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Research Article

Fungal Diversity and Dynamics during Grape Wine Fermentations with Different Sulfite Levels and Yeast Inoculations

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Abstract: Microbial communities during grape wine fermentations are diverse and dynamic. High-throughput sequencing (molecular methods enabling precise identification of microbial communities), was used to identify fungal diversity during fermentation of grape juice with different sulfite levels and yeast inoculations. Fermentation (0, 14, and 21 days) was evaluated on two grape varieties, Noble (*Vitis rotundifolia*) and Vignoles (*Vitis* hybrid) fermented at three sulfite levels (0, 10, and 20 mg/L) and three yeast inoculations (Uninoculated, *Saccharomyces cerevisiae,* and *Torulaspora delbrueckii*). Fungal taxonomy of both varieties included 6-7 phyla and 115-129 genera. Indigenous microbiota was impacted by sulfite levels and yeast inoculations but varied by grape variety. Sulfite levels had minimal impact on fungal communities but affected fermentation

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dynamics. Increasing sulfite additions did not impact fermentation performance of *S. cerevisiae* but impacted performance of uninoculated juice and *T. delbrueckii*-inoculated juice. Main fungal genera (*Podosphaera, Candida, Phialemoniopsis,* and *Meyerozyma*) present at a relative abundance > 1% were the same for both varieties but at different relative abundance. Similar fungal diversity patterns were observed for both varieties, with a decrease of fungal diversity at day 14 and increase at day 21 of fermentation. Juice inoculated with *T. delbrueckii* were rapidly colonized by *Torulaspora* at day 0 for both varieties, while *Saccharomyces* dominated by day 14 when inoculated with *S. cerevisiae* especially in Noble. The most abundant genera detected in uninoculated juice were *Hanseniaspora* and *Zygoascus* for Noble and *Hanseniaspora* and *Saccharomyces* for Vignoles. Understanding grape juice microbial communities and dynamics of communities during fermentation provide insight for wine production using spontaneous fermentations or non-*Saccharomyces* species and impact of sulfur dioxide on these novel fermentations.

Key words: fungi, high-throughput sequencing, *Saccharomyces cerevisiae*, spontaneous fermentation, sulfur dioxide, *Torulaspora delbrueckii*

Introduction

Grape wine fermentations have a microbial dynamic community that changes through the fermentation process. Commercial wine yeast strains are used to ensure completion of alcoholic fermentation of grape juice to wine. Winemakers use yeasts strains, such as *Saccharomyces cerevisiae,* selected for efficient production of alcohol and beneficial influence on wine flavor and aroma (Querol et al. 1992, Pretorius 2000, Jolly et al. 2014, Hirst and Richter 2016). *S.*

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cerevisiae out-compete non-*Saccharomyces* species due to higher fermentative efficiency, alcohol tolerance, resistance to low pH, scarce oxygen availability, or depletion of nutrients. Winemakers use multiple strategies to inhibit unwanted microorganisms, such as addition of sulfur dioxide (SO_2) or clarification and sterilization (Renouf and Lonvaud-Funel 2004, Umiker et al. 2013, Morgan et al. 2019a).

In contrast to use of commercial yeast strains, spontaneous fermentations are fermentations that occur "naturally" without addition of commercial yeast or bacteria. Thus, the fermentation is performed by microorganisms naturally present on the grapes as well as the harvest and winery equipment. The use of non-*Saccharomyces* yeasts and other indigenous yeasts isolated from vineyards for wine production has become more popular (Roudil et al. 2019, Ruiz et al. 2019, Morgan et al. 2020). These yeasts can provide characteristics of grape-growing regions, increase varietal aroma, enhance flavor and mouthfeel, reduce high alcohol levels, control wine acidity, and improve color of wines (Renouf et al. 2006, Jolly et al. 2014, Quirós et al. 2014). There are only a few non-*Saccharomyces* yeasts commercially available for wine production including *Torulaspora. delbrueckii* (Biodiva™), *Metschnikowia pulcherrima* (Flavia®), and *Metschnikowia* IVF (Gaïa™) (Lallemand Inc., Canada) and *Pichia kluyveri* (FrootZen™), *Lachancea. thermotolerans* (Concerto™), and *T. delbrueckii* (Prelude™) (Chr Hansen A/S, Denmark) (Roudil et al. 2019). However, these yeasts typically need to be co-inoculated with S. cerevisiae.

At harvest, the indigenous grape microbiota varies depending on conditions such as weather/climate, relative humidity, grape variety, vineyard management practices, soil composition, and grapevine health and age (Pretorius 2000, Cordero-Bueso et al. 2011, Bokulich

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et al. 2014, Pinto et al. 2014, Drumonde-Neves et al. 2016, Martins et al. 2016, Morrison-Whittle et al. 2017, Mezzasalma et al. 2018, Nadai et al. 2019). Fungi colonizing wineries vary depending on vintage, wines produced, and their capacities to adapt and survive the stressful conditions of winery environment (Abdo et al. 2020a, 2020b). These winery-associated fungal consortia can impact on grape/must juice microbiota. Consequently, initial grape juice microbiota will vary, which is why some studies found new bacterial or fungal species throughout fermentation compared to other studies (Marzano et al. 2016).

The indigenous grape mycobiota detected during early stages of fermentation generally involve the yeast genera *Hanseniaspora* (anamorph *Kloeckera*), *Metschnikowia, Candida*, *Pichia, Issatchenkia,* and filamentous fungi genera *Botrytis, Cladosporium,* and *Aspergillus* (Fleet 2003, Jolly et al. 2014, Pinto et al. 2015, De Filippis et al. 2017, Hall and Wilcox 2019). *Hanseniaspora* spp. and *Candida* spp. can grow well and co-dominate must/wine fermentation with *S. cerevisiae* if fermentation temperature is less than 15-20°C (Fleet 2003, Di Maro et al. 2007). *S. cerevisiae* is either undetected in early stages of fermentation or at a lower relative abundance (percent composition of *S. cerevisiae* relative to total number of yeast communities identified in a sample) but outcompete other yeasts and eventually dominate the fermentation (Fleet 2003, Pinto et al. 2015, De Filippis et al. 2017).

Previous research on identification of microbiota in wine fermentations used plating/culture methods where only a small percentage of microorganisms (<1%) can be cultivated and identified on media. Molecular methods, such as sequencing methods detect presence of both live and dead microorganisms without culture on media (culture-independent) but by DNA detection. Sequencing has traditionally been done using low throughput sequencing,

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whereas high-throughput sequencing (HTS) can sequence multiple DNA molecules in parallel, so that hundreds of millions of DNA molecules from different samples can be sequenced at a time (Mendoza et al. 2017, Morgan et al. 2017). It is important to understand the dynamics of indigenous yeasts during spontaneous and inoculated fermentations as they can impact organoleptic properties specific to grape-growing regions. While recently research has been done using HTS to identify grape/wine microbiota and study dynamics of microorganisms during wine fermentation (Bokulich et al. 2014, Portillo and Mas 2016, De Filippis et al. 2017, Mezzasalma et al. 2017, Chen et al. 2020, Guzzon et al. 2020, Mandakovic et al. 2020), only a few focused on impact of sulfites levels or yeast inoculations on grape juice/wine microbiota (Bokulich et al. 2015).

In this study, HTS of the Internal Transcribed Spacer (ITS) 1 region was used to provide insight into the fungal diversity and dynamics impacted by sulfite levels (0, 10, and 20 mg/L) and yeast inoculations (Uninoculated, *S. cerevisiae*, and *T. delbrueckii*) during fermentation (0, 14, and 21 days) of two grape varieties, a muscadine grape, Noble (*Vitis rotundifolia*), and a hybrid grape, Vignoles (Seibel 6905 x Pinot de Corton). The changes in composition of juice/wine were used to evaluate fermentation performance (via sugar conversion to ethanol) for both grape varieties as influenced by sulfite levels and yeast inoculations.

Materials and Methods

Juice production.

Vignoles and Noble grapes grown in Arkansas were hand harvested for this study in 2016. Vignoles grapes were harvested from a commercial vineyard and winery in Eureka

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Springs, AR, crushed, and pressed. Noble grapes were harvested from a commercial vineyard in Ozark, AR, and brought to the University of Arkansas System Division of Agriculture (UA System) Food Science Department, Fayetteville, AR, for crushing and pressing. The juice from both varieties was frozen (-10°C) to inhibit any spontaneous fermentation and indigenous yeast growth until the experiment was implemented. Sulfur dioxide was not added to Noble or Vignoles juice during processing or prior to freezing.

Sulfite additions and yeast inoculations of juice.

About 18 L of juice from each variety was used for wine production during fermentations. The juice was removed from the freezer and thawed at 2°C overnight for small-scale, microfermentations with different sulfite levels and yeast inoculations of the juice (Figure 1). All labware (flasks, bottles, cylinders, fermentation locks, corks, and caps) for fermentation were autoclaved prior to use.

Sulfite additions.

From the 18 L of juice, 2 L of juice was placed into six 3.7-L glass bottles (2 bottles for each SO_2 level) (Figure 1). Three concentrations of SO_2 (0, 10, and 20 mg/L) as potassium metabisulfite $K_2S_2O_6$ (57% SO_2) (Presque Isle Wine Cellar, North East, PA) were added to the bottles. These low levels of sulfites were chosen based on reported yeast SO_2 tolerance for yeast evaluated in this study. No addition of SO_2 (0 mg/L) was used as a control. The 10 mg/L of SO_2 was used because it inhibits indigenous microbiota growth but is below a level that impacts *T. delbrueckii* growth (15 mg/L). The 20 mg/L concentration inhibits both indigenous microbiota and *T. delbrueckii* but does not inhibit *S. cerevisiae*. After SO_2 additions, bottles were capped

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and shaken thoroughly. From each 3.7-L bottle, 500 mL of juice was placed into three 1.9-L glass bottles (18 1.9-L bottles in total).

Yeast species and inoculations.

Both varieties of juice at the different SO_2 levels were either uninoculated or inoculated with commercial yeast species. The uninoculated juice was used to evaluate indigenous yeasts and the resulting fermentation. Two commercial yeast species, *T. delbrueckii* (Biodiva™) (Lallemand Inc., Montreal, Quebec) and *S. cerevisiae* (var. *bayanus*) (Lalvin EC-1118) (Lallemand Inc., Montreal, Quebec) were used in this study. This specific *S. cerevisiae* strain was selected since it is used frequently for commercial wine production, whereas *T. delbrueckii* is naturally present on grapes' skin (van Breda et al. 2013). The yeasts were inoculated based on manufacturer recommendations. The yeasts were rehydrated with distilled water heated to 30°C (*T. delbrueckii*) or 40°C (*S. cerevisiae*), then settled for 15 and 20 min, respectively, and stirred for 5 sec. Following rehydration, yeasts were added to 1.9-L flasks containing each 500 mL of grape juice at room temperature (21°C). After inoculation, juice was shaken thoroughly for 1 min to ensure even distribution. Total yeast inoculation level was estimated as 4.10^5 viable cells/mL for *T. delbrueckii* and 8.10^5 viable cells/mL for *S. cerevisiae*. From each 1.9-L bottle, 200 mL of juice was placed into 250-mL Erlenmeyer glass flasks (36 flasks in total).

Fermentation and sampling.

Each flask was sealed with sterile rubber corks with fermentation airlocks. The flasks were stirred manually during fermentation for 1 min twice per day during the week and for 1 min once per day during weekends. The juice was fermented for 21 days at 24°C. A 2-mL sample from each flask containing juice/wine were collected aseptically at day 0, 14, and 21 and

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159 transferred into sterile 2 mL-tubes. Samples were centrifuged at 13,300 rpm for 3 min at 4°C.
160 The pellets were used for microbial analysis and juice/wine supernatants were used for
161 compositional analysis. The samples were frozen at -10°C until analysis.

162 **Compositional analysis.**

163 Compositional analysis of juice and wine samples were performed. The soluble solids,
164 pH, and titratable acidity of the juice was done prior to fermentation. The individual and total
165 sugars, individual and total organics acids, glycerol, and ethanol of the juice/wine was measured
166 prior to and during fermentation.

167 ***Soluble solids.***

168 Soluble solids of the juice were determined using an Abbe Mark II refractometer
169 (Bausch and Lomb, Scientific Instrument, Keene, NH) and expressed as percent.

170 ***pH and titratable acidity.***

171 The Titrino plus 862 compact titrosampler (Metrohm AG, Switzerland) was used to
172 measure pH and titratable acidity of juice/wine. Titratable acidity was determined using ~6 g of
173 juice/wine diluted with 50 mL deionized, degassed water with a titration using standardized 0.1
174 N sodium hydroxide to an endpoint of pH 8.2 (Garner et al. 2005). The results of titratable
175 acidity were expressed as percentage of tartaric acid.

176 ***Sugars, organic acids, ethanol, and glycerol.***

177 The sugars, organic acids, ethanol, and glycerol in juice/wine were identified and
178 quantified by High Performance Liquid Chromatography (HPLC). Samples were passed through
179 a 0.45-µm polytetrafluoroethylene (PTFE) syringe filter (Varian, Inc., Palo Alto, CA) before
180 injection onto an HPLC system consisting of a Waters 515 HPLC pump, a Waters 717 plus

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autosampler, and a Waters 410 differential refractometer detector connected in series with a Waters 996 photodiode array (PDA) detector (Waters Corporation, Milford, MA). Analytes were separated with a Bio-Rad HPLC Organic Acids Analysis Aminex HPX-87H ion exclusion column (300 x 7.8 mm) connected in series with a Bio-Rad HPLC column for fermentation monitoring (150 x 7.8 mm; Bio-Rad Laboratories, Hercules, CA). A Bio-Rad Micro-Guard Cation-H refill cartridge (30 x 4.5 mm) was used as a guard column. Columns were maintained at 65 ± 0.1°C by a temperature control unit. The isocratic mobile phase consisted of pH 2.28 aqueous sulfuric acid at a flow rate of 0.45 mL/min. Injection volumes of both 10 µL (for analysis of organic acids and sugars) and 5 µL (for ethanol and glycerol) were used to avoid overloading the detector. The total run time per sample was 60 minutes.

Citric, tartaric, malic, lactic, acetic, and succinic acids were detected at 210 nm by the PDA detector and glucose, fructose, ethanol, and glycerol were detected at 410 nm by the differential refractometer detector. Analytes in samples were identified and quantified using external calibration curves based on peak area estimation with baseline integration. Results were expressed as grams per liter wine for sugars, organic acids, glycerol, and ethanol. Total sugars were calculated as the sum of glucose and fructose. Total organic acids were calculated as the sum of citric, tartaric, malic, succinic, lactic, and acetic acids. As expected, no ethanol was detected at day 0 in both grape juices.

Fungal DNA extraction.

After centrifuging, tubes containing cell pellets were kept under a fume hood with the cap off for 30 min to evaporate residual alcohol. The cell pellet was washed three times with sterile water and centrifuged. Inhibitex buffer (1 mL) was added to the pellet and vortexed. The solution

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was heated at 70°C for 5 min, vortexed for 15 sec, and transferred into a screw-cap tube containing 0.1 g of 0.1-mm diameter and 0.1 g of 0.5-mm diameter zirconia-silica beads (BioSpec Products, Bartlesville, OK). The cell/bead mixture was homogenized in a FastPrep®-24 bead beater (MP Biomedicals, Santa Ana, CA) for 1 min at maximum speed. From this point, the DNA extraction was carried out with the QIAamp® Fast DNA Stool Mini Kit (Qiagen, Germany) starting at step 4 of the manufacturer's instructions. DNA quality was estimated spectrophotometrically using the NanoDrop^{TM} 1000 Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA). Extracted DNA was visualized by electrophoresis on a 2% agarose gel in 1X TAE buffer (AMRESCO®, Cleveland, OH). DNA extracts were stored at -20°C until further analysis.

Amplicon libraries preparation.

The suitability of DNA extracts for fungal ITS sequencing was checked by applying a universal PCR (primers ITS1 and ITS4).

An Index PCR targeting the fungal ITS 1 locus of 5.8S rRNA gene regions was performed with ITS1 and ITS2 primers using the dual-index strategy for primer design described by Kozich et al. (2013). Briefly, each primer consisted of the appropriate Illumina adapter (AAT GAT ACG GCG ACC ACC GAG ATC TAC AC for ITS1 and CAA GCA GAA GAC GGC ATA CGA GAT for ITS2), an 8 nt index sequence (each index being different from each other), a 10 nt pad sequence (TGT GGT GGC C for ITS1 and ACT GCG TCA T for ITS2), a 2 nt linker (GT for ITS1 and AT for ITS2) and the gene specific primer (CTT GGT CAT TTA GAG GAA GTA A and GCT GCG TTC TTC ATC GAT GC for ITS1 and for ITS2, respectively). PCR reactions (25 µL) were prepared containing 21 µL Master mix (Invitrogen, Carlsbad, CA) (2.5

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μL of Buffer II, 0.1 μL of AccuPrime™ Taq DNA Polymerase High Fidelity, 18.4 μL of water), 3 μL of template DNA, and 1 μL of each dual index primer combination. RNAse free water and *Escherichia coli* were used as negative controls, and *S. cerevisiae* was used as positive control. Reactions conditions consisted of an initial denaturation at 94°C for 2 min, followed by 35 cycles of (denaturation at 94°C for 30 sec, primer annealing at 55°C for 30 sec, and extension at 68 °C for 1 min) then a final extension at 72°C for 10 min using the Eppendorf Mastercycler pro S (Eppendorf, Germany). Random reactions (12 to 100%), containing positive and negative controls were chosen from the PCR plate and loaded on an agarose gel to confirm successful amplification.

The SequalPrep^{TM} Normalization Plate Kit (Invitrogen, Carlsbad, CA) was used to purify (elute short primers, unincorporated dNTPs, enzymes, short failed PCR products, and salts from PCR reactions) and to normalize the PCR products following manufacturer's protocol.

The pool concentration was analyzed with Qubit ds DNA High Sensitivity Assay Kit (Invitrogen**,** Carlsbad, CA). The size of the amplicon fragments was determined with an Agilent 2100 Tapestation Bioanalyzer (Agilent Technologies, Santa Clara, CA). The amplicon pools were then denatured and diluted with 0.1 N freshly diluted NaOH and diluted using HT1 buffer according to the MiSeq System denature and dilute user Guide. Denatured and diluted DNA was mixed with 20% denatured 12.5 pM PhiX control V3 and loaded on to Illumina MiSeq reagent V2 cartridge. The custom Index, Read 1, and Read 2 sequencing primers were also added (Kozich et al. 2013), and sequencing was performed using Illumina Miseq (Illumina Inc., San Diego, CA) platform.

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Statistical analysis for composition attributes.

The sulfite levels (0, 10, and 20 mg/L) and yeast inoculations (Uninoculated, *S. cerevisiae*, and *T. delbrueckii*) of the juice/wine of two grape varieties (Noble and Vignoles) were evaluated in duplicate during fermentation (0, 14, and 21 days). A univariate mixed-model with a first-order autoregressive covariance structure was used to conduct a repeated measures by time analysis, with individual experimental units (juice/wine) as the subjects in a repeated structure for fermentation time. For fixed effects (sulfites levels, yeast inoculations, and fermentation day), an analysis of variance (ANOVA) was used to determine significance of main factors and interactions. All factors were treated as categorial. Tukey's Honest Significant Difference (HSD) test was used to detect differences among means (p-value < 0.05) for the fixed effects. JMP Pro 15.1 (SAS, Cary, NC) software was used for statistical analysis. The error bars on the figures represented one standard error from the mean. The data analysis for composition attributes was carried out separately for each variety of grape juice.

Microbial data analysis.

Raw data generated by the Illumina Miseq instrument (Illumina Inc., San Diego, CA) were demultiplexed, quality filtered, and analyzed using PIPITS pipeline (Gweon et al. 2015). Shannon diversity index was calculated on PAST 3.18 to characterize species diversity in each sample. Mann-Whitney pairwise with Bonferroni-corrected p-values was performed on species richness to test the effect of the day, sulfite levels, and yeast inoculations on juice/wine mycobiota.

Non-metric multidimensional scaling (NMDS) plots and one-way Analysis of Similarities (ANOSIM), both based on Bray-Curtis similarity index, were also obtained in PAST

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3.18 to identify similarities/dissimilarities between the structures of mycobiota. The NMDS plots are shown as supplementary figures and used for results and discussion. Differences between fermentation time, sulfite levels, and yeast inoculations were considered significant when the *p*-value was < 0.05; however, statistical difference should be interpreted cautiously due to the low number of replications of each sample ($n = 4$).

Results and Discussion

Composition analysis of juice/wine.

The composition of Noble juice was 18.2% soluble solids, 0.3% titratable acidity, and 3.16 pH, while Vignoles juice was 24.2% soluble solids, 1.03% titratable acidity, and 3.02 pH. In general, *Vitis vinifera* grapes for commercial wine production have 20-23% soluble solids, titratable acidity of 0.6-0.7%, and pH < 3.3-3.5. The Noble and Vignoles juices had composition values outside of this range, but typical for these varieties when grown in Arkansas. Muscadine grapes often contain lower titratable acidity than other wine grapes (Barchenger et al. 2015, Zhang et al. 2017), whereas Vignoles have a higher soluble solids and titratable acidity (Howell et al. 1991, Wilker et al. 2004). Dry table wine contain 85-89% (w/w) water and 9-13% ethanol, with the remaining composition consisting of glycerol, acids, residual sugars, polyphenols, polysaccharides, minerals, and volatile compounds (Waterhouse et al. 2016). Wines typically have glycerol concentrations of 7-10 g/L (Waterhouse et al. 2016), and the glycerol levels of all Noble and Vignoles wines were near the typical range (5-6 g/L for Noble and 4-8 g/L for Vignoles) and did not differ greatly during and after fermentation. Vignoles (263 g/L) juice had a

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higher initial total sugars level than Noble (194 g/L) juice, which resulted in greater ethanol levels in the wine.

The three-way interaction between sulfite levels, yeast inoculations, and fermentation time was significant for both varieties (Figures 2 and 3). The type of yeast inoculation impacted fermentation performance (sugar conversion to ethanol) more than sulfite levels. Increasing sulfite levels did not impact fermentation performance of *S. cerevisiae* but did impact fermentation performance of uninoculated juice and juice inoculated with *T. delbrueckii.* Although there was a decrease in total sugars and increase in ethanol for both varieties, the impact of sulfite levels on fermentation performance differed between grape varieties. Sulfite levels impacted fermentation performance of Vignoles more than Noble.

The sugars, glycerol, ethanol, and organic acids levels of wines after fermentation at day 21 are presented in Table 1. Total sugars were presented as the sum of glucose and fructose, and these residual sugars varied by treatment. For both grape varieties and regardless of sulfite level, uninoculated juice and juice inoculated with *T. delbrueckii* had higher total residual sugars (~62 and ~51 g/L for Uninoculated and *T. delbrueckii*-inoculated juice, respectively) than juice inoculated with *S. cerevisiae* (~2 g/L), consequently resulting in a lower ethanol levels (~109 and ~117 g/L for Uninoculated and *T. delbrueckii*-inoculated juice, respectively compared to ~145 g/L for *S. cerevisiae*-inoculated juice). Glycerol levels of wine at 21 days of fermentation for both grape varieties were low, about 4-8 g/L and total organic acids ranged from 11-17 g/L. The total organics acids were the sum of citric, tartaric, malic, succinic, lactic, and acid acids, and these individual acids varied by treatment. Fermentation performance of Vignoles and Noble will be described in the following sections.

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Noble juice/wine.

Sugars. The initial total sugars of Noble juice prior to fermentation (day 0) were 191-198 g/L (Figure 2). The sulfite levels impacted total sugars in uninoculated juice/wine at days 14 and 21. At days 14 and 21, uninoculated juice with 0 mg/L SO_2 had lower total sugars than uninoculated juice with 20 mg/L SO_2. The uninoculated juice at day 14 had total sugar levels of 67-103 g/L (70, 67, and 103 g/L for uninoculated juice at 0, 10, and 20 mg/L sulfite, respectively). After 21 days of fermentation, uninoculated juice contained a total sugar concentration 49-71 g/L, indicating fermentation was incomplete or "stuck". Total sugars of wine at day 21 of uninoculated juice at 0, 10, and 20 mg/L sulfite were 49, 54, and 71 g/L, respectively. Table 1 shows that in uninoculated juice at day 21 regardless of SO_2 level, glucose and fructose levels of these residual sugars were about equal.

Most of the sugars were fermented in *S. cerevisiae*-inoculated juice after 14 days of fermentation with total sugars < 2 g/L. Total sugars of wine at day 21 of *S. cerevisiae*-inoculated juice was less than 0.4 g/L regardless of SO_2 level. *S. cerevisiae*-inoculated juice at day 21 regardless of SO_2 level had no fructose and very little glucose (<0.35 g/L) (Table 1).

For juice inoculated with *T. delbrueckii* at day 14, total sugars dropped to 64-67 g/L. At day 21, total sugars in juice inoculated with *T. delbrueckii* dropped slightly and reached 40-42 g/L, indicating the fermentation was also incomplete. Total sugars of wine at day 21 of *T. delbrueckii*-inoculated juice at the 0, 10, and 20 mg/L sulfite were 40, 42, and 42 g/L, respectively. The lower growth rates of *T. delbrueckii* species compared to *S. cerevisiae* and their inability to consume all available sugars was previously demonstrated (Bely et al. 2008). *T.*

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delbrueckii-inoculated juice at day 21 regardless of SO_2 level had more fructose (28-29 g/L) remaining than glucose (12-13 g/L) (Table 1).

Ethanol. The sulfite levels impacted ethanol in uninoculated juice/wine at days 14 and 21 (Figure 2). At day 14, juice with 0 and 10 mg/L SO_2 had higher ethanol levels than juice with 20 mg/L (SO_2 0 mg/L: 80 g/L, SO_2 10 mg/L: 82 g/L, and SO_2 20 mg/L: 58 g/L). At day 21, juice with 0 mg/L SO_2 had higher ethanol levels than juice with 20 mg/L. Ethanol levels at day 21 of uninoculated juice at 0, 10, and 20 mg/L sulfite were 102, 100, and 87 g/L, respectively.

For *S. cerevisiae*-inoculated juice, ethanol increased drastically from day 0 to day 14 (124-128 g/L) with similar levels at day 21 (131-133 g/L) with no significant difference between sulfites levels. On day 14, *S. cerevisiae*-inoculated juice contained more ethanol (124-128 g/L) compared to *T. delbrueckii* (78-83 g/L) and uninoculated (58-82 g/L) juices. Ethanol levels at day 21 of *S. cerevisiae*-inoculated juice at 0, 10, and 20 mg/L sulfite were 133, 131, and 131 g/L, respectively. The greater increase in ethanol and decrease in total sugars during fermentation confirmed that *S. cerevisiae* had the best efficiency for conversion.

Ethanol concentration increased progressively in juice inoculated with *T. delbrueckii* and reached 78-83 g/L at day 14 then 102-112 g/L at day 21 with no significant difference of fermentation performance between sulfite levels. Ethanol levels at day 21 of the *T. delbrueckii*-inoculated juice at 0, 10, and 20 mg/L sulfite were 109, 112, and 102 g/L, respectively.

Organic acids. Total organic acid levels of Noble juice increased during fermentation (Figure 2). At day 0, total organic acids were 7-8 g/L. From day 0 to day 14, total organic acids increased in all three inoculation treatments of the juices. A higher increase was noticed for uninoculated juice (11-13 g/L), followed by juice inoculated with *T. delbrueckii* (11-12 g/L) then

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S. cerevisiae (10-11 g/L). In uninoculated juice, the lower the sulfite levels, the greater the total organic acids produced at day 14. In uninoculated juice at day 21, there was no difference between total organic acid levels of the three sulfite levels (SO_2 0 mg/L: 13 g/L, SO_2 10 mg/L: 14 g/L, and SO_2 20 mg/L: 13 g/L). A similar pattern was found in juice inoculated with *S. cerevisiae*. However, at day 21 for juice inoculated with *T. delbrueckii,* total organics acids levels were greater with 10 and 20 mg/L sulfite (13 g/L) compared to juice without sulfite (12 g/L). Bokulich et al. (2015) found that low levels of SO_2 in uninoculated fermentations led to slower fermentations with higher levels of lactic and acetic acid bacteria. Thus, total organic acids can vary depending on the presence or absence of lactic and acetic acid bacteria, but these bacteria were not evaluated in our study. In all inoculated juices at day 21 regardless of SO_2 level, tartaric acid was the prominent acid (3.6-4.9 g/L), but in *T. delbrueckii* succinic acid levels were also high (3.1-3.3 g/L) (Table 1).

Vignoles juice/wine.

Sugars. At day 0, initial total sugar levels of Vignoles were 250-278 g/L (Figure 3).

Uninoculated juice had total sugar levels of 58-128 g/L at day 14. There were differences in total sugars between sulfite levels, with lower total sugars at higher sulfite levels for uninoculated juice (SO_2 0 mg/L: 128 g/L, SO_2 10 mg/L: 98 g/L, and SO_2 20 mg/L: 58 g/L). Total sugars of wine at day 21 of uninoculated juice at 0, 10, and 20 mg/L sulfite were 81, 69, and 50 g/L, respectively, indicating fermentation was incomplete. Similarly, Bokulich et al. (2015) also found lower sugar levels in uninoculated fermentations of Chardonnay at day 21, with 20 mg/L sulfite compared to 15 and 0 mg/L sulfite. In uninoculated juice at day 21 regardless of SO_2 level, fructose levels (43-60 g/L) were higher than glucose levels (7-21 g/L) (Table 1).

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Most of the sugars were fermented in the *S. cerevisiae*-inoculated juice after 14 days of fermentation with total sugars 2-10 g/L (Figure 3). At day 21, sugars were almost completely fermented (less than 7 g/L) for juice inoculated with *S. cerevisiae* (SO_2 0 mg/L: 7 g/L, SO_2 10 mg/L: 3 g/L, and SO_2 20 mg/L: 2 g/L). There were no differences in total sugars at days 14 and 21 between sulfite levels in juice inoculated with *S. cerevisiae.* In *S. cerevisiae*-inoculated juice at day 21 regardless of SO_2 level, glucose levels were very low (0-0.2 g/L) with low levels of fructose (2-6 g/L) (Table 1).

At day 14, total sugars of *T. delbrueckii*-inoculated juice at 0, 10, and 20 mg/L sulfite dropped (115, 87, and 50 g/L, respectively) (Figure 3). There were differences in total sugars between the three sulfite levels, with lower total sugars at higher sulfite levels at days 14 and 21. After 21 days of fermentation, total sugars of wine of *T. delbrueckii*-inoculated juice at 0, 10, and 20 mg/L sulfite were 81, 58, and 42 g/L, respectively, indicating the fermentation was also incomplete. In *T. delbrueckii*-inoculated juice at day 21 regardless of SO_2 level, fructose levels (36-56 g/L) were higher than glucose levels (5-25 g/L) (Table 1).

Ethanol. At day 14, there was an increase in ethanol concentration for three yeast inoculation treatments, with *S. cerevisiae* (153-162 g/L) having a better fermentation performance compared to *T. delbrueckii* (97-128 g/L) and uninoculated (79-119 g/L) juices (Figure 3).

At day 14, uninoculated juice had differences in ethanol levels for the three levels of sulfites (SO_2 0 mg/L: 79 g/L, SO_2 10 mg/L: 95 g/L, and SO_2 20 mg/L: 119 g/L). At day 21, higher levels of ethanol were observed with the highest level of sulfites for uninoculated juice (SO_2 0 mg/L: 115 g/L, SO_2 10 mg/L: 112 g/L, SO_2 20 mg/L: 137 g/L).

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For *S. cerevisiae*-inoculated juice, there was no difference in ethanol levels at day 14 between the three sulfite levels (SO_2 0 mg/L: 162 g/L, SO_2 10 mg/L: 153 g/L, SO_2 20 mg/L: 153 g/L) However, there was a difference at day 21 with lower ethanol levels observed with the lowest addition of sulfites (SO_2 0 mg/L: 163 g/L, SO_2 10 mg/L: 150 g/L, SO_2 20 mg/L: 164 g/L). As for Noble juice, the greater increase in ethanol and decrease in total sugars during fermentation confirmed that *S. cerevisiae* had the best efficiency for conversion in Vignoles juice.

Juice inoculated with *T. delbrueckii* had greater ethanol level when inoculated with the highest level of sulfite at days 14 (SO_2 0 mg/L: 98 g/L, SO_2 10 mg/L: 97 g/mL, and SO_2 20 mg/L: 128 g/L) and 21 (SO_2 0 mg/L: 115 g/L, SO_2 10 mg/L: 120 g/L, SO_2 20 mg/L: 142 g/L).

Organic acids. The total organic acids levels of Vignoles juice at day 0 was 14-18 g/L. The fermentation pattern of Vignoles was different than Noble as there was not an increase of total organic acids during fermentation. In general, regardless of yeast inoculation treatment, there was slightly higher total organic acids in juice/wine without sulfites compared to those with sulfite during fermentation, but not always significantly higher. This can be explained by the fact that the absence of sulfites allows lactic acid and acetic acid bacteria growth impacting organics acids (Bokulich et al. 2015). There were more differences in total organic acids in the Vignoles fermentation in term of impact of sulfites than in the Noble fermentation. In all inoculation treatments of juice at day 21 regardless of SO_2 level, malic acid was the prominent acid (5.2-6.4 g/L). The next highest levels of acids varied by inoculation treatment and was also impacted by SO_2 levels (Table1).

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Sequence analysis of juice/wine.

The fungal diversity analysis of Noble and Vignoles juices/wines generated 529 and 418 Operational Taxonomic Units (OTUs), respectively. About four samples from each variety were removed from the analysis due to either low number of sequence reads (< 400 sequences) or dissimilarities compared to replicates. The fungal taxonomic composition of Noble juice/wine included seven phyla (Ascomycota relative abundance 92.2% of the fungal communities, Basidiomycota 2.3%, and Chytridiomycota, Glomeromycota, Mortierellomycota, Olpidiomycita, and Rozellomycota combined represented 0.1%) and 129 genera. The fungal taxonomic composition of Vignoles juice/wine included six phyla (Ascomycota relative abundance 85.8% of the fungal communities, Basidiomycota 4.6%, and Chytridiomycota, Glomeromycota, Mortierellomycota, and Olpidiomycita combined represented 0.4%) and 115 genera. Unknown sequences (Fungi_unclassified) represented 5.4% and 9.1% of Noble and Vignoles, respectively, meaning that these sequences were not assigned to any fungi during the taxonomic assignment procedure (RDP Classifier against the UNITE fungal ITS reference data set). Morrison-Whittle et al. (2018) also found Ascomycota as a major phylum in juice and spontaneous wine fermentations in New Zealand (92.1% of all sequences), followed by Basidiomycota (0.4%) and the unknown sequences representing 7.5%, but did not identify more phyla. The percentage of unknown sequences were similar to the ones found in Vignoles and Noble. The data at the genus and phylum levels will be further discussed for the two grape varieties, but data for the phylum level is shown in Supplementary Figures.

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Indigenous fungal communities of juice from the two grape varieties.

The indigenous fungal communities of Noble and Vignoles grape varieties were identified from juice of grapes prior to fermentation. The fungal genera with a relative abundance higher than 1% in Noble and Vignoles are presented in Table 2. Grapes from both varieties were initially dominated by unclassified taxa: *Nectriaceae*_unclassified (40.7 and 45.2%) and Fungi_unclassified (15.9 and 17.6%) for Noble and Vignoles, respectively. Identifiable genera were represented in smaller relative abundance but were distinct between the two varieties. *Podosphaera* was present in both grape varieties but in a greater abundance in Vignoles (9.5%) than Noble (5.5%). *Candida* was also present in both grape varieties at a larger abundance in Noble (6.3%) than Vignoles (3.4%). Noble harbored abundant numbers of *Uwebraunia* (5.2%) and *Zygoascus* (1.8%). These two genera were not present or present at a low relative abundance in Vignoles (*Uwebraunia* 0.4% and *Zygoascus* not detected). On the other hand, Vignoles harbored larger relative abundance of *Filobasidium* (2.4%) compared to Noble (0.2%).

The other indigenous fungal genera (present at > 1% relative abundance for at least one of the two grape varieties) included *Phialemoniopsis, Meyerozyma, Penicillium, Cyberlindnera, Hanseniaspora,* and *Aspergillus* (Table 2). Interestingly, *Aspergillus* and *Penicillium* were present at a larger relative abundance in Noble (1.1 and 1.7%, respectively) than in Vignoles grapes (0.5 and 0.6%, respectively). The presence of these two filamentous fungi could be expected to be higher in Vignoles since the grapes are smaller and in tighter clusters than Noble grapes.

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462 A high percentage of *Necteriaceae*_unclassified was found in both grape varieties (>
463 40%). These results confirmed that a core of microorganisms was shared between varieties and
464 also that distinct microorganisms were found in each grape variety (e.g., *Zygoascus* in Noble).
465 This was observed in previous studies that demonstrated the impact of grape variety on
466 indigenous grape microbiota (Bokulich et al. 2014, Agarbati et al. 2019). Agarbati et al. (2019),
467 observed that *Aureobasidium pullulans* and *Hanseniaspora uvarum* were the most widespread
468 yeast species at harvest in two Italian grapes varieties, but they also identified specific
469 differences in yeast frequency between the two grapes varieties. For instance, *Pichia* spp. were
470 prevalent in Verdicchio, and *Lachancea thermotolerans* and *Zygoascus meyerae* were found in
471 Montepulciano variety. Bokulich et al. (2014) demonstrated that grape mycobiota of
472 Chardonnay, Zinfandel, and Cabernet Sauvignon grapes from different regions of California
473 were dependent of grape variety along with region and climatic factors, with for example
474 *Penicillium* significantly more abundant in Chardonnay, Dothideomycetes, Agaricomycetes,
475 Tremellomycetes, Microbotryomycetes, and *Saccharomycetaceae* in Cabernet Sauvignon,
476 Eurotiomycetes (*Aspergillus*), Leotiomycetes, and Saccharomycetes (notably *Candida*
477 *zemplinina*) in Zinfandel varieties. Either due to specific genetic features or due to vineyard
478 management that is specific to certain grape varieties, they demonstrated that distinct fungi were
479 found on different grape varieties. The high abundance of *C. zemplinina* (*Starmerella bacillaris*)
480 on Zinfandel grapes could be because Zinfandel berries have thin skins that allow juice and
481 nutrients to be more available for growth of these fermentative yeasts. Since a large proportion of
482 fungi present on grapes are unclassified more studies combining HTS and cultivation are needed
483 for further identification.

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Fungal diversity and successions during fermentation.

Regardless of yeast treatments, the Shannon Diversity Indices (Figure 4) showed similar patterns in fungal diversity for both varieties during fermentation. At day 0, there was high diversity, followed by a decrease in diversity at day 14 and then an increase in diversity at day 21. Surprisingly, this pattern was more notable for fermentation of Noble. As expected, uninoculated juice maintained higher fungal diversity compared with juices inoculated with *T. delbrueckii* and *S. cerevisiae*.

For Noble, diversity only increased slightly between day 14 and 21, whereas diversity returned to levels comparable to initial fermentation for Vignoles. This indicated that indigenous fungi of Vignoles were more resilient to fermentation processes, even in the presence of yeast inoculations. This observation may drive variety-specific organoleptic properties through secondary metabolic processes. Since indigenous grape microbiota and diversity of Noble and Vignoles juice/wine were different from each other, the dynamics of fungal communities during fermentation were analyzed separately.

Although the NMDS plots are typically used to visualize the level of similarity in a dataset, the significance of the points in the data set are hard to visualize when there are many factors (such as sulfite levels and yeast inoculations) and several plots in a figure. At each day of fermentation, the fungal communities tended to cluster apart by type of yeast inoculated (Uninoculated, *S. cerevisiae*, and *T. delbrueckii*) rather than by sulfite levels, more so in Noble than Vignoles (Supplementary Figures 1 and 2).

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Noble fungal communities' dynamics during fermentation.

Beginning fermentation. At day 0 of fermentation Noble juice fungal communities clustered by type of yeast inoculated (Uninoculated, *S. cerevisiae*, and *T. delbrueckii*) with *S. cerevisiae* and uninoculated clustered together and apart from juice inoculated with *T. delbrueckii* (Supplementary Figure 1).

The fungal profile at the phylum level were similar between the three types of inoculations and were dominated by the Ascomycota phylum (81.3% with Uninoculated, 81% with *S. cerevisiae,* and 89.2% with *T. delbrueckii*), followed by the Basidiomycota phylum (5.4% with Uninoculated, 5.7% with *S. cerevisiae,* and 2.9% with *T. delbrueckii*) (Supplementary Figure 3). Unclassified Fungi represented 13.2, 13.2, and 7.9% of the fungal communities of Uninoculated, *S. cerevisiae*, and *T. delbrueckii*-inoculated juices, respectively. A greater relative abundance of Ascomycota and smaller relative abundance of Fungi_unclassified and Basidiomycota was detected in *T. delbruecki*-inoculated juice, compared to *S. cerevisiae*-inoculated and uninoculated juices. There were no major dissimilarities between sulfite levels (0, 10, and 20 mg/L) for the three yeast inoculations of Noble juices. However, as sulfite levels increased, a small increase of Ascomycota and a small decrease of Fungi_unclassified and Basidiomycota were detected for both inoculations.

The fungal profile at the genus level presented dissimilarities between uninoculated and *S. cerevisiae*-inoculated juices together, compared to *T. delbrueckii*-inoculated juice (Table 3 and Supplementary Figure 4). At day 0, the dominant fungi identified in uninoculated and *S. cerevisiae*-inoculated juices were similar. For instance, regardless of sulfite levels, the predominant fungi were *Nectriaceae*_unclassified (42.5 and 44.3% for Uninoculated and *S.*

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cerevisiae-inoculated juices, respectively), followed by *Uwebraunia* (5.7 and 5.4%, respectively), *Candida* (5.4 and 5.1%, respectively), and *Podosphaera* (4.9 and 5.1%, respectively). Juice inoculated with *T. delbrueckii* was already dominated at day 0 by *Torulaspora*, representing 37.6% of the fungal communities, followed also by *Nectriaceae*_unclassified (31.7%), *Candida* (3%), *Uwebraunia* (2.9%), and *Podosphaera* (2.5%). Moreover, fungal profiles varied slightly between sulfite levels, mainly with slight differences in relative abundance of the main fungi and variation of fungi of smaller relative abundance.

Middle fermentation of Noble. At day 14 of fermentation, the fungal communities of Noble juice clustered by type of inoculation (Uninoculated*, S. cerevisiae*, and *T. delbrueckii*), with fungal communities of the three types of yeast inoculations clustered apart. The different sulfite levels for each type of yeast inoculations also clustered together (Supplementary Figure 1).

The fungal profiles at the phylum level were similar between the three types of inoculations (Supplementary Figure 3). Compared to the beginning of fermentation, the relative abundance of Ascomycota increased and represented 96.7, 98.5, and 99.5% of the fungal communities of juice/wine inoculated with Uninoculated, *S. cerevisiae*, and *T. delbrueckii*, respectively. No significant dissimilarities were observed between the three types of sulfite levels for the three types of inoculated juice.

However, the fungal profile at the genus level juice (Table 3 and Supplementary Figure 4) differed between the three types of inoculated juice/wine. Noble juice/wine inoculated with *T. delbrueckii* contained more than 97.8% of *Torulaspora* spp. No differences were observed

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549 between the three sulfites levels. Juice inoculated with *S. cerevisiae* contained more than 92% of
550 the genus *Saccharomyces*. The sulfite addition slightly modified the composition of fungal
551 communities with an increase of *Nectriaceae*_unclassified (0.95, 3.6, and 6.5% for 0, 10, and 20
552 mg/L of sulfites, respectively) and a decrease of *Saccharomyces* (97.1, 90.1, and 88.9%,
553 respectively) observed with increase of sulfite levels.

554 However, Noble juice uninoculated (representing spontaneous fermentation) was
555 dominated by *Hanseniaspora* (relative abundance 65.1%) followed by *Zygoascus* (11.8%) and
556 *Saccharomyces* (6.1%). Sulfite levels played an important part in this fermentation since the
557 greater the sulfite level, the greater the relative abundance of *Zygoascus* (0.21 to 32.5% for 0 and
558 20 mg/L of sulfites, respectively) and *Schizosaccharomyces* (0.1 to 7.4% for 0 and 20 mg/L of
559 sulfites, respectively), and smaller the relative abundance of *Hanseniaspora* (77.8 to 32.5% for 0
560 and 20 mg/L of sulfites, respectively). During the first stages of spontaneous fermentation,
561 *Hanseniaspora* spp. are known to be the dominant non-*Saccharomyces* yeast species along with
562 *Issatchenkia* spp. and *Candida* spp., and to be able to coexist with *S. cerevisiae* at later stages of
563 fermentation (Fleet 2003, Di Maro et al. 2007, Pinto et al. 2015, Portillo and Mas 2016, De
564 Filippis et al. 2017, Raymond Eder et al. 2018, Morgan et al. 2019b). *Hansenispora* genus can
565 represent up to 75% of the total initial microbiota and during fermentation can comprise up to
566 99% of the total yeast communities (Cioch-Skoneczny et al. 2018). This yeast is generally
567 undesirable in fermentation due to production of large concentrations of ethyl and amyl acetates,
568 glycerol, and acetic acid that negatively alter wine flavors and aroma (Johnson et al. 2020).
569 However, some *Hanseniaspora* species, such as *H. vineae*, when inoculated in mixed

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fermentation with *S. cerevisiae*, can positively impact organoleptic characteristics by increasing fruity aromas in wines (Domizio et al. 2011, Medina et al. 2013, Tristezza et al. 2016).

End fermentation of Noble. At day 21 of fermentation, fungal communities cluster by type of yeast inoculated (Uninoculated*, S. cerevisiae*, and *T. delbrueckii*) (Supplementary Figure 1). The three sulfites levels were clustered together for each type of yeast and overlapped for *T. delbrueckii*-inoculated wine. However, the fungal communities of *S. cerevisiae* and Uninoculated wines that did not receive sulfite treatment (SO_2 0 mg/L) clustered apart from wines with 10 and 20 mg/L of added sulfites.

The fungal profiles at the phylum level presented few dissimilarities between the three types of yeast inoculated (Supplementary Figure 3). An increase in relative abundance of Basidiomycota and Fungi_unclassified and a decrease of Ascomycota appeared in the three types of inoculations (Uninoculated*, S. cerevisiae*, and *T. delbrueckii*) especially in uninoculated wine and wine inoculated with *S. cerevisiae.* The sulfite additions had an impact fungal communities for uninoculated and inoculated with *S. cerevisiae* wines. The lower the sulfite level, the greater the relative abundance of Basidiomycota and Fungi_unclassified. Also, when sulfites were not added to juice for yeast treatments, other fungal phyla appeared. These results showed that sulfites inhibited other fungal growth in wines.

The fungal profile at the genus level (Table 3 and Supplementary Figure 4) presented some variation when comparing day 14 to day 21. Overall, at day 21 a decrease in the predominant fungi of day 14 (Uninoculated: decrease of *Hanseniaspora* 65.1 to 49.3%, *S. cerevisiae:* decrease of *Saccharomyces* 92 to 57.5%, *T. delbrueckii:* decrease of *Torulaspora* 97.8% to 90.3%) and an increase in the relative abundance of *Nectriaceae*_unclassified appeared

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592 in the three types of inoculated wines (from day 14 to day 21: 5.2 to 21.8% for Uninoculated, 3.7
593 to 25.5% for *S. cerevisiae*, and 0.75 to 5.4% for *T. delbrueckii*).

594 Wines inoculated with *T. delbrueckii* did not show significant dissimilarities in fungal
595 profiles between the three sulfite levels. However, wines inoculated with *S. cerevisiae* presented
596 an increase in other fungi, such as *Candida* (0.5 to 1.9%) and *Podosphaera* (0.8 to 1.8%).
597 Important dissimilarities in fungal profiles appeared between wines inoculated with *S. cerevisiae*
598 at different sulfite levels. Wine with no sulfites added, presented smaller relative abundance of
599 *Saccharomyces* (SO_2 0 mg/L: 10%, SO_2 10 mg/L: 83.6%, and SO_2 20 mg/L: 78.8%) and greater
600 relative abundance of *Nectriaceae*_unclassified (SO_2 0 mg/L54%, SO_2 10 mg/L: 10%, and SO_2
601 20 mg/L: 12.8%), *Phialemoniopsis* (SO_2 0 mg/L: 1.26%, SO_2 10 mg/L: 0.36% and SO_2 20 mg/L:
602 0.31%) and *Sarocladium* (SO_2 0 mg/L: 1.22%, SO_2 10 mg/L: 0.15%, and SO_2 20 mg/L: 0.11%)
603 compared to *S. cerevisiae*-inoculated wines with sulfites.

604 Uninoculated wines also presented dissimilarities in fungal profiles depending on the
605 sulfite levels added to juice. For instance, greater relative abundance of *Nectriaceae*_unclassified
606 (SO_2 0 mg/L 40.5%, SO_2 10 mg/L: 12.2%, and SO_2 20 mg/L: 12.5%) and lower relative
607 abundance of *Saccharomyces* genus (SO_2 0 mg/L: 0.11%, SO_2 10 mg/L: 3.9%, and SO_2 20
608 mg/L: 12.7%) were detected in uninoculated wines with no addition of sulfites.

609 Higher levels of sulfites promoted *Saccharomyces* growth in uninoculated
610 and inoculated with *S. cerevisiae* wines. Wine inoculated with *T. delbrueckii* maintained a high
611 relative abundance of *Torulaspora* throughout fermentation (day 0 to day 21), with a greater
612 relative abundance than *Saccharomyces* in wine inoculated with *S. cerevisiae*. This confirmed
613 that *T. delbrueckii*, a yeast naturally found in vineyards can be a good candidate for producing

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wines with specific terroir flavors (Azzolini et al. 2012, 2015, Cordero-Bueso et al. 2013, Roudil et al. 2019). However, the genus *Torulaspora* was not detected in uninoculated wines. It would be interesting to co-inoculate Noble juice with both *S. cerevisiae* and *T. delbrueckii* to observe dynamics of these two yeasts and indigenous grape microbiota using HTS.

Table 1 shows the main fungal genera of Noble wine at 21 days of fermentation. The main fungi detected in uninoculated juice at 0 mg/L SO_2 and 10 and 20 mg/L SO_2 were *Nectriaceae*_unclassified *and Hanseniaspora*, respectfully. The main fungi detected in *S. cerevisiae*-inoculated juice at 0 mg/L SO_2 and 10 and 20 mg/L SO_2 were *Nectriaceae*_unclassified *and Saccharomyces*, respectfully. The main fungal genera of *T. delbrueckii*-inoculated juice at all SO_2 levels was *Torulaspora*.

Vignoles fungal communities' dynamics during fermentation.

Beginning fermentation of Vignoles. At day 0 of fermentation, fungal communities of Vignoles juice clustered by type of inoculation (Uninoculated*, S. cerevisiae*, and *T. delbrueckii*) (Supplementary Figure 2).

The fungal profiles at the phylum level varied between the three types of inoculations and three sulfites levels (Supplementary Figure 5). Overall, similar to Noble juice, fungal communities of the three inoculation types of Vignoles juice were dominated by the Ascomycota phylum (76.6, 76.1, and 82.8% for Uninoculated, *S. cerevisiae*, and *T. delbrueckii*, respectively) followed by Basidiomycota phylum (6.1, 7.3, and 6%, for Uninoculated, *S. cerevisiae*, and *T. delbrueckii*, respectively). Unclassified fungi represented 16.2, 16, and 11% of the fungal communities of uninoculated and inoculated with *S. cerevisiae* and *T. delbrueckii* Vignoles

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635 wines. Greater relative abundance of Ascomycota was observed for the three types of
636 inoculations when sulfites were added to the juices.

637 The fungal profile at the genus level juice (Table 4 and Supplementary Figure 6),
638 presented dissimilarities between the three types of inoculated Vignoles juices and variation
639 appeared between sulfite levels for each type of inoculated juices. A day 0, the five most
640 abundant fungi identified in uninoculated Vignoles juice, regardless of sulfite levels, were
641 *Nectriaceae*_unclassified (48.3%), *Podosphaera* (8%), *Candida* (4.7%), *Meyerozyma* (2%) and
642 *Penicillium* (2%). The difference in sulfite levels slightly affected the relative abundance of
643 fungal communities, mainly those present at lower abundance in uninoculated juice. Vignoles
644 juice had a high soluble solids (24.8%) that could have impacted the initial microbial
645 communities.

646 For juice inoculated with *S. cerevisiae,* a clear distinction between fungal profiles
647 appeared at day 0 depending on sulfite levels. Juice inoculated with *S. cerevisiae* without sulfites
648 was dominated by *Nectriaceae*_unclassified (42.3%), followed by
649 *Sporidiobolaceae*_unclassified (6.6%), Tremellales_unclassified (2.6%), *Phialemoniopsis* (2%),
650 and Saccharomycetales_unclassified (1.7%). With the addition of sulfites (SO_2 10 mg/L and SO_2
651 20 mg/L), the presence of *Saccharomyces* was apparent (SO_2 0 mg/L: 0.13%, SO_2 10 mg/L:
652 48.2%, and SO_2 20 mg/L: 29.3%). Intriguingly, relative abundance of *Saccharomyces* was
653 greater when sulfite level was 10 mg/L compared to 20 mg/L. The addition of sulfites promoted
654 higher relative abundance of *Saccharomyces*. The relative abundance of other fungi also varied
655 between the no sulfite and the two levels of sulfites added, such as a greater relative abundance
656 of *Podosphaera* (SO_2 0 mg/L: 1.3%, SO_2 10 mg/L: 3.5%, and SO_2 20 mg/L: 4.9%) and *Candida*

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(SO_2 0 mg/L: 0.5%, SO_2 10 mg/L: 2.5%, and SO_2 20 mg/L: 3.8%), and a lower relative abundance in *Phialemoniopsis* (SO_2 0 mg/L: 2%, SO_2 10 mg/L: 1%, and SO_2 20 mg/L: 1.2%), *Nectriaceae*_unclassified (SO_2 0 mg/L: 42.3%, SO_2 10 mg/L: 23.7%, and SO_2 20 mg/L: 37.6%), and *Sporidiobolaceae*_unclassified (SO_2 0 mg/L: 6.6%, SO_2 10 mg/L: 0.6%, and SO_2 20 mg/L: 0.3%).

Juice inoculated with *T. delbrueckii*, regardless of sulfite levels, was dominated by *Torulaspora* (40.3%), *Nectriaceae*_unclassified (28.1%), *Podosphaera* (3.1%), *Candida* (2.4%), and *Sporidiobolaceae*_unclassified (1.5%). The addition of sulfites altered fungal communities of juice inoculated with *T. delbrueckii*. For instance, a decrease of the genus *Torulaspora* (SO_2 0 mg/L: 59.7%, SO_2 10 mg/L: 42.6%, and SO_2 20 mg/L: 18.7%) and an increase of *Nectriaceae*_unclassified (SO_2 0 mg/L: 7.8%, SO_2 10 mg/L: 30.8%, and SO_2 20 mg/L: 45.6%), and *Candida* (SO_2 0 mg/L: 0.7%, SO_2 10 mg/L: 3.4%, and SO_2 20 mg/L: 3%) were observed when sulfites were added. Overall, for Vignoles juice a clear pattern of fungal profile appeared distinct to each inoculation (Uninoculated, *S. cerevisiae*, and *T. delbrueckii*) and to sulfite levels.

Middle fermentation of Vignoles. At day 14 of fermentation, fungal communities for each type of inoculation (Uninoculated*, S. cerevisiae*, and *T. delbrueckii*) clustered apart except clusters of Vignoles that received 20 mg/L of sulfites (Supplementary Figure 2).

Fungal profiles at the phylum level were similar between the three types of inoculations (Supplementary Figure 5). Compared to the beginning of fermentation, the relative abundance of Ascomycota increased and represented 95.7, 96.7, and 98.7% of the fungal communities of uninoculated, *S. cerevisiae,* and *T. delbrueckii*-inoculated juices, respectively. The relative abundance of Basidiomycota decreased at day 14 and was greater in uninoculated juice (SO_2 0

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679 mg/L: 2%, SO_2 10 mg/L: 0.9%, and SO_2 0 mg/L: 0.4%). The relative abundance of
680 Fungi_unclassified also decreased at day 14 for the three type of inoculations (SO_2 0 mg/L:
681 2.2%, SO_2 10 mg/L: 2.3%, and SO_2 20 mg/L: 0.9%). No significant dissimilarities were observed
682 between the three types of sulfites levels for the three types of inoculated juice.
683 However, the fungal profile at the genus level (Table 4 and Supplementary Figure 6)
684 presented strong dissimilarities between the three types of inoculated juice. Overall, a decrease
685 of *Nectriaceae*_unclassified and an increase of *Saccharomyces* were observed for the three types
686 of inoculated juice.
687 Vignoles juice inoculated with *S. cerevisiae* was dominated by the genus *Saccharomyces*
688 (85.8%) followed by *Nectriaceae*_unclassified (6.7%). The sulfite additions did not affect fungal
689 profiles. However, sulfite levels modified the relative abundance of fungi in uninoculated juice
690 and juice inoculated with *T. delbrueckii*. For instance, uninoculated juice with no sulfites and
691 SO_2 at 10 mg/L presented a different fungal profile compared to uninoculated juice with SO_2 at
692 20 mg/L. A greater relative abundance of the genera *Hanseniaspora* was detected in no sulfite
693 and SO_2 at 10 mg/L in uninoculated juice (SO_2 0 mg/L: 56.8%, SO_2 10 mg/L: 45.8%, and SO_2 20
694 mg/L: 0.02%), *Candida* (SO_2 0 mg/L: 1.6%, SO_2 10 mg/L: 1.1%, and SO_2 20 mg/L: 0.6%) while
695 a larger relative abundance of the genus *Saccharomyces* was observed in uninoculated juice with
696 20 mg/L of sulfites (SO_2 0 mg/L: 23%, SO_2 10 mg/L: 35.5%, and SO_2 20 mg/L: 93.2%). The
697 increase in relative abundance of *Hanseniaspora* in low sulfite additions or no sulfite
698 fermentation was previously described (Morgan et al. 2019b).
699 Juice inoculated with *T. delbrueckii* was dominated by the genus *Torulaspora* when no
700 sulfite was added and 10 mg/L (SO_2 0 mg/L: 96.3%, SO_2 10 mg/L: 95.8%, and SO_2 20 mg/L:

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16.1%), while when a higher level of sulfites (20 mg/L) was added, the genus *Saccharomyces* dominated (SO_2 0 mg/L: 0.8%, SO_2 10 mg/L: 0.5%, and SO_2 20 mg/L: 74.1%) followed by *Torulaspora* (16.1%).

At day 14 of fermentation, each inoculation presented a dominant yeast with *Hanseniaspora* and *Saccharomyces* in inoculated juice, *Saccharomyces* for juice inoculated with *S. cerevisiae*, and *Torulaspora* or *Saccharomyces* when higher levels of sulfites added for juice inoculated with *T. delbrueckii*. The increase in sulfite levels had a significant impact on fungal profiles of the three types of inoculated juice.

End fermentation of Vignoles. At day 21 of fermentation, fungal communities clustered by type of yeast inoculation with fungal communities of uninoculated and inoculated with *S. cerevisiae* juices closer to each other (Supplementary Figure 2).

The fungal profiles at the phylum level presented slight dissimilarities between the three types of inoculations and different sulfite levels (Supplementary Figure 5). An increase of Basidiomycota (Uninoculated: 5.5%, *S. cerevisiae*: 9.5%, and *T. delbrueckii*: 4%) and Fungi_unclassified (Uninoculated: 9.6%, *S. cerevisiae*: 17.5%, and *T. delbrueckii*: 6.7%) appeared in the three types of yeast inoculation, and the increase was especially greater in uninoculated wines and wines inoculated with *S. cerevisiae*.

The fungal profile at the genus level (Table 4 and Supplementary Figure 6) presented some variation compared to day 14. Overall, from day 14 to day 21 an increase of *Nectriaceae*_unclassified (Uninoculated: 31.8%, *S. cerevisiae*: 40.2%, and *T. delbrueckii*: 34.5%) and a decrease of *Saccharomyces* (Uninoculated: 14.7%, *S. cerevisiae*: 19.3, and *T. delbrueckii*: 1.6%) for the three types of inoculations and sulfite additions were observed. The

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723 relative abundance of fungi of smaller abundance appeared at day 21, such as *Aspergillus,*
724 *Lachancea,* and *Zygoascus.*

725 The emergence of new fungi at day 21 may be explained by the fact that yeasts present at
726 high relative abundance throughout fermentation died and autolyzed, releasing nutrients (amino
727 acids and vitamins) allowing other yeast species (such as *Necteriaceae*_unclassified and
728 Fungi_unclassified in this study) that were previously outcompeted for growth to proliferate
729 (Fleet 2003). The initial sugar level impacts the microbiota, which could influence differences
730 between initial microbiota of Vignoles versus Noble juice. The higher soluble solids of Vignoles
731 juice resulted in a higher ethanol level that could also have impacted microbiota, selecting
732 microbial communities capable of surviving at higher ethanol levels.

733 The main fungi detected in uninoculated juice at 0 and 10 mg/L SO_2 and 20 mg/L SO_2
734 were *Hanseniaspora* and *Nectriaceae*_unclassified, respectfully. The main fungi identified in *S.*
735 *cerevisiae*-inoculated juice at 0 mg/L SO_2 and 10 and 20 mg/L SO_2 were *Saccharomyces* and
736 *Nectriaceae*_unclassified, respectfully. The main fungi detected in *T. delbrueckii*-inoculated
737 juice at 0 and 10 mg/L SO_2 and 20 mg/L SO2 were *Torulaspora* and *Nectriaceae*_unclassified,
738 respectfully (Table 1).

739 **Overall impact of sulfite additions and yeast inoculations.**

740 In terms of sulfite additions, highest levels of sulfites significantly affected fermentation
741 dynamics. For uninoculated juice, *Hanseniaspora* was strongly inhibited. Intriguingly,
742 *Hanseniaspora* was replaced by *Saccharomyces* for Vignoles and by *Zygoascus* and
743 *Schizosaccharomyces* for Noble. For Vignoles, the higher level of sulfites promoted
744 *Saccharomyces* but inhibited other fungi. Even in *T. delbrueckii*-inoculated juice at higher sulfite

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levels, *Saccharomyces* growth was promoted over *Torulaspora.* This can be a beneficial property in terms of *Saccharomyces*-driven wine production, but it is important to note that the initial inoculation might be reduced when adding too much sulfite at the beginning of fermentation. This should be taken into consideration when using commercial yeast strains of non-*Saccharomyces* yeasts in mixed culture fermentations with *S. cerevisiae* strains. As mentioned by the manufacturers, these commercially available non-*Saccharomyces* yeasts are sensitive to different SO_2 levels and need to be first inoculated without sulfites additions before a second inoculation with selected *S. cerevisiae* strains. At lower sulfite concentrations and without the presence of *S. cerevisiae*, non-*Saccharomyces* yeasts can grow and produce beneficial chemical compounds that can enhance wine complexity or inhibit spoilage microorganisms (Roudil et al. 2019). To further complete fermentation, *S. cerevisiae* strains are later added to the fermentation.

*Nectriaceae*_unclassified were stimulated at day 21 when high levels of sulfites were used for Vignoles juice, while for Noble juice their growth were inhibited. In terms of yeast inoculations, uninoculated Noble and Vignoles juices were dominated by *Hanseniaspora* and *Saccharomyces* genera. However, the relative abundance of these two genera varied by sulfite levels and inversely for the two grape varieties. While relative abundance of *Saccharomyces* increased with higher sulfite levels in uninoculated Noble juice, it decreased for uninoculated Vignoles juice. Moreover, a third genera, *Zygoascus*, was identified at a large relative abundance only in uninoculated Noble juice with sulfite additions, while it was not identified in uninoculated Vignoles juice, possibly because the two grape varieties had different compositions (e.g., more total sugars in Vignoles juice). In Noble juice inoculated with *S. cerevisiae,* *Saccharomyces* growth and dominance took a few days, but was detected at day 0 during

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767 fermentation in Vignoles juice. However, *Saccharomyces* genus retained a larger relative
768 abundance in Noble juice than in Vignoles juice at day 21. Both juices inoculated with *T.*
769 *delbrueckii* showed a dominant *Torulaspora* relative abundance from day 0 to day 21, with
770 higher relative abundances in Noble juice at day 21 compared to Vignoles juice in which a
771 decrease in *Torulaspora* relative abundance was observed at day 21. These results confirmed that
772 grape variety impacted indigenous juice/wine mycobiota and performance of commercial yeasts.

773 ## Conclusion

774 This manuscript is novel because the HTS approach was used to determine the impact of
775 the sulfite levels and yeast inoculations on wine fungal diversity and dynamics during
776 fermentation (0, 14, and 21 days) of two grape varieties, a muscadine grape (Noble) and a hybrid
777 grape (Vignoles). The fungal taxonomy of both varieties included 6-7 phyla and 115-129 genera.
778 It was demonstrated that while the most abundant fungi (relative abundance > 1%) in juice were
779 the same, their relative abundances varied by grape variety. The fungal diversity pattern
780 throughout fermentation was similar for the two grape varieties, but sulfite additions and yeast
781 inoculations impacted juice/wine mycobiota differently. These results confirm the importance of
782 indigenous grape mycobiota and grape variety in shaping juice/wine mycobiota. The presence of
783 these specific fungi can impact wine enological characteristics. Since indigenous fungi react
784 differently to sulfites or yeast inoculations, knowing initial mycobiota and their behavior during
785 fermentation can help winemakers interested in producing wines with less sulfites and
786 encouraging use of spontaneous fermentations. Understanding grape juice microbial
787 communities and dynamics of the communities during fermentation can provide more insight for

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788 wines production using spontaneous fermentations or fermentation with non-*Saccharomyces*
789 species.

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Table 1 Composition[a] (g/L) and five main fungi at day 21 of fermentation of Noble and Vignoles wines with different sulfite levels (0, 10, and 20 mg/L) and yeast inoculations (Uninoculated, *Saccharomyces cerevisiae*, and *Torulaspora delbrueckii*).

	Uninoculated			***S. cerevisiae***			***T. delbrueckii***		
	0 mg/L	**10 mg/L**	**20 mg/L**	**0 mg/L**	**10 mg/L**	**20 mg/L**	**0 mg/L**	**10 mg/L**	**20 mg/L**
NOBLE									
Glucose	21.17 ± 7.45	24.23 ± 2.41	34.18 ± 11.04	0.35 ± 0.00	0.33 ± 0.02	0.35 ± 0.01	11.57 ± 0.21	12.56 ± 0.91	12.7 ± 0.77
Fructose	27.71 ± 3.49	29.39 ± 3.64	36.6 ± 5.06	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	28.21 ± 0.55	29.56 ± 1.92	29.2 ± 1.81
Total sugars[b]	*48.88 ± 10.86*	*53.62 ± 4.56*	*70.79 ± 16.1*	*0.35 ± 0.0*	*0.33 ± 0.02*	*0.35 ± 0.01*	*39.78 ± 0.75*	*42.12 ± 2.83*	*41.9 ± 2.58*
Glycerol	5.12 ± 0.26	5.23 ± 0.26	6.16 ± 0.23	5.18 ± 0.10	5.13 ± 0.26	5.32 ± 0.04	4.98 ± 0.11	5.29 ± 0.37	5.25 ± 0.37
Ethanol	101.68 ± 2.65	99.98 ± 2.27	86.72 ± 13.5	133.39 ± 4.42	130.78 ± 7.68	130.95 ± 5.75	108.8 ± 2.28	111.74 ± 3.96	101.63 ± 7.50
Citric acid	1.39 ± 0.06	1.51 ± 0.03	1.73 ± 0.06	1.34 ± 0.05	1.3 ± 0.10	1.57 ± 0.06	1.19 ± 0.03	1.38 ± 0.02	1.41 ± 0.13
Tartaric acid	4.56 ± 0.11	4.45 ± 0.24	4.85 ± 0.15	3.65 ± 0.19	4.15 ± 0.19	3.94 ± 0.39	3.86 ± 0.13	4.19 ± 0.20	4.25 ± 0.20
Malic acid	1.47 ± 0.07	1.56 ± 0.04	1.32 ± 0.05	1.19 ± 0.04	1.22 ± 0.14	1.28 ± 0.04	1.48 ± 0.02	1.53 ± 0.03	1.49 ± 0.06
Succinic acid	2.11 ± 0.03	2.27 ± 0.13	1.94 ± 0.17	2.55 ± 0.07	2.79 ± 0.45	2.79 ± 0.04	3.06 ± 0.08	3.32 ± 0.13	3.33 ± 0.13
Lactic acid	2.08 ± 0.40	2.1 ± 0.18	1.67 ± 0.09	0.71 ± 0.02	0.75 ± 0.07	0.76 ± 0.04	0.84 ± 0.01	1.05 ± 0.01	1.23 ± 0.09
Acetic acid	1.8 ± 0.11	2.07 ± 0.70	1.72 ± 0.15	1.28 ± 0.04	1.32 ± 0.17	1.31 ± 0.03	1.39 ± 0.02	1.62 ± 0.06	1.44 ± 0.12
Total organic acids[b]	*13.41 ± 0.66*	*13.96 ± 0.73*	*13.24 ± 0.47*	*10.71 ± 0.31*	*11.52 ± 0.79*	*11.66 ± 0.54*	*11.82 ± 0.13*	*13.09 ± 0.17*	*13.15 ± 0.60*
Main fungi[c]	*Nectriaceae*_unclassified (40.5%)	*Hanseniaspora* (70.2%)	*Hanseniaspora* (41.6%)	*Nectriaceae*_unclassified (54%)	*Saccharomyces* (83.6%)	*Saccharomyces* (78.8%)	*Torulaspora* (92.3%)	*Torulaspora* (89.4%)	*Torulaspora* (89.4%)

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	Hanseniaspora (36.1%) *Candida* (2.7%) *Podosphaera* (1.5%) Saccharomycetales_unclassified (1.3%)	*Nectriaceae*_unclassified (12.2%) *Saccharomyces* (3.9%) *Candida* (1.4%) *Zygoascus* (1.2%) *Podosphaera* (1.2%)	*Zygoascus* (14.3%) *Saccharomyces* (12.7%) *Nectriaceae*_unclassified (12.5%) *Schizosaccharomyces* (2.5%)	*Saccharomyces* (10%) *Candida* (3.7%) *Podosphaera* (3.6%) *Phialemoniopsis* (1.3%)	*Nectriaceae*_unclassified (9.9%)	*Nectriaceae*_unclassified (12.8%) *Candida* (1.2%) *Podosphaera* (1%)	*Nectriaceae*_unclassified (5.4%)	*Nectriaceae*_unclassified (5.1%)	*Nectriaceae*_unclassified (5.5%)
VIGNOLES									
Glucose	20.92 ± 2.82	16.42 ± 0.71	7.33 ± 0.92	0.24 ± 0.24	0.24 ± 0.42	0.00 ± 0.00	25.21 ± 2.43	16.4 ± 1.49	5.18 ± 1.13
Fructose	60.39 ± 4.2	52.43 ± 0.8	42.55 ± 1.86	6.46 ± 3.47	2.73 ± 2.25	1.63 ± 0.28	55.76 ± 2.68	41.32 ± 2.22	36.32 ± 3.87
Total sugars[b]	*81.31 ± 7.00*	*68.85 ± 1.49*	*49.89 ± 2.78*	*6.7 ± 3.70*	*2.98 ± 2.67*	*1.63 ± 0.28*	*80.97 ± 5.10*	*57.72 ± 3.70*	*41.5 ± 4.99*
Glycerol	5.29 ± 0.15	4.27 ± 0.08	4.94 ± 0.02	7.4 ± 0.14	6.69 ± 0.39	7.19 ± 0.16	4.9 ± 0.08	4.76 ± 0.11	4.95 ± 0.13
Ethanol	115.27 ± 4.48	112.26 ± 2.17	136.74 ± 2.06	163.39 ± 2.07	149.55 ± 7.17	163.58 ± 4.99	114.87 ± 1.84	120.3 ± 1.57	141.7 ± 3.50
Citric acid	3.53 ± 0.2	1.02 ± 0.01	1.26 ± 0.06	1.03 ± 0.04	0.91 ± 0.03	1.02 ± 0.02	1.03 ± 0.12	0.91 ± 0.01	1.27 ± 0.03
Tartaric acid	0.91 ± 0.07	3.11 ± 0.12	2.97 ± 0.14	3.04 ± 0.24	2.64 ± 0.17	2.71 ± 0.08	3.47 ± 0.25	2.91 ± 0.12	2.97 ± 0.13
Malic acid	5.51 ± 0.08	5.24 ± 0.05	5.88 ± 0.09	6.43 ± 0.11	5.84 ± 0.15	5.97 ± 0.04	6.02 ± 0.09	5.83 ± 0.10	5.87 ± 0.13
Succinic acid	2.98 ± 0.05	2.59 ± 0.04	2.98 ± 0.02	3.81 ± 0.07	3.0 ± 0.19	3.21 ± 0.12	3.32 ± 0.14	3.6 ± 0.08	3.16 ± 0.10
Lactic acid	1.43 ± 0.10	1.1 ± 0.06	1.17 ± 0.09	1.62 ± 0.02	1.14 ± 0.06	1.23 ± 0.00	1.27 ± 0.07	1.45 ± 0.06	1.29 ± 0.09

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Acetic acid	0.54 ± 0.00	0.42 ± 0.05	0.57 ± 0.03	0.58 ± 0.01	0.41 ± 0.10	0.53 ± 0.02	0.5 ± 0.07	0.86 ± 0.47	0.66 ± 0.11
Total organic acids[b]	*14.9 ± 0.39*	*13.48 ± 0.12*	*14.83 ± 0.26*	*16.5 ± 0.20*	*13.94 ± 0.66*	*14.66 ± 0.15*	*15.61 ± 0.39*	*15.56 ± 0.58*	*15.22 ± 0.31*
Main fungi[c]	*Hanseniaspora* (40.6%)	*Hanseniaspora* (30.8%)	*Nectriaceae*_unclassified (51.1%)	*Saccharomyces* (35.1%)	*Nectriaceae*_unclassified (41.2%)	*Nectriaceae*_unclassified (53.7%)	*Torulaspora* (62.4%)	*Torulaspora* (51.9%)	*Nectriaceae*_unclassified (50%)
	Saccharomyces (24.1%)	*Nectriaceae*_unclassified (23.9%)	*Candida* (4.3%)	*Nectriaceae*_unclassified (25.8%)	*Saccharomyces* (21.2%)	*Sporidiobolaceae*_unclassified (7.4%)	*Nectriaceae*_unclassified (22%)	*Nectriaceae*_unclassified (31.6%)	*Torulaspora* (17.6%)
	*Nectriaceae*_unclassified (20.4%)	*Saccharomyces* (16.3%)	*Saccharomyces* (3.7%)	*Sporidiobolaceae*_unclassified (4.7%)	*Candida* (3.1%)	*Candida* (2.5%)	Microbotryomycetes_unclassified (2%)	*Candida* (1.9%)	*Saccharomyces* (4.3%)
	Lachancea (3.5%)	*Sporidiobolaceae*_unclassified (5.8%)	*Sporidiobolaceae*_unclassified (3.3%)	Microbotryomycetes_unclassified (3.3%)	*Podosphaera* (2.9%)	*Podosphaera* (2.2%)	Saccharomycetales_unclassified (1.3%)	*Podosphaera* (1.7%)	*Podosphaera* (2.6%)
		Candida (1.8%)	*Aspergillus* (2.7%)	*Candida* (1.4%)	*Penicillium* (2.1%)	*Saccharomyces* (1.7%)	*Candida* (1.1%)	*Trichosporonaceae*_unclassified (1.2%)	*Candida* (2.3%)

[a]Mean of 4 replicates ± standard deviation.
[b]Total sugars calculated as sum of glucose and fructose. Total organic acids calculated as sum of citric, tartaric, malic, succinic, lactic, and acetic acids.
[c]Five main fungi present at a relative abundance > 1%.

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T**able 2** Relative abundance (> 1%) of fungi at the genus level recovered in juice from Arkansas-grown Noble and Vignoles prior to fermentation.

Fungi[a]	**Variety of grape**	
	Noble	**Vignoles**
*Nectriaceae*_unclassified	40.66	45.21
Fungi_unclassified	15.91	17.62
Podosphaera	5.47	9.47
Candida	6.33	3.42
Uwebraunia	5.24	0.39
*Sporidiobolaceae*_unclassified	0.09	4.70
Saccharomycetales_unclassified	2.43	0.73
Phialemoniopsis	1.66	1.38
Meyerozyma	1.28	1.62
Filobasidium	0.23	2.40
Penicillium	1.71	0.56
Cyberlindnera	1.23	0.73
Hanseniaspora	1.01	0.82
Zygoascus	1.75	0.00
Aspergillus	1.07	0.48
Mortierellales_unclassified	0.01	1.51
Ascomycota_unclassified	1.07	0.44
Microbotryomycetes_unclassified	1.20	0.20

[a]Relative abundance > 1% recovered in Noble and Vignoles juice highlighted. When the assignment to genus rank failed, the nearest taxonomic level with assignment was reported.

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Table 3 Relative abundance (> 1%) of fungi at the genus level in Arkansas-grown Noble juice/wine during fermentation at 0, 14, and 21 days with different sulfite levels and yeast inoculations.

		Treatments								
		Uninoculated[b]			*S. cerevisiae*			*T. delbrueckii*		
Day	**Fungi[a]**	**NS[c]**	**S10**	**S20**	**NS**	**S10**	**S20**	**NS**	**S10**	**S20**
Day 0	*Torulaspora*	0.0	0.0	0.0	0.0	0.0	0.0	48.6	33.2	31.1
	*Nectriaceae*_unclassified	40.7	32.4	54.4	43.9	42.4	46.5	18.0	31.0	46.2
	Saccharomyces	0.0	0.0	0.0	1.4	0.7	0.2	0.0	0.0	0.0
	Hanseniaspora	1.0	5.0	1.7	0.5	0.8	1.6	2.2	0.6	0.7
	Fungi_unclassified	15.9	12.7	10.9	13.7	14.0	12.0	8.0	9.4	6.2
	Zygoascus	1.7	3.8	2.7	2.2	2.4	2.3	1.6	1.8	0.9
	Candida	6.3	5.9	3.8	5.6	5.1	4.7	2.9	3.6	2.4
	Podosphaera	5.5	5.8	3.3	5.2	5.3	4.6	2.4	2.8	2.2
	Uwebraunia	5.2	7.0	4.8	5.1	5.8	5.1	3.6	3.5	1.6
	Saccharomycetales_unclassified	2.4	3.3	1.5	1.9	2.2	1.6	1.4	1.3	0.6
	Penicillium	1.7	2.6	1.6	1.5	1.6	2.0	1.0	1.6	0.9
	Phialemoniopsis	1.7	0.9	1.5	1.3	1.4	1.0	0.5	0.6	0.7
	Ascomycota_unclassified	1.1	1.9	1.8	1.3	1.6	1.6	0.7	1.0	0.4
	Microbotryomycetes_unclassified	1.2	1.8	0.8	1.0	1.1	1.2	0.9	0.5	0.4
	Meyerozyma	1.3	1.2	0.4	1.1	1.1	1.1	0.7	0.5	0.3
	Cyberlindnera	1.2	1.0	0.6	1.0	1.0	0.9	0.6	0.5	0.5
	*Trichosporonaceae*_unclassified	1.0	1.0	0.3	0.9	1.0	0.8	0.5	0.7	0.4
	Aspergillus	1.1	0.9	0.6	0.7	0.9	1.0	0.4	0.6	0.5
	Talaromyces	0.2	0.6	0.4	0.2	0.5	1.2	0.0	0.5	0.0
Day 14	*Torulaspora*	0.1	0.1	0.2	0.1	0.3	0.6	98.5	97.4	97.5
	*Nectriaceae*_unclassified	5.3	6.3	4.1	0.9	3.6	6.5	0.4	1.0	0.8
	Saccharomyces	7.8	0.6	9.9	97.1	90.1	88.8	0.0	0.0	0.1
	Hanseniaspora	77.8	80.3	37.1	0.1	0.1	0.2	0.1	0.1	0.1
	Fungi_unclassified	2.1	2.3	1.9	0.4	1.5	1.3	0.2	0.4	0.4

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	Zygoascus	0.2	2.6	32.5	0.0	0.0	0.0	0.0	0.0	0.0
	Candida	0.7	1.1	0.8	0.2	0.9	0.3	0.1	0.2	0.2
	Podosphaera	1.5	1.2	1.3	0.3	1.2	0.8	0.2	0.3	0.3
	Schizosaccharomyces	0.1	0.0	7.4	0.0	0.0	0.0	0.0	0.0	0.0
Day 21	*Torulaspora*	0.2	0.1	0.1	0.1	0.1	0.1	92.3	89.4	89.4
	*Nectriaceae*_unclassified	40.5	12.2	12.5	54.0	9.9	12.8	5.4	5.1	5.5
	Saccharomyces	0.1	3.9	12.7	10.0	83.6	78.8	0.0	0.1	0.1
	Hanseniaspora	36.1	70.2	41.6	0.4	0.3	0.2	0.0	0.1	0.1
	Fungi_unclassified	6.5	3.6	4.3	9.3	2.0	2.4	0.7	1.6	1.6
	Zygoascus	0.9	1.2	14.3	0.7	0.0	0.1	0.1	0.0	0.0
	Candida	2.7	1.4	1.9	3.7	0.8	1.2	0.3	1.0	0.7
	Podosphaera	1.5	1.2	1.7	3.6	0.6	1.0	0.2	0.7	0.6
	Saccharomycetales_unclassified	1.3	0.4	0.9	1.0	0.2	0.4	0.1	0.3	0.2
	Phialemoniopsis	1.2	0.3	0.5	1.3	0.4	0.3	0.1	0.1	0.1
	Schizosaccharomyces	0.0	0.0	2.5	0.0	0.0	0.0	0.0	0.0	0.0
	*Trichosporonaceae*_unclassified	0.3	0.2	0.2	1.3	0.1	0.1	0.0	0.1	0.1
	Sarocladium	0.4	0.1	0.3	1.2	0.1	0.1	0.1	0.1	0.1

[a] Relative abundance > 1% recovered in Noble juice/wine is highlighted. When the assignment to the genus rank failed, the nearest taxonomic level with assignment was reported.
[b] Yeast inoculations were Uninoculated, *Saccharomyces cerevisiae,* and *Torulaspora delbrueckii.*
[c] Sulfur dioxide (as potassium metabisulfite) levels were 0 mg/L (NS), 10 mg/L (S10), and 20 mg/L (S20).

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Table 4 Relative abundance (> 1%) of fungi at the genus level in Arkansas-grown Vignoles juice/wine during fermentation at 0, 14, and 21 days with different sulfite levels and yeast inoculations.

		Treatments								
		Uninoculated[b]			*S. cerevisiae*			*T. delbrueckii*		
Day	**Fungi**[a]	**NS**[c]	**S10**	**S20**	**NS**	**S10**	**S20**	**NS**	**S10**	**S20**
Day 0	*Nectriaceae*_unclassified	45.2	44.8	54.9	42.3	23.8	37.6	7.8	30.8	45.6
	Saccharomyces	0.0	0.0	0.0	0.1	48.2	29.3	0.0	0.0	0.0
	Torulaspora	0.0	0.1	0.4	0.0	0.0	0.0	59.7	42.6	18.7
	Fungi_unclassified	17.6	16.7	14.5	28.7	9.0	10.4	12.0	8.6	12.0
	Podosphaera	9.5	8.6	5.9	1.3	3.5	4.9	1.8	4.2	3.3
	Candida	3.4	4.9	5.7	0.5	2.5	3.8	0.7	3.4	3.0
	*Sporidiobolaceae*_unclassified	4.7	0.1	0.8	6.6	0.6	0.3	3.4	0.1	1.1
	Saccharomycetales_unclassified	0.7	1.2	1.5	1.7	0.5	1.3	0.3	0.7	2.8
	Penicillium	0.6	3.3	2.1	0.3	1.7	0.9	0.7	1.1	0.5
	Phialemoniopsis	1.4	1.3	1.7	2.0	1.0	1.2	0.6	1.0	1.1
	Meyerozyma	1.6	3.7	0.7	0.3	0.6	0.8	0.3	0.6	1.1
	Aspergillus	0.5	1.0	1.1	0.2	0.6	0.5	0.3	0.6	0.5
	*Trichosporonaceae*_unclassified	0.4	0.6	0.6	0.3	0.4	0.6	0.2	0.5	1.7
	Tremellales_unclassified	0.1	0.3	0.3	2.6	0.5	0.6	2.5	0.0	0.4
	Microbotryomycetes_unclassified	0.2	0.1	0.2	1.6	0.4	0.1	0.0	0.1	0.0
	Filobasidium	2.4	0.1	0.2	1.2	0.1	0.1	1.3	0.1	0.9
	Talaromyces	0.0	2.1	1.3	0.0	1.2	0.5	0.0	0.9	0.0
	Trichoderma	0.8	2.6	0.5	0.1	0.5	0.5	0.2	0.5	0.6
	Cyberlindnera	0.7	1.1	0.9	0.0	0.5	0.8	0.0	0.6	0.5
	Hannaella	0.3	0.4	0.8	0.1	0.3	0.6	1.2	0.2	0.9
	Mortierellales_unclassified	1.5	0.2	0.5	1.1	0.0	0.0	0.0	0.0	0.3
	Ascomycota_unclassified	0.4	1.4	0.3	0.2	0.3	0.3	0.6	0.2	0.3
	Didymella	0.2	0.1	0.2	0.3	0.0	0.1	1.2	0.0	0.1
	Papiliotrema	0.4	0.0	0.1	1.0	0.1	0.1	0.0	0.0	0.0
Day 14	*Nectriaceae*_unclassified	5.8	7.2	2.1	6.9	7.6	5.6	0.8	2.0	3.8
	Saccharomyces	23.0	35.5	93.2	84.7	86.3	86.5	0.8	0.5	74.1
	Torulaspora	0.3	0.6	0.2	0.2	0.3	0.0	96.3	95.8	16.1
	Fungi_unclassified	3.1	2.3	1.1	2.3	2.2	2.4	0.5	0.5	1.8

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	Hanseniaspora	56.8	45.8	0.0	0.2	0.0	0.0	0.5	0.1	0.1
	Podosphaera	1.7	1.3	0.3	0.9	0.3	1.1	0.2	0.1	0.9
	Candida	1.6	1.1	0.6	1.0	0.4	1.0	0.2	0.1	0.6
	Lachancea	2.4	0.5	0.0	0.1	0.0	0.0	0.1	0.1	0.0
Day 21	*Nectriaceae*_unclassified	20.4	23.9	51.1	25.8	41.2	53.7	22.0	31.6	50.0
	Saccharomyces	24.1	16.3	3.7	35.1	21.2	1.7	0.3	0.1	4.3
	Torulaspora	0.7	0.2	0.6	0.3	0.4	0.1	62.4	51.9	17.6
	Fungi_unclassified	4.3	10.4	14.1	20.7	16.1	15.5	6.3	5.4	8.3
	Hanseniaspora	40.6	30.8	0.8	0.7	0.3	0.1	0.2	0.0	0.1
	Podosphaera	0.4	1.4	2.1	0.4	2.9	2.2	1.0	1.7	2.6
	Candida	0.5	1.8	4.3	1.4	3.1	2.5	1.1	1.9	2.3
	*Sporidiobolaceae*_unclassified	0.4	5.8	3.3	4.7	1.4	7.4	0.6	0.1	1.5
	Saccharomycetales_unclassified	0.1	0.1	1.5	0.1	1.1	0.6	1.3	0.9	0.6
	Penicillium	0.3	0.9	0.4	0.3	2.1	0.5	0.1	0.5	0.3
	Phialemoniopsis	0.9	0.2	1.5	0.1	0.8	1.0	0.1	0.4	0.9
	Meyerozyma	0.5	1.3	0.7	0.3	1.1	0.2	0.0	0.3	0.4
	Aspergillus	0.1	0.3	2.7	0.3	1.2	0.8	0.0	0.3	0.5
	*Trichosporonaceae*_unclassified	0.0	0.0	0.8	0.0	0.6	0.1	0.0	1.2	1.4
	Microbotryomycetes_unclassified	0.2	0.0	0.7	3.3	0.1	1.0	2.0	0.1	0.2
	Filobasidium	0.0	0.5	0.5	0.7	0.0	1.4	0.1	0.0	0.7
	Lachancea	3.5	1.0	0.1	0.3	0.0	0.1	0.4	0.1	0.1
	Uwebraunia	0.1	0.0	1.6	0.7	0.0	0.3	0.0	0.1	0.0
	Trigonopsis	0.5	0.0	0.1	0.2	0.7	1.5	0.1	0.2	0.2
	Didymella	0.0	1.3	1.0	0.0	0.1	0.1	0.0	0.0	0.5
	Pleosporales_unclassified	0.0	0.0	0.0	0.3	0.3	1.2	0.1	0.0	0.2
	Zygoascus	0.0	0.0	1.1	0.0	0.0	0.0	0.0	0.0	0.6
	Naganishia	0.0	0.0	0.1	1.0	0.0	0.0	0.0	0.3	0.0

[a] Relative abundance > 1% recovered in Vignoles juice/wine is highlighted. When the assignment to the genus rank failed, the nearest taxonomic level with assignment was reported.
[b] Yeast inoculations were Uninoculated, *Saccharomyces cerevisiae,* and *Torulaspora delbrueckii.*
[c] Sulphur dioxide (as potassium metabisulfite) levels were 0 mg/L (NS), 10 mg/L (S10), and 20 mg/L (S20).

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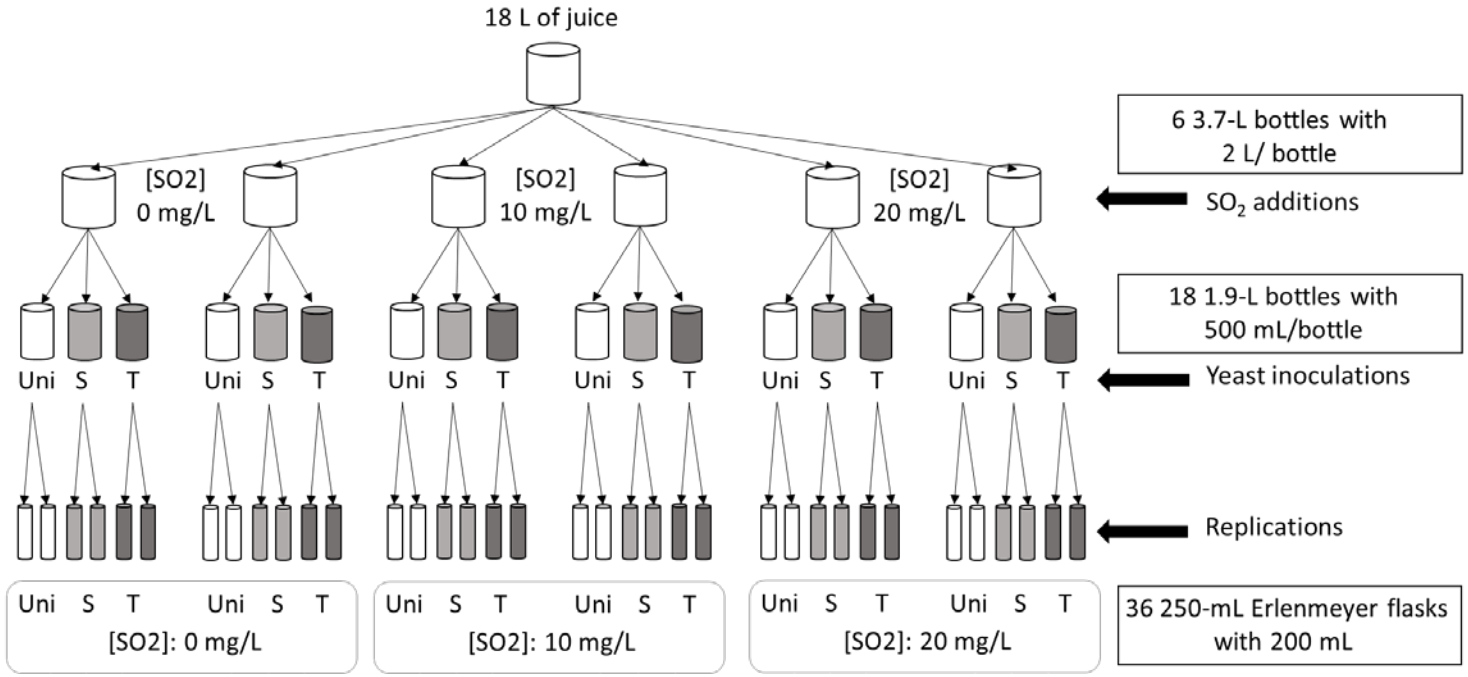

Figure 1 Flow chart presenting the sulfite levels (0, 10, and 20 mg/L) and yeast inoculations (uninoculated, *Saccharomyces cerevisiae*, and *Torulaspora delbrueckii*) added to Noble and Vignoles juices.
Uni: Uninoculated juice, S: *Saccharomyces cerevisiae*-inoculated juice, T: *Torulaspora delbrueckii*-inoculated juice, [SO_2]: 0, 10, and 20 mg/L of sulfur dioxide as potassium metabisulfite.

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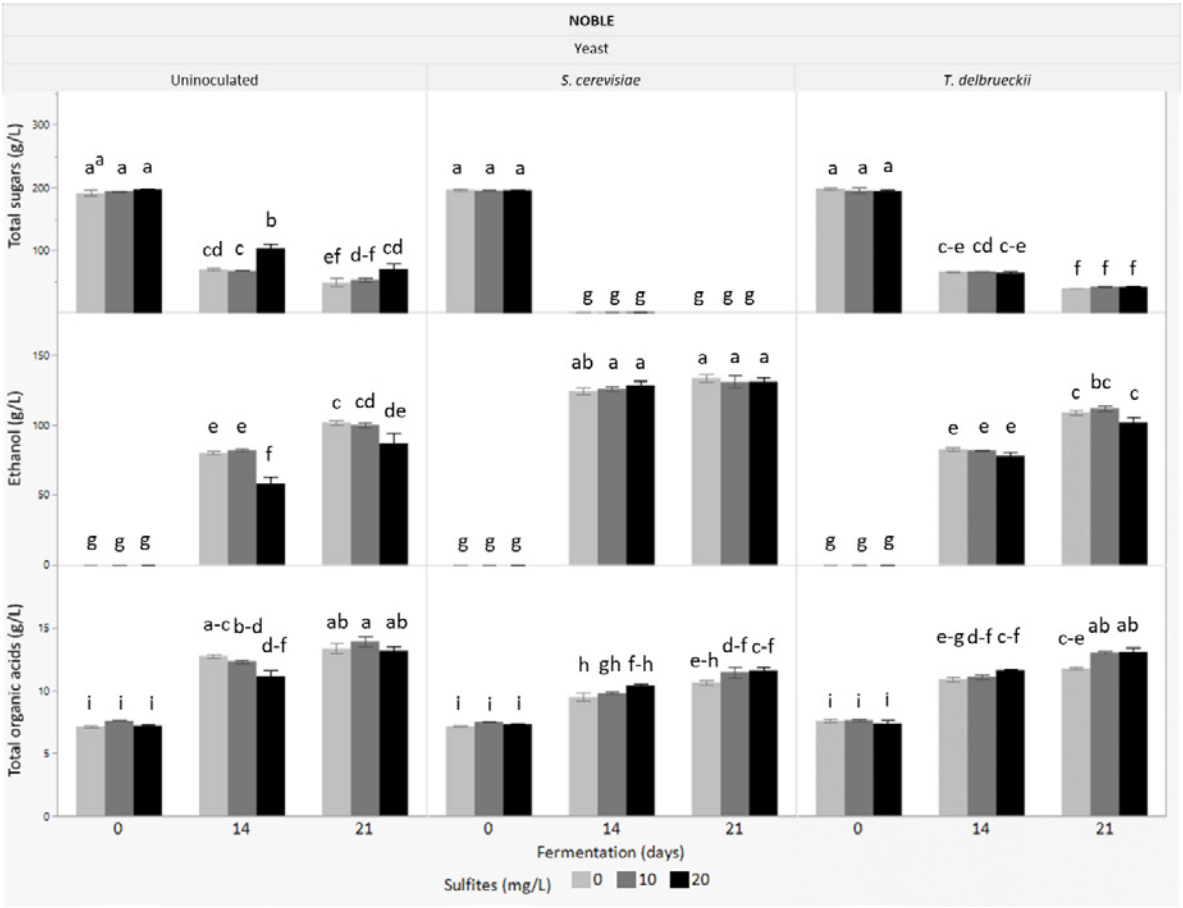

Figure 2 Effects of sulfite levels (SO_2: 0, 10, and 20 mg/L), yeast inoculations (Uninoculated, *Saccharomyces cerevisiae*, and *Torulaspora delbrueckii*), and fermentation time (0, 14, and 21 days) on total sugars, ethanol, and total organic acids in Arkansas-grown Noble juice/wine. [a] Each standard error bar was constructed using 1 standard error from the mean. Means with different letters for each attribute are significantly different (p-value < 0.05) according to Tukey's Honest Significant Difference (HSD) test. Total sugars were calculated as the sum of glucose and fructose. Total organic acids were calculated as the sum of citric, tartaric, malic, succinic, lactic, and acetic acids.

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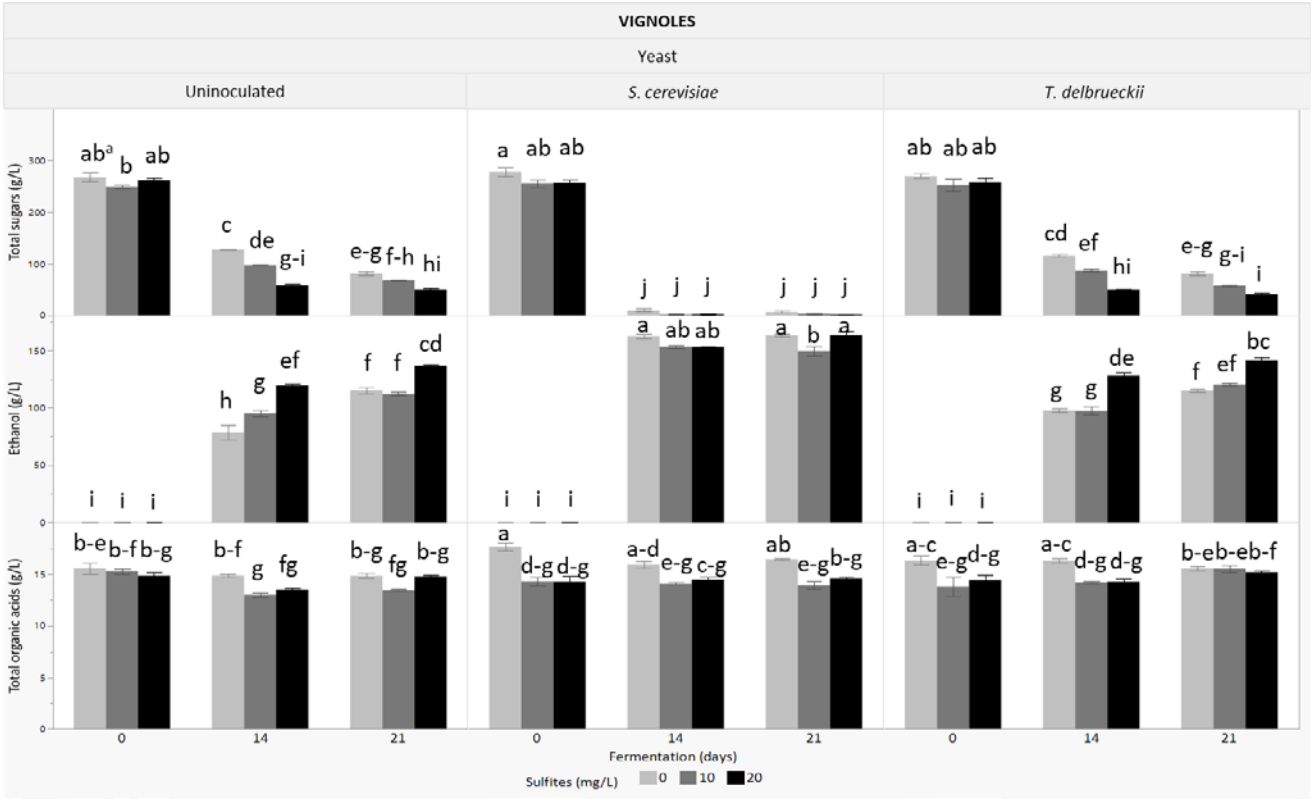

Figure 3 Effects of sulfite levels (SO_2 0, 10, and 20 mg/L), yeast inoculations (Uninoculated, *Saccharomyces cerevisiae*, and *Torulaspora delbrueckii*), and fermentation time (0, 14, and 21 days) on total sugars, ethanol, and total organic acids in Arkansas-grown Vignoles juice/wine. [a] Each standard error bar was constructed using 1 standard error from the mean. Means with different letters for each attribute are significantly different (p-value < 0.05) according to Tukey's Honest Significant Difference (HSD) test. Total sugars were calculated as the sum of glucose and fructose. Total organic acids were calculated as the sum of citric, tartaric, malic, succinic, lactic, and acetic acids.

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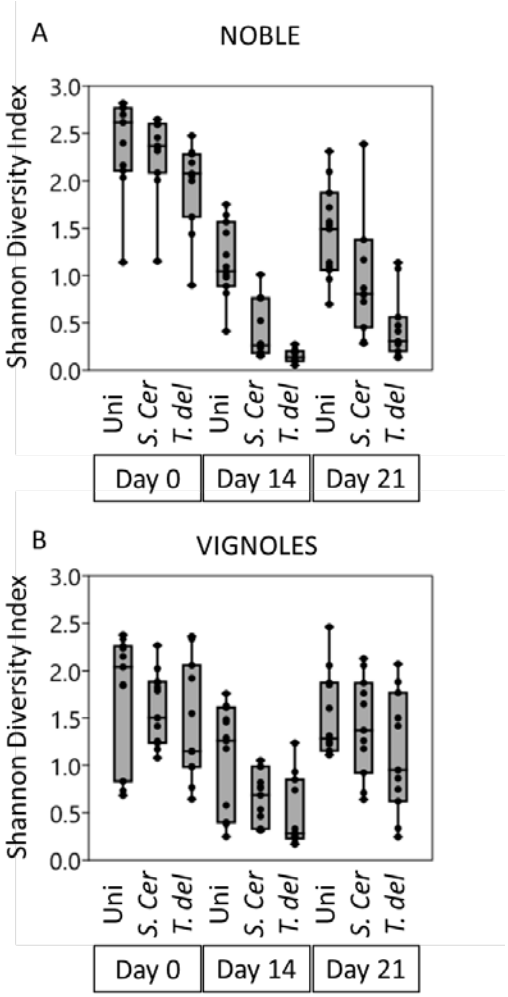

Figure 4 Shannon diversity indices of Arkansas-grown Noble and Vignoles juice/wine at day 0, 14, and 21 of fermentation.
Uni: Uninoculated juice, *S. cer*: *Saccharomyces cerevisiae*-inoculated juice, and *T. del*: *Torulaspora delbrueckii*-inoculated juice.

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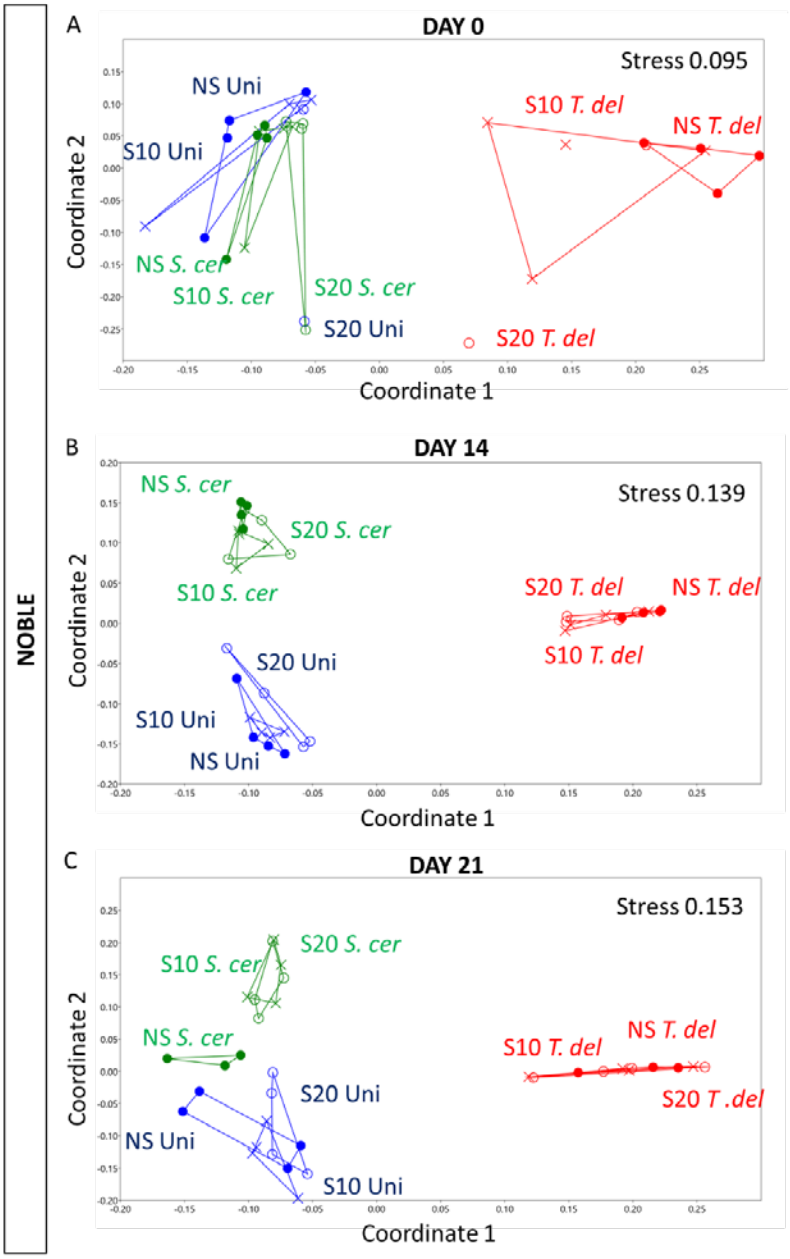

Supplementary Figure 1 Non-metric multidimensional scaling (NMDS) plots based on Bray-Curtis similarity index of the fungal community structures of Noble juice/wine samples at the genus level at day 0 (A), day 14 (B), and day 21 (C) of fermentation.
blue: Uni (Uninoculated juice), green: *S.cer* (*Saccharomyces cerevisiae*-inoculated juice), red: *T.del* (*Torulaspora delbrueckii*-inoculated juice), filled circle: NS (0 mg/L SO_2), cross: S10 (10 mg/L SO_2), circle: S20 (20 mg/L SO_2). One-way ANOSIM based on Bray-Curtis similarity index, Bonferroni-corrected *p*-values: *p*-value = 0.0001 for the three NMDS plots.

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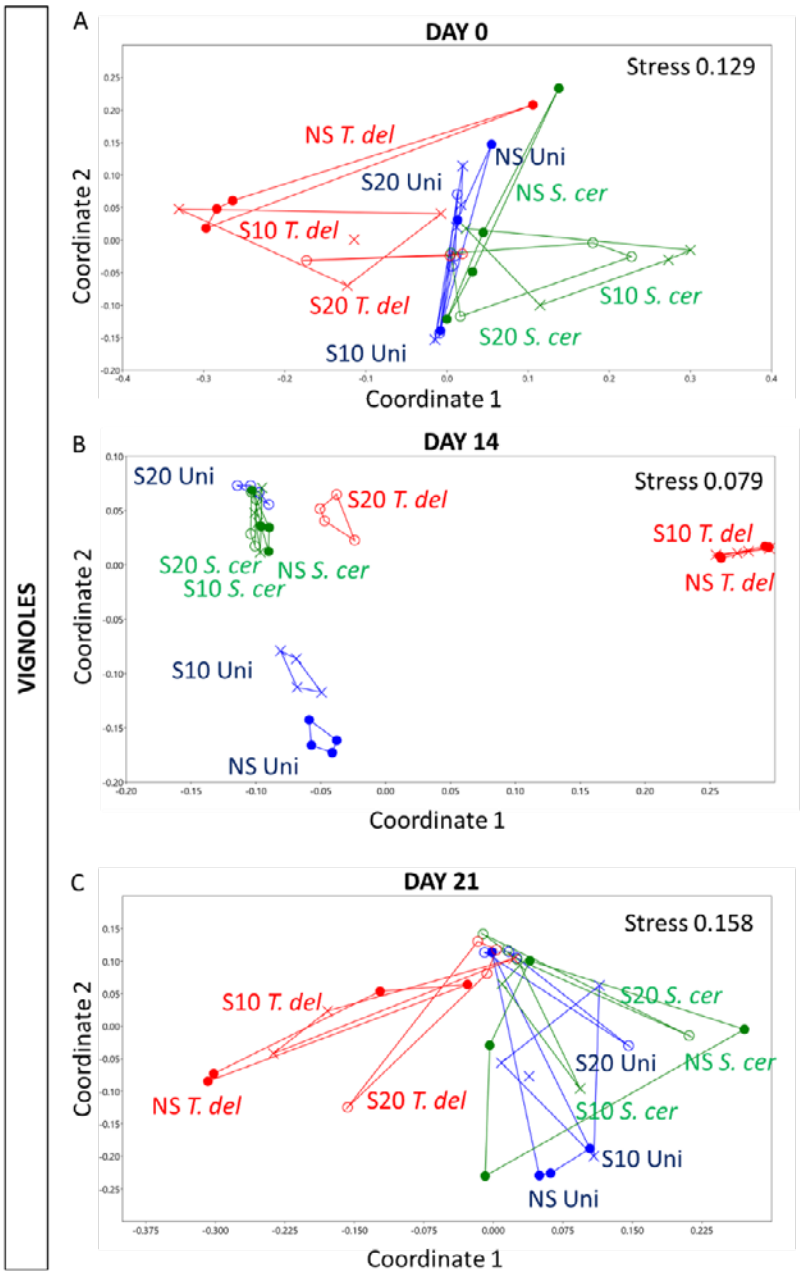

Supplementary Figure 2 Non-metric multidimensional scaling (NMDS) plots based on Bray-Curtis similarity index of the fungal community structures of Vignoles juice/wine samples at the genus level at day 0 (A), day 14 (B), and day 21 (C) of fermentation.
blue: Uni (Uninoculated juice), green: *S.cer* (*Saccharomyces cerevisiae*-inoculated juice), red: *T.del* (*Torulaspora delbrueckii*-inoculated juice), filled circle: NS (0 mg/L SO_2), cross: S10 (10 mg/L SO_2), circle: S20 (20 mg/L SO_2). One-way ANOSIM based on Bray-Curtis similarity index, Bonferroni-corrected *p*-values: *p*-value = 0.0001 for the three NMDS plots.

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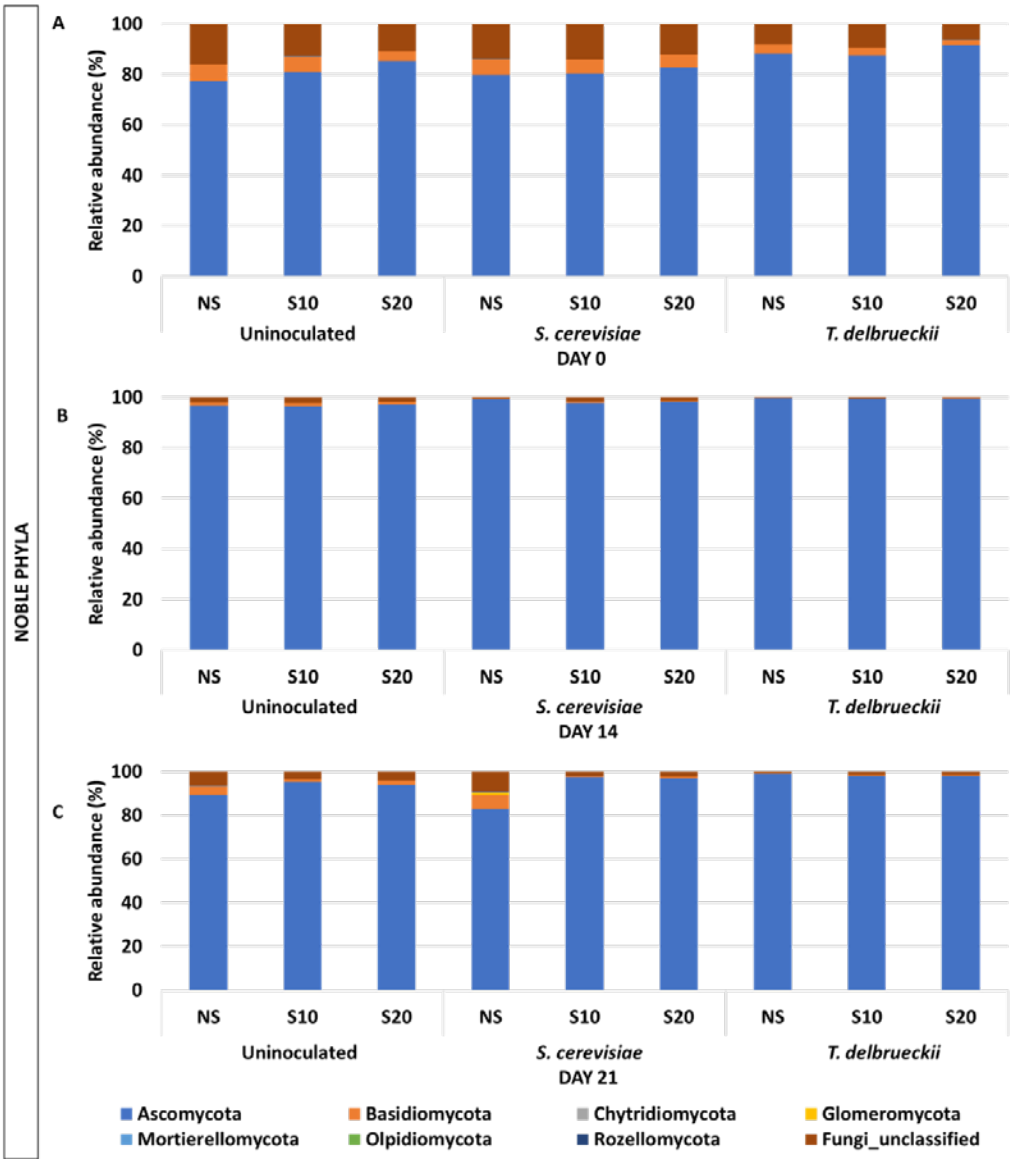

Supplementary Figure 3 Fungal community distribution at the phylum level (relative abundance > 1%) recovered in Noble juice/wine at day 0 (A), day 14 (B), and day 21 (C) of fermentation.
NS: 0 mg/L SO_2, S10: 10 mg/L SO_2, S20: 20 mg/L SO_2, Uninoculated: Uninoculated juice, *S. cerevisiae*: *Saccharomyces cerevisiae*-inoculated juice, *T. delbrueckii*: *Torulaspora delbrueckii*-inoculated juice.

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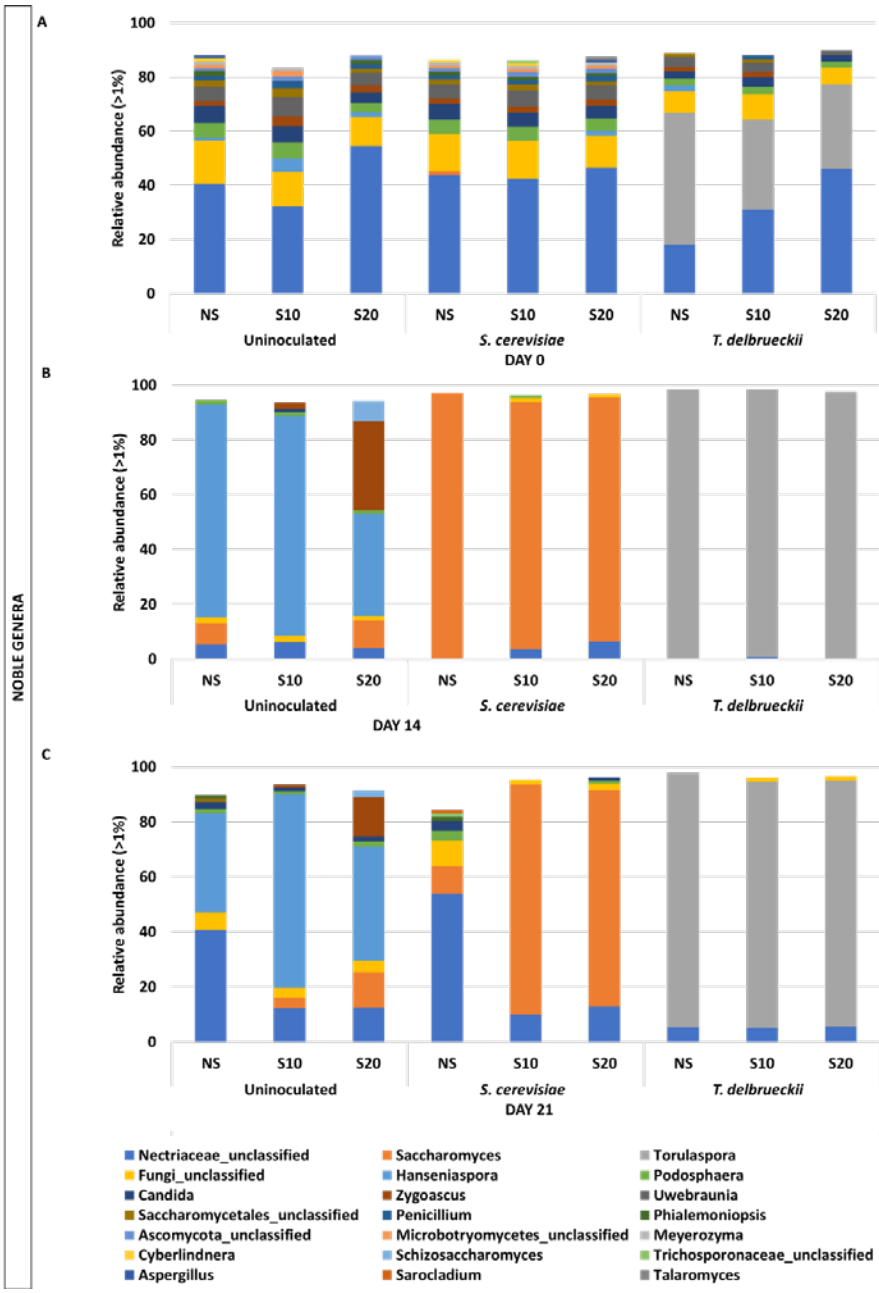

Supplementary Figure 4 Fungal community distribution at the genus level (relative abundance > 1%) recovered in Uninoculated, inoculated with *S. cerevisiae*, and inoculated with *T. delbrueckii* Noble juice/wine at day 0 (A), day 14 (B), and day 21 (C) of fermentation.
NS: 0 mg/L SO_2, S10: 10 mg/L SO_2, S20: 20 mg/L SO_2, Uninoculated: Uninoculated juice, *S. cerevisiae*: *Saccharomyces cerevisiae*-inoculated juice, *T. delbrueckii*: *Torulaspora delbrueckii*-inoculated juice.
Where the assignment to the genus rank failed, the nearest taxonomic level with assignment was reported.

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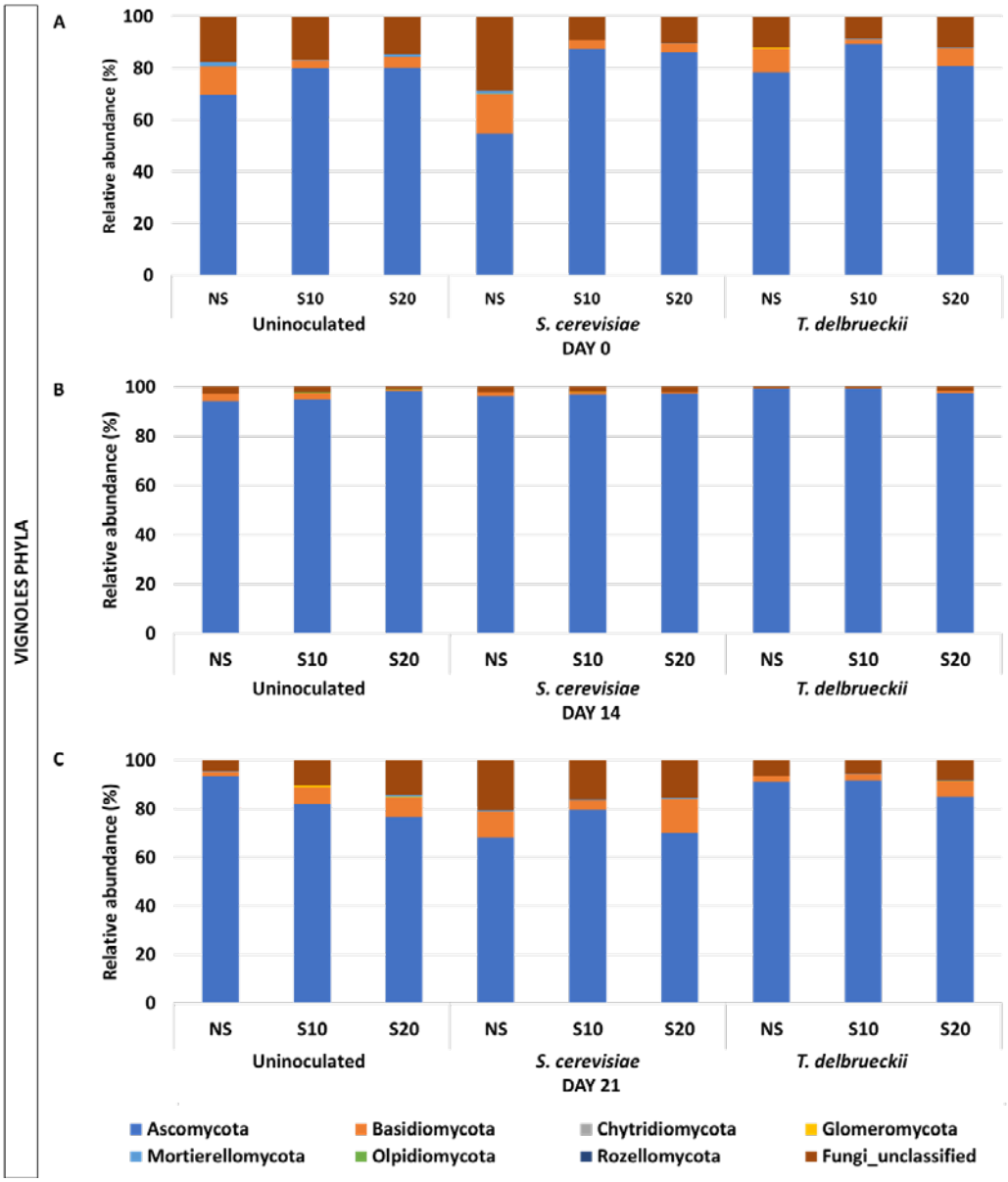

Supplementary Figure 5 Fungal community distribution at the phylum level (relative abundance > 1%) recovered in Vignoles juice/wine at day 0 (A), day 14 (B), and day 21 (C) of fermentation.
NS: 0 mg/L SO_2, S10: 10 mg/L SO_2, S20: 20 mg/L SO_2, Uninoculated: Uninoculated juice, *S. cerevisiae*: *Saccharomyces cerevisiae*-inoculated juice, *T. delbrueckii*: *Torulaspora delbrueckii*-inoculated juice.

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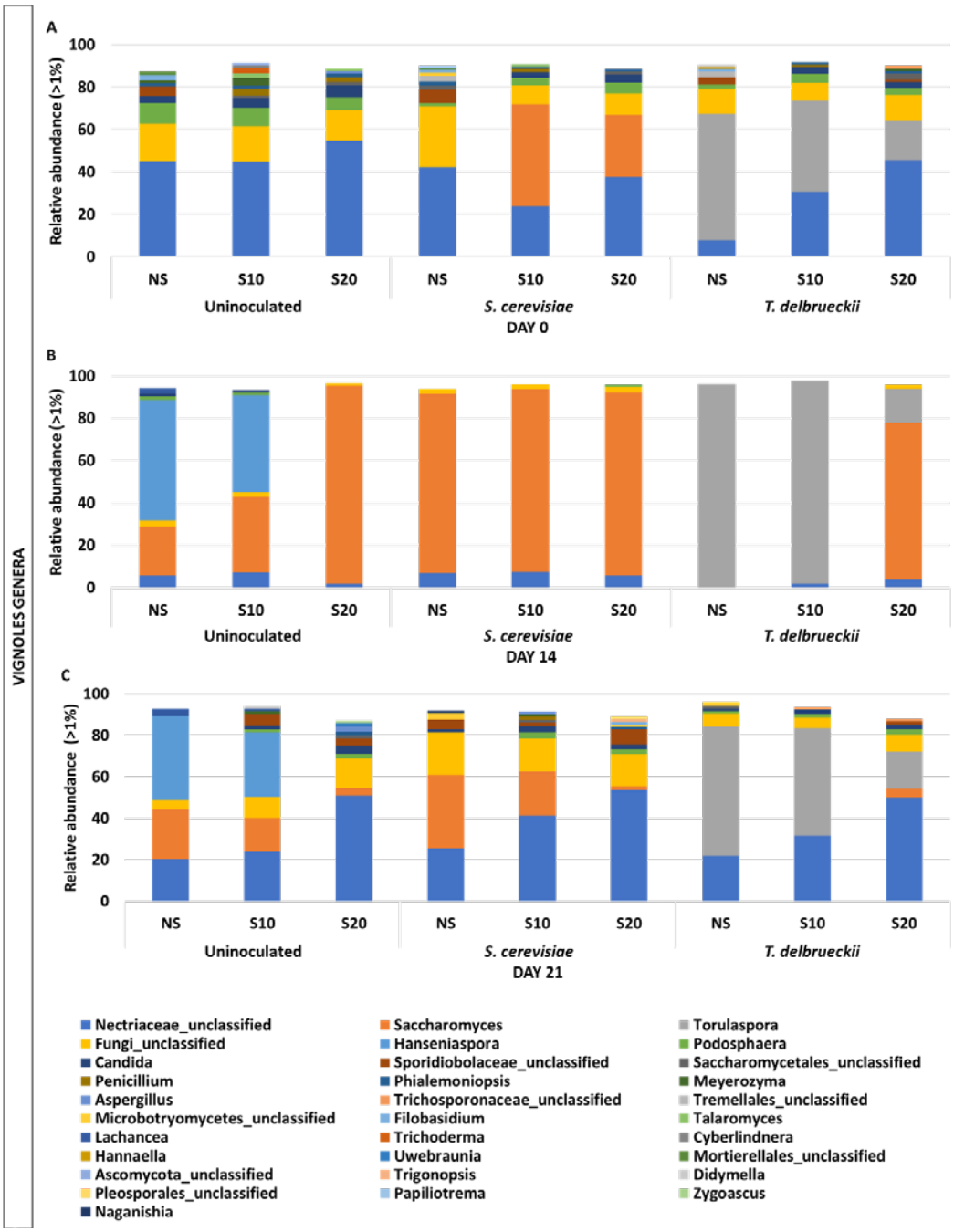

Supplementary Figure 6 Fungal community distribution at the genus level (relative abundance > 1%) recovered in Uninoculated, inoculated with *S. cerevisiae*, and inoculated with *T. delbrueckii* Vignoles juice/wine at day 0 (A), day 14 (B), and day 21 (C) of fermentation.
NS: 0 mg/L SO_2, S10: 10 mg/L SO_2, S20: 20 mg/L SO_2, Uninoculated: Uninoculated juice, *S. cerevisiae*: *Saccharomyces cerevisiae*-inoculated juice, *T. delbrueckii*: *Torulaspora delbrueckii*-inoculated juice. Where the assignment to the genus rank failed, the nearest taxonomic level with assignment was reported.