 865–876. [[CrossRef](#)]
22. Granchi, L.; Ganucci, D.; Messini, A.; Vincenzini, M. Oenological properties of and from wines produced by spontaneous fermentations of normal and dried grapes. *FEMS Yeast Res.* **2002**, *2*, 403–407. [[CrossRef](#)]
23. Hierro, N.; Gonzalez, A.; Mas, A.; Guillamon, J.M. Diversity and evolution of non-*Saccharomyces* yeast populations during wine fermentation: Effect of grape ripeness and cold maceration. *FEMS Yeast Res.* **2006**, *6*, 102–111. [[CrossRef](#)] [[PubMed](#)]
24. Bagheri, B.; Bauer, F.F.; Cardinali, G.; Setati, M.E. Ecological interactions are a primary driver of population dynamics in wine yeast microbiota during fermentation. *Sci. Rep.* **2020**, *10*, 1–12. [[CrossRef](#)] [[PubMed](#)]
25. Bokulich, N.A.; Swadener, M.; Sakamoto, K.; Mills, D.A.; Bisson, L.F. Sulfur dioxide treatment alters wine microbial diversity and fermentation progression in a dose-dependent fashion. *Am. J. Enol. Vitic.* **2015**, *66*, 73–79. [[CrossRef](#)]
26. Divol, B.; du Toit, M.; Duckitt, E. Surviving in the presence of sulphur dioxide: Strategies developed by wine yeasts. *Appl. Microbiol. Biotechnol.* **2012**, *95*, 601–613. [[CrossRef](#)]
27. Park, H.; Bakalinsky, A.T. *SSU1* mediates sulphite efflux in *Saccharomyces cerevisiae*. *Yeast* **2000**, *16*, 881–888. [[CrossRef](#)]
28. Varela, C.; Bartel, C.; Roach, M.; Borneman, A.; Curtin, C. *Brettanomyces bruxellensis SSU1* haplotypes confer different levels of sulfite tolerance when expressed in a *Saccharomyces cerevisiae SSU1* null mutant. *Appl. Environ. Microbiol.* **2019**, *85*, e02429-18. [[CrossRef](#)]
29. Sternes, P.R.; Lee, D.; Kutyna, D.R.; Borneman, A.R. Genome sequences of three species of *Hanseniaspora* isolated from spontaneous wine fermentations. *Genome Announc.* **2016**, *4*, e01287-16. [[CrossRef](#)]
30. Seixas, I.; Barbosa, C.; Mendes-Faia, A.; Güldener, U.; Tenreiro, R.; Mendes-Ferreira, A.; Mira, N.P. Genome sequence of the non-conventional wine yeast *Hanseniaspora guilliermondii* UTAD222 unveils relevant traits of this species and of the *Hanseniaspora* genus in the context of wine fermentation. *DNA Res.* **2019**, *26*, 67–83. [[CrossRef](#)]
31. Čadež, N.; Bellora, N.; Ulloa, R.; Hittinger, C.T.; Libkind, D. Genomic content of a novel yeast species *Hanseniaspora gamundiae* sp. nov. from fungal stromata (*Cyttaria*) associated with a unique fermented beverage in Andean Patagonia, Argentina. *PLoS ONE* **2019**, *14*, e0210792. [[CrossRef](#)]

32. Viana, F.; Gil, J.V.; Genovés, S.; Vallés, S.; Manzanares, P. Rational selection of non-*Saccharomyces* wine yeasts for mixed starters based on ester formation and enological traits. *Food Microbiol.* **2008**, *25*, 778–785. [[CrossRef](#)] [[PubMed](#)]
33. Viana, F.; Gil, J.V.; Vallés, S.; Manzanares, P. Increasing the levels of 2-phenylethyl acetate in wine through the use of a mixed culture of *Hanseniaspora osmophila* and *Saccharomyces cerevisiae*. *Int. J. Food Microbiol.* **2009**, *135*, 68–74. [[CrossRef](#)] [[PubMed](#)]
34. Bolger, A.M.; Lohse, M.; Usadel, B. Trimmomatic: A flexible trimmer for Illumina sequence data. *Bioinform. Oxf. Engl.* **2014**, *30*, 2114–2120. [[CrossRef](#)] [[PubMed](#)]
35. Martin, M. Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMB-Net. J.* **2011**, *17*, 10–12. [[CrossRef](#)]
36. Magoč, T.; Salzberg, S.L. FLASH: Fast length adjustment of short reads to improve genome assemblies. *Bioinform. Oxf. Engl.* **2011**, *27*, 2957–2963. [[CrossRef](#)] [[PubMed](#)]
37. Edgar, R.C. Search and clustering orders of magnitude faster than BLAST. *Bioinform. Oxf. Engl.* **2010**, *26*, 2460–2461. [[CrossRef](#)] [[PubMed](#)]
38. Mahé, F.; Rognes, T.; Quince, C.; de Vargas, C.; Dunthorn, M. Swarm: Robust and fast clustering method for amplicon-based studies. *PeerJ* **2014**, *2*, e593. [[CrossRef](#)]
39. Caporaso, J.G.; Kuczynski, J.; Stombaugh, J.; Bittinger, K.; Bushman, F.D.; Costello, E.K.; Fierer, N.; Peña, A.G.; Goodrich, J.K.; Gordon, J.I.; et al. QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods* **2010**, *7*, 335–336. [[CrossRef](#)]
40. Gump, B.H.; Zoeklein, B.W.; Fugelsang, K.C.; Whiton, R.S. Comparison of analytical methods for prediction of prefermentation nutritional status of grape juice. *Am. J. Enol. Vitic.* **2002**, *53*, 325–329.
41. Vermeir, S.; Nicolai, B.M.; Jans, K.; Maes, G.; Lammertyn, J. High-throughput microplate enzymatic assays for fast sugar and acid quantification in Apple and Tomato. *J. Agric. Food Chem.* **2007**, *55*, 3240–3248. [[CrossRef](#)] [[PubMed](#)]
42. Siebert, T.E.; Smyth, H.E.; Capone, D.L.; Neuwöhner, C.; Pardon, K.H.; Skouroumounis, G.K.; Herderich, M.J.; Sefton, M.A.; Pollnitz, A.P. Stable isotope dilution analysis of wine fermentation products by HS-SPME-GC-MS. *Anal. Bioanal. Chem.* **2005**, *381*, 937–947. [[CrossRef](#)] [[PubMed](#)]
43. Holt, H.; Cozzolino, D.; McCarthy, J.; Abrahamse, C.; Holt, S.; Solomon, M.; Smith, P.; Chambers, P.J.; Curtin, C. Influence of yeast strain on Shiraz wine quality indicators. *Int. J. Food Microbiol.* **2013**, *165*, 302–311. [[CrossRef](#)] [[PubMed](#)]
44. Bekker, M.Z.; Day, M.P.; Holt, H.; Wilkes, E.; Smith, P.A. Effect of oxygen exposure during fermentation on volatile sulfur compounds in Shiraz wine and a comparison of strategies for remediation of reductive character: Macro-oxygenation: Effect on sulfurous off-odours. *Aust. J. Grape Wine Res.* **2016**, *22*, 24–35. [[CrossRef](#)]
45. Rankine, B.; Pocock, K. Alkalimetric determination of sulphur dioxide in wine. *Aust. Wine Brew. Spirit Rev.* **1970**, *88*, 40–44.

