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

Natacha Cureau,[1] Renee Threlfall,[1]* Franck Carbonero,[1,2] Luke Howard,[1] and Laura Lavefve[1]

**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 in two grape varieties, Noble (*Vitis rotundifolia*) and Vignoles (*Vitis* hybrid), fermented at three sulfite levels (0, 10, and 20 mg/L) with three yeast inoculations (uninoculated, *Saccharomyces cerevisiae*, and *Torulaspora delbrueckii*). Fungal taxonomy of both varieties included six to seven phyla and 115 to 129 genera. The indigenous microbiota was affected by sulfite level and yeast inoculation and varied by grape variety. Sulfite levels had minimal effect on fungal communities but did affect fermentation dynamics. Increasing sulfite additions did not affect the fermentation performance of *S. cerevisiae* but did affect the fermentation of uninoculated juice and *T. delbrueckii*-inoculated juice. The primary fungal genera (*Podosphaera*, *Candida*, *Phialemoniopsis*, and *Meyerozyma*)—those 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 in diversity at day 14 and an increase at day 21 of fermentation. Juices inoculated with *T. delbrueckii* were rapidly colonized by *Torulaspora* spp. at day 0 for both varieties, whereas *Saccharomyces* spp. dominated by day 14 when inoculated with *S. cerevisiae*, especially in Noble. The most abundant genera in uninoculated juice were *Hanseniaspora* and *Zygoascus* for Noble and *Hanseniaspora* and *Saccharomyces* for Vignoles. Understanding grape juice microbial communities and the dynamics of these communities during fermentation provides insight for wine production using spontaneous fermentations or non-*Saccharomyces* species and information on the effect of sulfur dioxide on these novel fermentations.

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

Grape wine fermentations have a dynamic microbial community that changes throughout the fermentation process. In 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. cerevisiae* outcompetes non-*Saccharomyces* species due to higher fermentative efficiency, alcohol tolerance, resistance to low pH, scarce oxygen availability, or depletion of nutrients. To inhibit unwanted microorganisms, winemakers use multiple strategies, such as the 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 fermentations by added commercial yeast strains, spontaneous fermentations occur "naturally" without the addition of commercial yeast or bacteria. Thus, the fermentation is performed by microorganisms naturally present on the grapes, as well as on 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 (Ruiz et al. 2019, Morgan et al. 2020, Roudil et al. 2020). These yeasts can provide characteristics of grapegrowing 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) from

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Acknowledgments: The authors would like to thank Scott Laboratories (Petaluma, CA), Keels Creek Winery (Eureka Springs, AR), and Four Dogs Ranch (Ozark, AR) for the donation of yeasts, grapes, and juice for this study.

Supplemental data is freely available with the online version of this article at www.ajevonline.org.

Manuscript submitted Sept 2020, revised Jan 2021, accepted Feb 2021

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doi: 10.5344/ajev.2021.20054

Lallemand Inc. and *Pichia kluyveri* (FrootZen), *Lachancea thermotolerans* (Concerto), and *T. delbrueckii* (Prelude) from Chr Hansen A/S (Roudil et al. 2020). However, these yeasts typically need to be co-inoculated with *S. cerevisiae*.

At harvest, 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 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 fungi capacities to adapt and survive the stressful conditions of the winery environment (Abdo et al. 2020a, 2020b). These winery-associated fungal consortia can affect grape/must/juice microbiota. Consequently, initial grape juice microbiota will vary, which is why some studies have found different bacterial or fungal species throughout fermentation compared to other studies (Marzano et al. 2016).

The indigenous grape mycobiota detected during the early stages of fermentation generally involve the yeast genera *Hanseniaspora* (anamorph *Kloeckera*), *Metschnikowia*, *Candida*, *Pichia*, and *Issatchenkia*, as well as the 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 codominate must/wine fermentation with *S. cerevisiae* if the fermentation temperature is less than 15 to 20°C (Fleet 2003, Di Maro et al. 2007). *S. cerevisiae* is either undetected in the early stages of fermentation or is detected at a lower relative abundance (percent composition of *S. cerevisiae* relative to total number of yeast communities identified in a sample), but it then outcompetes other yeasts and eventually dominates 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; however, only a small percentage of microorganisms (<1%) can be cultivated and identified on media. Molecular methods, such as sequencing methods, determine the presence of both live and dead microorganisms by DNA detection without the need to culture on media (culture independent). Sequencing has traditionally involved low-throughput sequencing. However, high-throughput sequencing (HTS) can sequence multiple DNA molecules in parallel, allowing the simultaneous sequencing of hundreds of millions of DNA molecules from different samples (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 affect organoleptic properties specific to grapegrowing regions. Recent research has been done using HTS to identify grape/wine microbiota and study the 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). However, only a few studies have focused on the effect of sulfite 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 affected 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 × Pinot de Corton). The changes in juice/wine composition 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 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 performed. $SO_2$ was not added to the 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 the 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 (Figure 1). All labware (flasks, bottles, cylinders, fermentation locks, corks, and caps) for fermentation was autoclaved prior to use.

*Sulfite additions.* From the 18 L of juice, 2 L was placed into each of six 3.7-L glass bottles (two bottles for each $SO_2$ level) (Figure 1). Three concentrations of $SO_2$ (0, 10, and 20 mg/L potassium metabisulfite $K_2S_2O_6$; 57% $SO_2$; Presque Isle Wine Cellar) were added to the bottles. These low levels of sulfites were chosen based on reported $SO_2$ tolerance for the yeasts evaluated in this study. No addition of $SO_2$ (0 mg/L) was used as a control. A concentration of 10 mg/L $SO_2$ was used because it inhibits indigenous microbiota growth but remains below the level that affects *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, the bottles were capped and shaken thoroughly. From each 3.7-L bottle, 500 mL of juice was placed into each of 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 left uninoculated or were 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.) and *S. cerevisiae* (var. *bayanus*) (Lalvin EC-1118; Lallemand Inc.) were used. This specific

*S. cerevisiae* strain was selected because it is used frequently for commercial wine production, whereas *T. delbrueckii* is naturally present on grape skin (van Breda et al. 2013). The yeasts were inoculated according to manufacturers' 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, the yeasts were added to 1.9-L flasks, each containing 500 mL of grape juice at room temperature (24°C). After inoculation, the juice was shaken thoroughly for 1 min to ensure even distribution. The total yeast inoculation levels were 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 a 250-mL Erlenmeyer glass flask (36 flasks in total).

**Fermentation and sampling.** Each flask was sealed with a sterile rubber cork 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. Samples (2 mL) from each flask containing juice/wine were collected aseptically at day 0, 14, and 21 and transferred into sterile 2-mL tubes. These samples were centrifuged at 13,300 rpm for 3 min at 4°C. The pellets were used for microbial analysis, and the juice/wine supernatants were used for compositional analysis. The samples were frozen at -10°C until analysis.

**Composition analysis.** Composition analysis of juice and wine samples was performed. The soluble solids, pH, and titratable acidity (TA) of the juice were measured prior to fermentation. The individual and total sugars, individual and total organic acids, glycerol, and ethanol of the juice/wine were measured prior to and during fermentation.

*Soluble solids.* The levels of soluble solids in the juice were determined using an Abbe Mark II refractometer (Bausch and Lomb, Scientific Instruments) and expressed as percentages.

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

*Sugars, organic acids, ethanol, and glycerol.* The sugars, organic acids, ethanol, and glycerol in the juice/wine were identified and quantified by high-performance liquid chromatography (HPLC). Samples were passed through a 0.45-µm polytetrafluoroethylene syringe filter (Varian, Inc.) before injection onto an HPLC system consisting of a Waters 515 HPLC pump, a Waters 717 plus autosampler, and a Waters 410 differential refractometer detector connected in series with a Waters 996 photodiode array detector (Waters Corporation). Analytes were separated with a Bio-Rad HPLC Organic Acids Analysis Aminex HPX-87H ion-exclusion column (300 × 7.8 mm) connected in series with a Bio-Rad HPLC column for fermentation monitoring (150 × 7.8 mm; Bio-Rad Laboratories). A Bio-Rad Micro-Guard Cation-H refill cartridge (30 × 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 min.

Citric, tartaric, malic, lactic, acetic, and succinic acids were detected at 210 nm by the photodiode array detector. Glucose, fructose, ethanol, and glycerol were detected at 410 nm by the differential refractometer detector. Analytes in

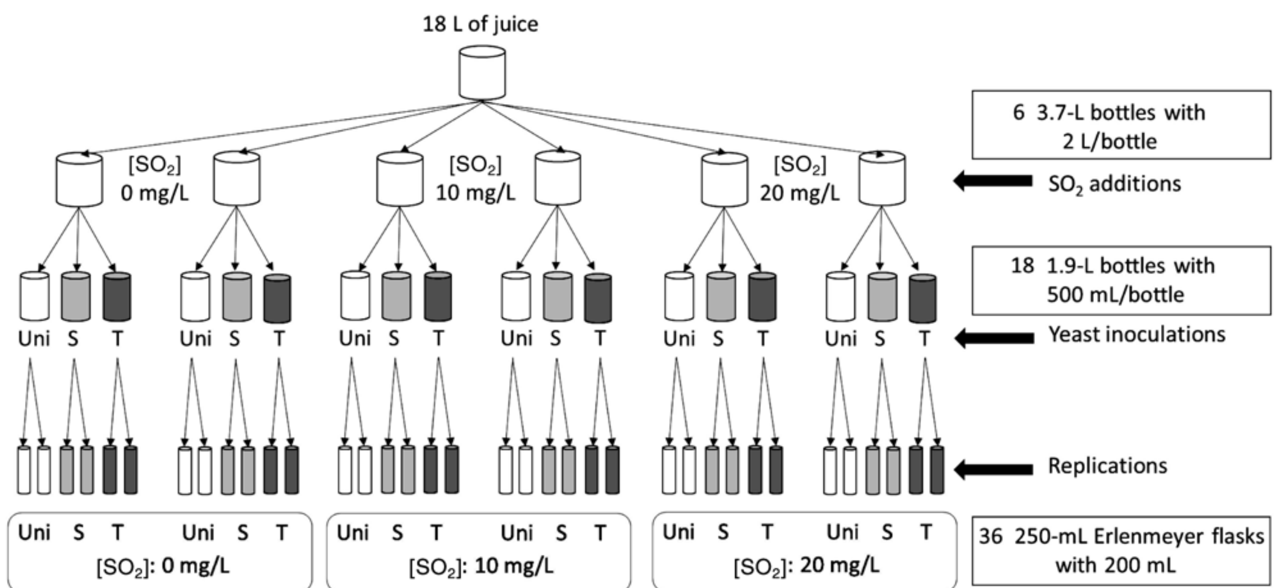


**Figure 1** Flow chart presenting the sulfite levels (0, 10, and 20 mg/L) and yeast inoculation types (uninoculated, *Saccharomyces cerevisiae*, and *Torulaspora delbrueckii*) added to Noble and Vignoles juices. Uni: uninoculated juice, S: *S. cerevisiae*-inoculated juice, T: *T. delbrueckii*-inoculated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

samples were identified and quantified using external calibration curves based on peak area estimation with baseline integration. Results were expressed as g/L of 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 was heated at 70°C for 5 min, vortexed for 15 sec, and transferred into a screwcap tube containing zirconia-silica beads, 0.1 g of 0.1-mm diam beads, and 0.1 g of 0.5-mm-diam beads (BioSpec Products). The cell/bead mixture was homogenized in a FastPrep-24 bead beater (MP Biomedicals) for 1 min at maximum speed. From this point, the DNA extraction was carried out with the QIAamp Fast DNA Stool Mini Kit (Qiagen) starting at step 4 of the manufacturer's instructions. DNA quality was estimated spectrophotometrically using the NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Inc.). Extracted DNA was visualized by electrophoresis on a 2% agarose gel in 1X TAE buffer (AMRESCO). 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 universal PCR (primers ITS1 and ITS4).

An Index PCR targeting the fungal ITS1 locus of 5.8S rRNA gene regions was performed with ITS1 and ITS2 primers using the dual-index strategy for primer design as described (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 for ITS1 and GCT GCG TTC TTC ATC GAT GC for ITS2). PCR reactions (25 µL) were prepared containing 21 µL Master mix (2.5 µL of Buffer II, 0.1 µL of AccuPrime Taq DNA Polymerase High Fidelity, 18.4 µL of water; Invitrogen), 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 a positive control. Reaction conditions consisted of an initial denaturation at 94°C for 2 min, followed by 35 cycles (denaturation at 94°C for 30 sec, primer annealing at 55°C for 30 sec, and extension at 68°C for 1 min), and a final extension at 72°C for 10 min using the Eppendorf Mastercycler pro S (Eppendorf). 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 Normalization Plate Kit (Invitrogen) was used to purify (by eluting short primers, unincorporated dNTPs, enzymes, short failed PCR products, and salts from the PCR reactions) and to normalize the PCR products following the manufacturer's protocol.

The pool concentration was analyzed with Qubit ds DNA High Sensitivity Assay Kit (Invitrogen). The size of the amplicon fragments was determined with an Agilent 2100 Tapestation Bioanalyzer (Agilent Technologies). The amplicon pools were 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 the Illumina Miseq (Illumina, Inc.) platform.

**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 (sulfite levels, yeast inoculations, and fermentation day), an analysis of variance was used to determine the significance of the main factors and interactions. All factors were treated as categorial. Tukey's honest significant difference test was used to detect differences among means ($p <$ 0.05) for the fixed effects. JMP Pro 15.1 (SAS Institute, Inc.) software was used for statistical analysis. The error bars on the figures represent 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 were demultiplexed, quality filtered, and analyzed using the PIPITS pipeline (Gweon et al. 2015). The Shannon Diversity Index was calculated on PAST 3.18 to characterize species diversity in each sample. A Mann-Whitney pairwise test with Bonferroni-corrected $p$ values was performed on species richness to test the effect of the day, sulfite level, and yeast inoculation on juice/wine mycobiota.

Non-metric multidimensional scaling (NMDS) plots and one-way analysis of similarities, both based on the Bray-Curtis similarity index, were also obtained in PAST 3.18 to identify similarities and dissimilarities between the structures of mycobiota. The NMDS plots are presented as supplemental figures and used for Results and Discussion. Differences in fermentation time, sulfite levels, and yeast inoculations were considered significant when $p < 0.05$; 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 analysis of the Noble juice showed 18.2% soluble solids, 0.3% TA, and pH 3.16, whereas the Vignoles juice had 24.2%

soluble solids, 1.03% TA, and a pH of 3.02. In general, *Vitis vinifera* grapes for commercial wine production have 20 to 23% soluble solids, a TA of 0.6 to 0.7%, and a pH < 3.3 to 3.5. The Noble and Vignoles juices had composition values that fall outside of this range but are typical for these varieties when grown in Arkansas. Muscadine grapes often contain lower TA than other winegrapes (Barchenger et al. 2015, Zhang et al. 2017), whereas Vignoles had higher soluble solids and TA (Howell et al. 1991, Wilker et al. 2004). Dry table wine contains 85 to 89% (w/w) water and 9 to 13% ethanol, with the remainder consisting of glycerol, acids, residual sugars, polyphenols, polysaccharides, minerals, and volatile compounds (Waterhouse et al. 2016). Wines typically have glycerol concentrations of 7 to 10 g/L (Waterhouse et al. 2016), and the glycerol levels of all Noble and Vignoles wines were near the typical range of 5 to 6 g/L for Noble and 4 to 8 g/L for Vignoles and did not differ greatly during and after fermentation. The Vignoles juice had a higher level of initial total sugars (263 g/L) than the Noble juice (194 g/L), which resulted in higher 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 had a larger effect than sulfite level on fermentation performance (sugar conversion to ethanol). 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 an increase in ethanol for both varieties, the effect of sulfite levels on fermentation performance was larger for Vignoles than for Noble.

The levels of sugars, glycerol, ethanol, and organic acids in wines after fermentation at day 21 are presented in Table 1. Total sugars are presented as the sum of glucose and fructose, and these residual sugars varied by treatment. For both grape varieties, 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), resulting in 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). The glycerol levels of wine at 21 days of fermentation for both grape varieties were low (~4 to 8 g/L), and total organic acids ranged from 11 to 17 g/L. Total organic acids consisted of the sum of citric, tartaric, malic, succinic, lactic, and acetic acids, and these individual acids varied by treatment. The fermentation performance of Vignoles and Noble juice/wine will be described in the following sections.

*Noble juice/wine.* Sugars of Noble. The initial total sugar levels of the Noble juice prior to fermentation (day 0) were

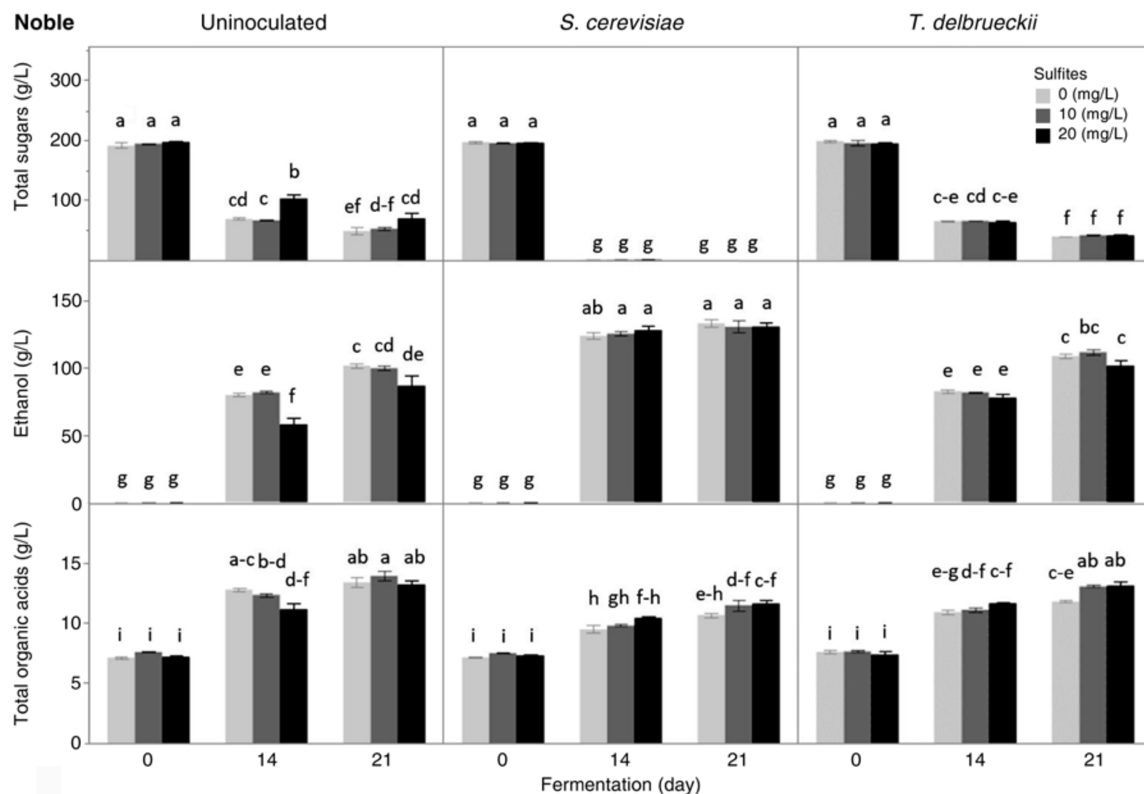


**Figure 2** Effects of sulfite levels ($SO_2$: 0, 10, and 20 mg/L), yeast inoculation type (uninoculated, *Saccharomyces cerevisiae*, and *Torulaspora delbrueckii*), and fermentation time (0, 14, and 21 days) on total sugars, ethanol, and total organic acids in Arkansas-grown Noble juice/wine. Each standard error bar was constructed using 1 standard error from the mean. Means with different letters for each attribute are significantly different ($p < 0.05$) according to Tukey's honest significant difference 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.

191 to 198 g/L (Figure 2). The sulfite levels affected total sugars in the uninoculated juice/wine at days 14 and 21. On both days, 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 to 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 of 49 to 71 g/L, indicating that fermentation was incomplete or "stuck". Total sugar levels of wine at day 21 in uninoculated juice with 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, the 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 (total sugars < 2 g/L). The total sugar content of *S. cerevisiae*-inoculated juice/wine at day 21 was less than 0.4 g/L regardless of $SO_2$ level. At day 21, *S. cerevisiae*-inoculated juice had no fructose and very little glucose (<0.35 g/L) regardless of $SO_2$ level (Table 1).

For juice inoculated with *T. delbrueckii*, total sugars had dropped to 64 to 67 g/L at day 14. At day 21, total sugars in *T. delbrueckii*-inoculated juice/wine had further dropped to 40 to 42 g/L, indicating that the fermentation was incomplete. Total sugar levels of *T. delbrueckii*-inoculated juice/wine at day 21 at sulfite levels of 0, 10, and 20 mg/L were 40, 42, and 42 g/L, respectively. The lower growth rates of *T. delbrueckii* species compared to *S. cerevisiae*, as well as their inability to consume all available sugars, have previously been demonstrated (Bely et al. 2008). Regardless of $SO_2$ level, *T. delbrueckii*-inoculated juice at day 21 had more fructose (28 to 29 g/L) remaining than glucose (12 to 13 g/L) (Table 1).

<u>Ethanol of Noble.</u> The sulfite levels showed an effect on 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 $SO_2$ ($SO_2$ 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 in 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 to 128 g/L) with similar levels at day 21 (131 to 133 g/L) and no significant difference between sulfite levels. On day 14, *S. cerevisiae*-inoculated juice contained more ethanol (124 to 128 g/L) than *T. delbrueckii*-inoculated juice (78 to 83 g/L) and uninoculated (58 to 82 g/L) juice. Ethanol levels at day 21 in *S. cerevisiae*-inoculated juice at 0, 10, and 20 mg/L sulfite were 133, 131, and 131 g/L, respectively. The larger increase in ethanol and decrease in total sugars during fermentation confirmed that *S. cerevisiae* had better conversion efficiency.

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

<u>Organic acids of Noble.</u> Total organic acid levels of the Noble juice increased during fermentation (Figure 2). At day 0, total organic acid levels were 7 to 8 g/L. From day 0 to day 14, total organic acids increased in all three inoculation treatments of the juices. The largest increase was observed for uninoculated juice (11 to 13 g/L), followed by

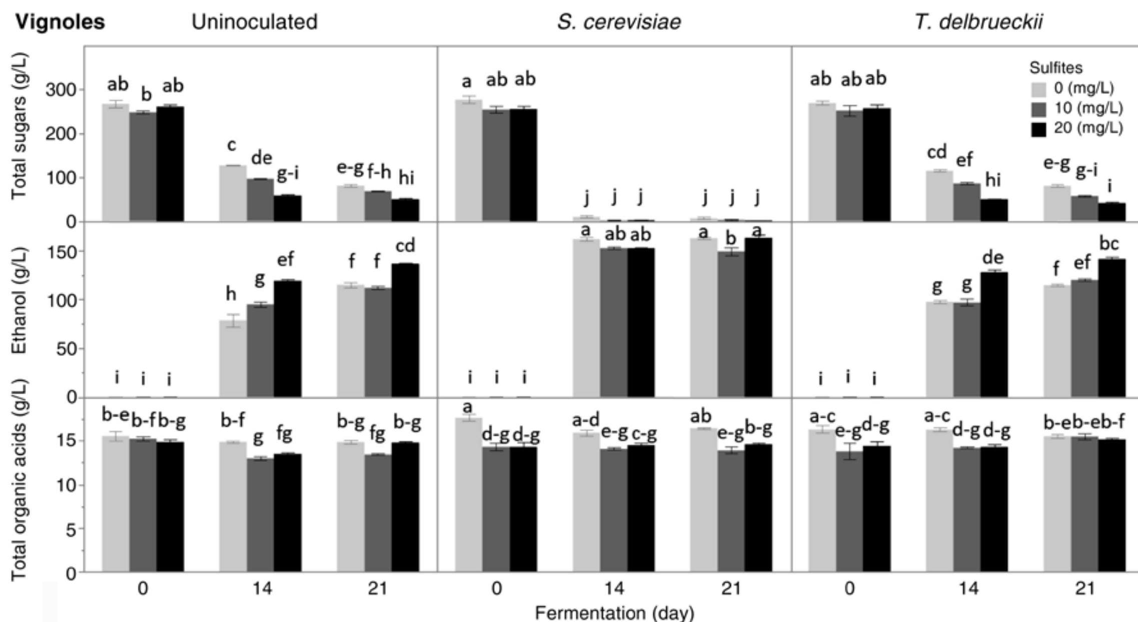


**Figure 3** Effects of sulfite levels ($SO_2$ 0, 10, and 20 mg/L), yeast inoculation types (uninoculated, *Saccharomyces cerevisiae*, and *Torulaspora delbrueckii*), and fermentation time (0, 14, and 21 days) on total sugars, ethanol, and total organic acids in Arkansas-grown Vignoles juice/wine. Each standard error bar was constructed using 1 standard error from the mean. Means with different letters for each attribute are significantly different ($p <$ 0.05) according to Tukey's honest significant difference 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.

**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 types (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** | | | | | | | | | |
| ***Composition*** | | | | | | | | | |
| 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 | 12.70 ± 0.77 |
| Fructose | 27.71 ± 3.49 | 29.39 ± 3.64 | 36.60 ± 5.06 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 | 28.21 ± 0.55 | 29.56 ± 1.92 | 29.20 ± 1.81 |
| Total sugars[b] | 48.88 ± 10.86 | 53.62 ± 4.56 | 70.79 ± 16.1 | 0.35 ± 0.00 | 0.33 ± 0.02 | 0.35 ± 0.01 | 39.78 ± 0.75 | 42.12 ± 2.83 | 41.90 ± 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 | 108.80 ± 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 | 1.30 ± 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 | 2.10 ± 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 | 1.80 ± 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%) |
| | *Hanseniaspora* (36.1%) | *Nectriaceae_ unclassified* (12.2%) | *Zygoascus* (14.3%) | *Saccharomyces* (10%) | *Nectriaceae_ unclassified* (9.9%) | *Nectriaceae_ unclassified* (12.8%) | *Nectriaceae_ unclassified* (5.4%) | *Nectriaceae_ unclassified* (5.1%) | *Nectriaceae_ unclassified* (5.5%) |
| | *Candida* (2.7%) | *Saccharomyces* (3.9%) | *Saccharomyces* (12.7%) | *Candida* (3.7%) | | *Candida* (1.2%) | | | |
| | *Podosphaera* (1.5%) | *Candida* (1.4%) | *Nectriaceae_ unclassified* (12.5%) | *Podosphaera* (3.6%) | | *Podosphaera* (1%) | | | |
| | Saccharomycetales_ unclassified (1.3%) | *Zygoascus* (1.2%) *Podosphaera* (1.2%) | *Schizosaccharomyces* (2.5%) | *Phialemoniopsis* (1.3%) | | | | | |
| **Vignoles** | | | | | | | | | |
| ***Composition*** | | | | | | | | | |
| 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 | 16.40 ± 1.49 | 5.18 ± 1.13 |
| Fructose | 60.39 ± 4.20 | 52.43 ± 0.80 | 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 | 6.70 ± 3.70 | 2.98 ± 2.67 | 1.63 ± 0.28 | 80.97 ± 5.10 | 57.72 ± 3.70 | 41.50 ± 4.99 |
| Glycerol | 5.29 ± 0.15 | 4.27 ± 0.08 | 4.94 ± 0.02 | 7.40 ± 0.14 | 6.69 ± 0.39 | 7.19 ± 0.16 | 4.90 ± 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 | 141.70 ± 3.50 |
| Citric acid | 3.53 ± 0.20 | 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 | 3.00 ± 0.19 | 3.21 ± 0.12 | 3.32 ± 0.14 | 3.60 ± 0.08 | 3.16 ± 0.10 |
| Lactic acid | 1.43 ± 0.10 | 1.10 ± 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 |
| 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 | 0.50 ± 0.07 | 0.86 ± 0.47 | 0.66 ± 0.11 |
| Total organic acids[b] | 14.90 ± 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 four 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%.

juice inoculated with *T. delbrueckii* (11 to 12 g/L), and then *S. cerevisiae* (10 to 11 g/L). In uninoculated juice, the lower the sulfite levels, the higher the total organic acids produced at day 14. In uninoculated juice at day 21, there were no differences among the 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 organic acid levels were higher in juice with 10 and 20 mg/L sulfite (13 g/L) than in 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 most prominent acid (3.6 to 4.9 g/L); in the *T. delbrueckii*-inoculated juices, succinic acid levels were also high (3.1 to 3.3 g/L) (Table 1).

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

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

Most of the sugars were fermented in the *S. cerevisiae*-inoculated juice after 14 days of fermentation, with total sugar levels of 2 to 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 sugar levels 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 to 0.2 g/L), as were fructose levels (2 to 6 g/L) (Table 1).

At day 14, total sugar levels of *T. delbrueckii*-inoculated juice at 0, 10, and 20 mg/L sulfite had decreased (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 sugar levels of wine from *T. delbrueckii*-inoculated juice with 0, 10, and 20 mg/L sulfite were 81, 58, and 42 g/L, respectively, indicating that the fermentation was incomplete. In *T. delbrueckii*-inoculated juice at day 21, regardless of $SO_2$ level, fructose levels (36 to 56 g/L) were higher than glucose levels (5 to 25 g/L) (Table 1).

Ethanol of Vignoles. At day 14, ethanol concentration had increased for all three yeast inoculations, with *S. cerevisiae* (153 to 162 g/L) having better fermentation performance than *T. delbrueckii* (97 to 128 g/L) and uninoculated (79 to 119 g/L) juices (Figure 3).

At day 14, ethanol levels in uninoculated juice differed 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 for uninoculated juice, higher levels of ethanol were observed at the highest level of sulfites ($SO_2$ 0 mg/L: 115 g/L, $SO_2$ 10 mg/L: 112 g/L, $SO_2$ 20 mg/L: 137 g/L).

For *S. cerevisiae*-inoculated juice, there were no differences 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, a larger increase in ethanol and decrease in total sugar levels during fermentation confirmed that *S. cerevisiae* had the best conversion efficiency in Vignoles juice.

Juice inoculated with *T. delbrueckii* had its highest level of ethanol when inoculated with the highest level of sulfite at day 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 day 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 of Vignoles. The level of total organic acids in Vignoles juice at day 0 was 14 to 18 g/L. The fermentation pattern of Vignoles was different from that of Noble as there was not an increase of total organic acids during fermentation. In general, regardless of yeast inoculation treatment, total organic acid levels were slightly higher in juices/wines without sulfites compared to those with sulfites during fermentation, but this difference was not always significant. This can be explained by the fact that the absence of sulfites allows the growth of lactic and acetic acid bacteria, which affect organic acid levels (Bokulich et al. 2015). Total organic acid levels differed more with sulfite level in the Vignoles fermentation than in the Noble fermentation. In all inoculation treatments of juice at day 21, regardless of $SO_2$ level, malic acid was the most prominent acid (5.2 to 6.4 g/L). The second-highest acids varied by inoculation treatment and was also impacted by $SO_2$ level (Table 1).

**Sequence analysis of juice/wine.** The fungal diversity analysis of the Noble and Vignoles juices/wines generated 529 and 418 Operational Taxonomic Units, respectively. About four samples from each variety were removed from the analysis due to either a low number of sequence reads (<400 sequences) or dissimilarities compared to replicates. The fungal taxonomic composition of the Noble juice/wine included seven phyla (relative abundance of the fungal communities: Ascomycota, 92.2%; Basidiomycota, 2.3%; and Chytridiomycota, Glomeromycota, Mortierellomycota, Olpidiomycita, and Rozellomycota combined, 0.1%) and 129 genera. The fungal taxonomic composition of the Vignoles juice/wine included six phyla (relative abundance of the fungal communities: Ascomycota, 85.8%; Basidiomycota,

4.6%; and Chytridiomycota, Glomeromycota, Mortierellomycota, and Olpidiomycita combined, 0.4%) and 115 genera. Unknown sequences (Fungi _unclassified) represented 5.4% and 9.1% in Noble and Vignoles, respectively, meaning 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 to be a major phylum in juice and spontaneous wine fermentations in New Zealand (92.1% of all sequences), followed by Basidiomycota (0.4%), and unknown sequences (7.5%) but did not identify more phyla. The percentage of unknown sequences were similar to those found in Vignoles and Noble. The data at the genus and phylum levels will be further discussed for the two grape varieties, and data for the phylum level is shown in the supplemental figures.

**Indigenous fungal communities of juice from the two grape varieties.** The indigenous fungal communities of the Noble and Vignoles grape varieties were identified from the juice of grapes prior to fermentation. The fungal genera with a relative abundance higher than 1% in each variety 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 higher abundance in Vignoles (9.5%) than in Noble (5.5%). *Candida* was also present in both grape varieties, with a higher abundance in Noble (6.3%) than in Vignoles (3.4%). Noble juice harbored abundant numbers of *Uwebraunia* (5.2%) and *Zygoascus* (1.8%). These two genera were either not present or present at a low relative abundance in Vignoles (*Uwebraunia*, 0.4% and *Zygoascus*, not detected). In contrast, Vignoles juice harbored a higher relative abundance of *Filobasidium* (2.4%) compared to Noble (0.2%).

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

| | Variety of grape | |
|---|---|---|
| Fungi[a] | 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 is highlighted. When the assignment to genus rank failed, the nearest taxonomic level with assignment was reported.

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 higher 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 because the grapes are smaller and in tighter clusters than Noble grapes.

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

**Fungal diversity and successions during fermentation.** Regardless of yeast treatment, 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 increased only slightly between days 14 and 21, whereas for Vignoles, diversity returned to levels comparable to initial fermentation. This indicated that the indigenous fungi of Vignoles were more resilient to the fermentation processes, even in the presence of yeast inoculations. This observation may drive variety-specific organoleptic properties through secondary metabolic processes. Because the indigenous grape microbiota and diversity of the Noble and Vignoles juices/wines differed, the dynamics of the fungal communities during fermentation were analyzed separately.

Although NMDS plots are typically used to visualize the level of similarity in a data set, 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 by type of yeast inoculation (uninoculated, *S. cerevisiae*, and *T. delbrueckii*) rather than by sulfite level, more so in Noble than Vignoles (Supplemental Figures 1 and 2).

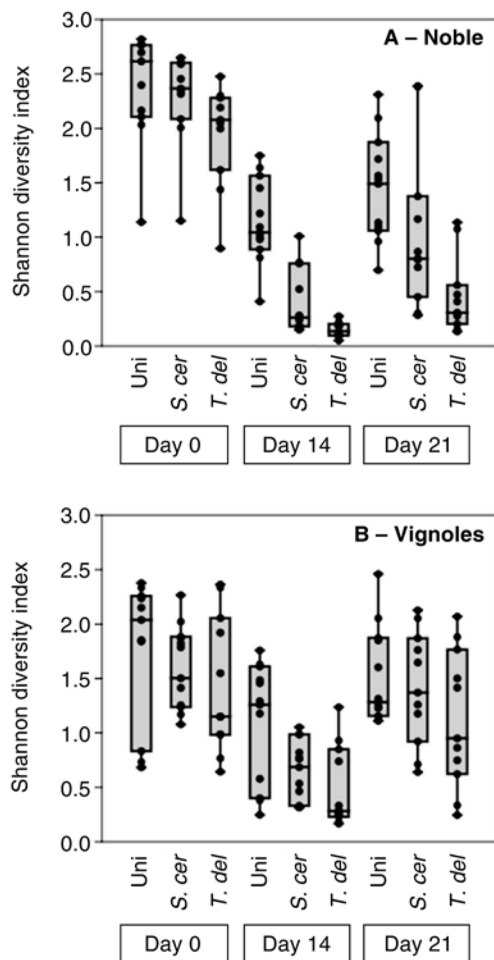


**Figure 4** Shannon Diversity Indices of Arkansas-grown Noble (**A**) and Vignoles (**B**) juice/wine at day 0, 14, and 21 of fermentation. Uni: uninoculated juice, *S. cer*: *Saccharomyces cerevisiae*-inoculated juice, and *T. del*: *Torulaspora delbrueckii*-inoculated juice.

*Noble fungal community dynamics during fermentation.* Beginning fermentation of Noble. At day 0 of fermentation, the fungal communities in Noble juice clustered by the type of yeast inoculation (uninoculated, *S. cerevisiae*, and *T. delbrueckii*), with *S. cerevisiae* and uninoculated clustered together and apart from juice inoculated with *T. delbrueckii* (Supplemental Figure 1).

The fungal profiles at the phylum level were similar among the three types of inoculations and were dominated by the Ascomycota phylum (81.3% for uninoculated, 81% for *S. cerevisiae*, and 89.2% for *T. delbrueckii*), followed by the Basidiomycota phylum (5.4% for uninoculated, 5.7% for *S. cerevisiae*, and 2.9% for *T. delbrueckii*) (Supplemental Figure 3). Unclassified Fungi represented 13.2, 13.2, and 7.9% of the fungal communities of uninoculated, *S. cerevisiae*-inoculated, and *T. delbrueckii*-inoculated juices, respectively. A higher relative abundance of Ascomycota and smaller relative abundance of Fungi_unclassified and Basidiomycota were detected in *T. delbrueckii*-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 profiles at the genus level presented dissimilarities between the *T. delbrueckii*-inoculated juice and both the uninoculated and the *S. cerevisiae*-inoculated juices (Table 3 and Supplemental Figure 4). At day 0, the dominant fungi identified in the uninoculated and *S. cerevisiae*-inoculated juices were similar. For instance, regardless of sulfite level, the predominant fungi were *Nectriaceae*_unclassified (42.5 and 44.3% for uninoculated and *S. 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 by *Nectriaceae*_unclassified (31.7%), *Candida* (3%), *Uwebraunia* (2.9%), and *Podosphaera* (2.5%). Moreover, fungal profiles varied slightly between sulfite levels, 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 inoculation clustered apart from one another. The three sulfite levels for each type of yeast inoculation also clustered together (Supplemental Figure 1).

The fungal profiles at the phylum level were similar among the three types of inoculations (Supplemental 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 uninoculated juice/wine, juice/wine inoculated with *S. cerevisiae*, and juice/wine inoculated with *T. delbrueckii*, respectively. No

significant dissimilarities were observed between the three sulfite levels for the three types of inoculated juice.

However, the fungal profile at the genus level (Table 3 and Supplemental Figure 4) differed between the three inoculation types. Noble juice/wine inoculated with *T. delbrueckii* contained more than 97.8% *Torulaspora* spp. No differences were observed between the three sulfite levels. Juice inoculated with *S. cerevisiae* contained more than 92% of the genus *Saccharomyces*. Sulfite addition slightly modified the composition of fungal communities, with an increase in

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

| | Treatments | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Uninoculated[a] | | | *Saccharomyces cerevisiae* | | | *Torulaspora delbrueckii* | | |
| **Day/Fungi**[b] | **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 |
| *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]Yeast inoculation types were uninoculated, *S. cerevisiae*, and *T. delbrueckii.*
[b]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.
[c]Sulfur dioxide (as potassium metabisulfite) levels were 0 mg/L (NS), 10 mg/L (S10), and 20 mg/L (S20).

*Nectriaceae*_unclassified (0.95, 3.6, and 6.5% for 0, 10, and 20 mg/L of sulfites, respectively) and a decrease in *Saccharomyces* (97.1, 90.1, and 88.9%, respectively) observed with increasing sulfite levels.

However, uninoculated Noble juice (representing spontaneous fermentation) was dominated by *Hanseniaspora* (65.1%), followed by *Zygoascus* (11.8%) and *Saccharomyces* (6.1%). Sulfite levels played an important part in this fermentation; the higher the sulfite level, the higher the relative abundance of *Zygoascus* (0.21 to 32.5% for 0 and 20 mg/L of sulfites, respectively) and *Schizosaccharomyces* (0.1 to 7.4% for 0 and 20 mg/L of sulfites, respectively), and the smaller the relative abundance of *Hanseniaspora* (77.8 to 32.5% for 0 and 20 mg/L of sulfites, respectively). During the first stages of spontaneous fermentation, *Hanseniaspora* spp. are known to be the dominant non-*Saccharomyces* yeast species, along with *Issatchenkia* spp. and *Candida* spp., and to be able to coexist with *S. cerevisiae* at later stages of fermentation (Fleet 2003, Di Maro et al. 2007, Pinto et al. 2015, Portillo and Mas 2016, De Filippis et al. 2017, Raymond Eder et al. 2018, Morgan et al. 2019b). The *Hansenispora* genus can represent up to 75% of the total initial microbiota and, during fermentation, can be up to 99% of the total yeast communities (Cioch-Skoneczny et al. 2018). This yeast is generally undesirable in fermentation due to the production of large concentrations of ethyl and amyl acetates, glycerol, and acetic acid that negatively alter wine flavors and aroma (Johnson et al. 2020). However, some *Hanseniaspora* species, such as *H. vineae*, when inoculated in mixed fermentation with *S. cerevisiae*, can positively effect 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 clustered by type of yeast inoculation (uninoculated, *S. cerevisiae*, and *T. delbrueckii*) (Supplemental Figure 1). The three sulfites levels 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 inoculation (Supplemental Figure 3). An increase in relative abundance of Basidiomycota and Fungi_unclassified and a decrease of Ascomycota appeared in the three types of inoculation (uninoculated, *S. cerevisiae*, and *T. delbrueckii*), especially in uninoculated wine and wine inoculated with *S. cerevisiae.* The sulfite additions had an impact on fungal communities for uninoculated and *S. cerevisiae*-inoculated wines. The lower the sulfite level, the higher 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 Supplemental Figure 4) presented some variation between days 14 and 21. Overall, day 21 showed a decrease in the predominant fungi of day 14 (uninoculated: decrease of *Hanseniaspora* from 65.1 to 49.3%, *S. cerevisiae*-inoculated: decrease of *Saccharomyces* from 92 to 57.5%, *T. delbrueckii*-inoculated: decrease of *Torulaspora* from 97.8 to 90.3%) and an increase in the relative abundance of *Nectriaceae*_unclassified (from day 14 to day 21: 5.2 to 21.8% for uninoculated, 3.7 to 25.5% for *S. cerevisiae*, and 0.75 to 5.4% for *T. delbrueckii*).

Wines inoculated with *T. delbrueckii* did not show significant dissimilarities in fungal profiles between the three sulfite levels. However, wines inoculated with *S. cerevisiae* showed an increase in other fungi, such as *Candida* (0.5 to 1.9%) and *Podosphaera* (0.8 to 1.8%). Important dissimilarities in fungal profiles appeared between wines inoculated with *S. cerevisiae* at different sulfite levels. Compared to *S. cerevisiae*-inoculated wines with sulfites, wine with no added sulfites had a lower relative abundance of *Saccharomyces* ($SO_2$ 0 mg/L: 10%, $SO_2$ 10 mg/L: 83.6%, and $SO_2$ 20 mg/L: 78.8%) and a higher relative abundance of *Nectriaceae*_unclassified ($SO_2$ 0 mg/L: 54%, $SO_2$ 10 mg/L: 10%, and $SO_2$ 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: 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%).

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

Higher levels of sulfites promoted *Saccharomyces* growth in both uninoculated and *S. cerevisiae*-inoculated wines. Wine inoculated with *T. delbrueckii* maintained a high relative abundance of *Torulaspora* throughout fermentation (day 0 to day 21), with a higher relative abundance than that of *Saccharomyces* in wine inoculated with *S. cerevisiae*. This confirmed that *T. delbrueckii*, a yeast naturally found in vineyards, can be a good candidate for producing wines with specific terroir flavors (Azzolini et al. 2012, 2015, Cordero-Bueso et al. 2013, Roudil et al. 2020). 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 the 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 at 10 and 20 mg/L $SO_2$ were *Nectriaceae*_unclassified and *Hanseniaspora*, respectively. The main fungi detected in *S. cerevisiae*-inoculated juice at 0 mg/L $SO_2$ and at 10 and 20 mg/L $SO_2$ were *Nectriaceae*_unclassified and *Saccharomyces*, respectively. The main fungal genera of *T. delbrueckii*-inoculated juice at all $SO_2$ levels was *Torulaspora*.

*Vignoles fungal community 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*) (Supplemental Figure 2).

The fungal profiles at the phylum level varied between the three types of inoculations and the three sulfite levels (Supplemental Figure 5). Overall, as in Noble juice, fungal communities of the three inoculation types in 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 the 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, *S. cerevisiae*-inoculated, and *T. delbrueckii*-inoculated Vignoles wines, respectively. A higher relative abundance of Ascomycota was observed for all three types of inoculation when sulfites were added to the juices.

The fungal profiles at the genus level (Table 4 and Supplemental Figure 6) presented dissimilarities between the three types of inoculated Vignoles juices and varied with sulfite level for each type of inoculated juice. A day 0, the five most abundant fungi identified in uninoculated Vignoles juice, regardless of sulfite level, were *Nectriaceae*_unclassified (48.3%), *Podosphaera* (8%), *Candida* (4.7%), *Meyerozyma* (2%), and *Penicillium* (2%). Differences in sulfite level slightly affected the relative abundance of fungal communities, mainly those present at lower abundance in uninoculated juice. The Vignoles juice had a high soluble solids level (24.8%) that could have impacted the initial microbial communities.

For juice inoculated with *S. cerevisiae*, a clear distinction between fungal profiles appeared at day 0 and was dependent on sulfite level. Juice inoculated with *S. cerevisiae* without sulfites was dominated by *Nectriaceae*_unclassified (42.3%), followed by *Sporidiobolaceae*_unclassified (6.6%), Tremellales_unclassified (2.6%), *Phialemoniopsis* (2%), and Saccharomycetales_unclassified (1.7%). With the addition of sulfites ($SO_2$ 10 mg/L and $SO_2$ 20 mg/L), the presence of *Saccharomyces* was apparent ($SO_2$ 0 mg/L: 0.13%, $SO_2$ 10 mg/L: 48.2%, and $SO_2$ 20 mg/L: 29.3%). The addition of sulfites promoted a higher relative abundance of *Saccharomyces*. Intriguingly, the relative abundance of *Saccharomyces* was higher at a sulfite level of 10 mg/L compared to 20 mg/L. The relative abundance of other fungi also varied between no sulfite addition and the two levels of added sulfites, such as a higher relative abundance 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* ($SO_2$ 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 of *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 level, 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 did alter the fungal communities of juice inoculated with *T. delbrueckii*. For instance, sulfite addition caused 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 increases 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%). Overall, for Vignoles juice, a clear pattern of fungal profiles appeared distinct to each inoculation (uninoculated, *S. cerevisiae*, and *T. delbrueckii*) and to sulfite level.

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 for the clusters of Vignoles that received 20 mg/L of sulfites (Supplemental Figure 2).

Fungal profiles at the phylum level were similar among the three types of inoculations (Supplemental Figure 5). Compared to the relative abundance at 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 had decreased at day 14 and was higher in uninoculated juice ($SO_2$ 0 mg/L: 2%, $SO_2$ 10 mg/L: 0.9%, and $SO_2$ 20 mg/L: 0.4%). The relative abundance of Fungi_unclassified had also decreased at day 14 for the three type of inoculations ($SO_2$ 0 mg/L: 2.2%, $SO_2$ 10 mg/L: 2.3%, and $SO_2$ 20 mg/L: 0.9%). No significant dissimilarities were observed among the three sulfite levels for the three types of inoculated juice.

However, the fungal profile at the genus level (Table 4 and Supplemental Figure 6) presented strong dissimilarities among the three types of inoculated juice. Overall, for all three types of inoculated juice, *Nectriaceae*_unclassified decreased and *Saccharomyces* increased.

Vignoles juice inoculated with *S. cerevisiae* was dominated by the genus *Saccharomyces* (85.8%), followed by *Nectriaceae*_unclassified (6.7%). The sulfite additions did not affect fungal profiles. However, sulfite levels modified the relative abundance of fungi in uninoculated juice and juice inoculated with *T. delbrueckii*. For instance, uninoculated juice with no added sulfite and with 10 mg/L $SO_2$ presented a different fungal profile from uninoculated juice with 20 mg/L $SO_2$. *Hanseniaspora* was detected at a higher relative abundance in uninoculated juice with no added sulfite and with 10 mg/L $SO_2$ than in juice with more sulfite ($SO_2$ 0 mg/L: 56.8%, $SO_2$ 10 mg/L: 45.8%, and $SO_2$ 20 mg/L: 0.02%). This was also true for *Candida* ($SO_2$ 0 mg/L: 1.6%, $SO_2$ 10 mg/L: 1.1%, and $SO_2$ 20 mg/L: 0.6%). In contrast, a higher relative abundance of *Saccharomyces* was observed in uninoculated juice with 20 mg/L of sulfites compared to juice with no added sulfite or with 10 mg/L $SO_2$ ($SO_2$ 0 mg/L: 23%, $SO_2$ 10 mg/L: 35.5%, and $SO_2$ 20 mg/L: 93.2%). The increase in relative abundance of *Hanseniaspora* in low-sulfite additions or no-sulfite fermentation has been previously described (Morgan et al. 2019b).

Juice inoculated with *T. delbrueckii* was dominated by the genus *Torulaspora* when no sulfite was added and when 10 mg/L $SO_2$ was added ($SO_2$ 0 mg/L: 96.3%, $SO_2$ 10 mg/L: 95.8%, and $SO_2$ 20 mg/L: 16.1%). When a higher level of sulfite (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%).

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

| | Treatments | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Uninoculated[a] | | | *Saccharomyces cerevisiae* | | | *Torulaspora delbrueckii* | | |
| **Day/Fungi**[b] | **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 |
| *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]Yeast inoculations were uninoculated, *S. cerevisiae*, and *T. delbrueckii.*

[b]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.

[c]Sulfur dioxide (as potassium metabisulfite) levels were 0 mg/L (NS), 10 mg/L (S10), and 20 mg/L (S20).

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

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

The fungal profiles at the phylum level presented slight dissimilarities between the three types of inoculation and the three sulfite levels (Supplemental 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 all 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 Supplemental 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 was observed. Fungi of smaller relative abundance appeared at day 21, including *Aspergillus*, *Lachancea*, and *Zygoascus.*

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

The primary fungi detected in uninoculated juice without sulfite addition and with addition of 10 mg/L or 20 mg/L $SO_2$ were *Hanseniaspora* and *Nectriaceae*_unclassified, respectively. The main fungi identified in *S. cerevisiae*-inoculated juice without sulfite addition and with addition of 10 mg/L or 20 mg/L $SO_2$ were *Saccharomyces* and *Nectriaceae*_unclassified, respectively. The main fungi detected in *T. delbrueckii*-inoculated juice at 0 mg/L $SO_2$ and at 10 or 20 mg/L $SO_2$ were *Torulaspora* and *Nectriaceae*_unclassified, respectively (Table 1).

**Overall impact of sulfite additions and yeast inoculations.** The highest level of sulfites significantly affected fermentation dynamics. For uninoculated juice, *Hanseniaspora* was strongly inhibited. Intriguingly, *Hanseniaspora* was replaced by *Saccharomyces* for Vignoles and by *Zygoascus* and *Schizosaccharomyces* for Noble. For Vignoles, the high level of sulfites promoted *Saccharomyces* but inhibited other fungi. Even in *T. delbrueckii*-inoculated juice at higher sulfite 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 if too much sulfite is added 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 $SO_2$ levels and need to be first inoculated without sulfite 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. 2020). To further complete fermentation, *S. cerevisiae* strains are later added to the fermentation.

*Nectriaceae*_unclassified growth in Vignoles juice was stimulated at day 21 when higher levels of sulfites were added, whereas for Noble juice, their growth was inhibited. Uninoculated Noble and Vignoles juices were dominated by the *Hanseniaspora* and *Saccharomyces* genera. However, the relative abundance of these two genera varied by sulfite level and shifted in opposite directions for the two grape varieties. While the relative abundance of *Saccharomyces* increased with higher sulfite levels in uninoculated Noble juice, it decreased for uninoculated Vignoles juice. Moreover, a third genus, *Zygoascus*, was identified at a high relative abundance only in uninoculated Noble juice with sulfite additions; 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 its growth was detected at day 0 during fermentation in Vignoles juice. However, the *Saccharomyces* genus retained a higher relative abundance in Noble juice than in Vignoles juice at day 21. Both juices inoculated with *T. delbrueckii* showed a dominant *Torulaspora* relative abundance from day 0 to day 21, with higher relative abundance in Noble juice at day 21 compared to Vignoles juice, in which a decrease in *Torulaspora* relative abundance was observed at day 21. These results confirmed that grape variety affected indigenous juice/wine mycobiota and the performance of commercial yeasts.

## Conclusion

This article is novel because the HTS approach was used to determine the impact of sulfite levels and yeast inoculations on wine fungal diversity and dynamics during fermentation (0, 14, and 21 days) of two grape varieties, a muscadine grape (Noble) and a hybrid grape (Vignoles). The fungal taxonomy of both varieties included six to seven phyla and 115 to 129

genera. Although the most abundant fungi (relative abundance >1%) in the juices were the same, their relative abundances varied by grape variety. The fungal diversity pattern throughout fermentation was similar for the two grape varieties, but sulfite additions and yeast inoculations affected the juice/wine mycobiota differently. These results confirm the importance of indigenous grape mycobiota and grape variety in shaping juice/wine mycobiota. The presence of these specific fungi can impact wine enological characteristics. Because indigenous fungi react differently to sulfite addition or yeast inoculations, knowing the initial mycobiota and their behavior during fermentation can help winemakers interested in producing wines with less sulfite and in using spontaneous fermentations. Understanding the microbial communities in grape juice and the dynamics of these communities during fermentation can provide more insight into wine production using spontaneous fermentation or fermentation with non-*Saccharomyces* species.

## Literature Cited


Abdo H, Catacchio CR, Ventura M, D'Addabbo P, Alexandre H, Guilloux-Bénatier M and Rousseaux S. 2020a. The establishment of a fungal consortium in a new winery. Sci Rep 10:7962.

Abdo H et al. 2020b. Colonization of wild *Saccharomyces cerevisiae* strains in a new winery. Beverages 6:9.

Agarbati A, Canonico L, Ciani M and Comitini F. 2019. The impact of fungicide treatments on yeast biota of Verdicchio and Montepulciano grape varieties. PLoS ONE 14:e0217385.

Azzolini M, Fedrizzi B, Tosi E, Finato F, Vagnoli P, Scrinzi C and Zapparoli G. 2012. Effects of *Torulaspora delbrueckii* and *Saccharomyces cerevisiae* mixed cultures on fermentation and aroma of Amarone wine. Eur Food Res Technol 235:303-313.

Azzolini M, Tosi E, Lorenzini M, Finato F and Zapparoli G. 2015. Contribution to the aroma of white wines by controlled *Torulaspora delbrueckii* cultures in association with *Saccharomyces cerevisiae*. World J Microbiol Biotechnol 31:277-293.

Barchenger DW, Clark JR, Threlfall RT, Howard LR and Brownmiller CR. 2015. Evaluation of physicochemical and storability attributes of muscadine grapes (*Vitis rotundifolia* Michx.). HortScience 50:104-111.

Bely M, Stoeckle P, Masneuf-Pomarède I and Dubourdieu D. 2008. Impact of mixed *Torulaspora delbrueckii-Saccharomyces cerevisiae* culture on high-sugar fermentation. Int J Food Microbiol 122:312-320.

Bokulich NA, Thorngate JH, Richardson PM and Mills DA. 2014. Microbial biogeography of wine grapes is conditioned by cultivar, vintage, and climate. Proc Natl Acad Sci USA 111:E139-E148.

Bokulich NA, Swadener M, Sakamoto K, Mills DA and Bisson LF. 2015. Sulfur dioxide treatment alters wine microbial diversity and fermentation progression in a dose-dependent fashion. Am J Enol Vitic 66:73-79.

Chen Y, Zhang W, Yi H, Wang B, Xiao J, Zhou X, Jiankun X, Jiang L and Shi X. 2020. Microbial community composition and its role in volatile compound formation during the spontaneous fermentation of ice wine made from Vidal grapes. Process Biochem 92:365-377.

Cioch-Skoneczny M, Satora P, Skotniczny M and Skoneczny S. 2018. Quantitative and qualitative composition of yeast microbiota in spontaneously fermented grape musts obtained from cool climate grape varieties 'Rondo' and 'Regent.' FEMS Yeast Res 18:foy089.

Cordero-Bueso G, Arroyo T, Serrano A, Tello J, Aporta I, Vélez MD and Valero E. 2011. Influence of the farming system and vine variety on yeast communities associated with grape berries. Int J Food Microbiol 145:132-139.

Cordero-Bueso G, Esteve-Zarzoso B, Cabellos JM, Gil-Díaz M and Arroyo T. 2013. Biotechnological potential of non-*Saccharomyces* yeasts isolated during spontaneous fermentations of Malvar (*Vitis vinifera* cv. L.). Eur Food Res Technol 236:193-207.

De Filippis F, La Storia A and Blaiotta G. 2017. Monitoring the mycobiota during Greco di Tufo and Aglianico wine fermentation by 18S rRNA gene sequencing. Food Microbiol 63:117-122.

Di Maro E, Ercolini D and Coppola S. 2007. Yeast dynamics during spontaneous wine fermentation of the Catalanesca grape. Int J Food Microbiol 117:201-210.

Domizio P, Romani C, Lencioni L, Comitini F, Gobbi M, Mannazzu I and Ciani M. 2011. Outlining a future for non-*Saccharomyces* yeasts: Selection of putative spoilage wine strains to be used in association with *Saccharomyces cerevisiae* for grape juice fermentation. Int J Food Microbiol 147:170-180.

Drumonde-Neves J, Franco-Duarte R, Lima T, Schuller D and Pais C. 2016. Yeast biodiversity in vineyard environments is increased by human intervention. PLoS ONE 11:e0160579.

Fleet GH. 2003. Yeast interactions and wine flavour. Int J Food Microbiol 86:11-22.

Garner D, Crisosto CH, Wiley P and Crisosto GM. 2005. Measurement of pH and titratable acidity. http://fruitandnuteducation.ucdavis.edu/files/162035.pdf.

Guzzon R, Malacarne M, Larcher R, Franciosi E and Toffanin A. 2020. The impact of grape processing and carbonic maceration on the microbiota of early stages of winemaking. J Appl Microbiol 128:209-224.

Gweon HS, Oliver A, Taylor J, Booth T, Gibbs M, Read DS, Griffiths RI and Schonrogge K. 2015. PIPITS: An automated pipeline for analyses of fungal internal transcribed spacer sequences from the Illumina sequencing platform. Methods Ecol Evol 6:973-980.

Hall ME and Wilcox WF. 2019. Identification and frequencies of endophytic microbes within healthy grape berries. Am J Enol Vitic 70:212-219.

Hirst MB and Richter CL. 2016. Review of aroma formation through metabolic pathways of *Saccharomyces cerevisiae* in beverage fermentations. Am J Enol Vitic 67:361-370.

Howell GS, Miller DP, Edson CE and Striegler RK. 1991. Influence of training system and pruning severity on yield, vine size, and fruit composition of Vignoles grapevines. Am J Enol Vitic 42:191-198.

Johnson J, Fu M, Qian M, Curtin C and Osborne JP. 2020. Influence of select non-*Saccharomyces* yeast on *Hanseniaspora uvarum* growth during prefermentation cold maceration. Am J Enol Vitic 71:278-287.

Jolly NP, Varela C and Pretorius IS. 2014. Not your ordinary yeast: Non-*Saccharomyces* yeasts in wine production uncovered. FEMS Yeast Res 14:215-237.

Kozich JJ, Westcott SL, Baxter NT, Highlander SK and Schloss PD. 2013. Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. Appl Environ Microbiol 79:5112-5120.

Mandakovic D, Pulgar R, Maldonado J, Mardones W, González M, Cubillos FA and Cambiazo V. 2020. Fungal diversity analysis of grape musts from Central Valley-Chile and characterization of potential new starter cultures. Microorganisms 8:956.

Martins G, Miot-Sertier C, Lonvaud-Funel A and Masneuf-Pomarède I. 2016. Grape berry bacterial inhibition by different copper fungicides. BIO Web Conf 7:01043.

Marzano M et al. 2016. Complexity and dynamics of the winemaking bacterial communities in berries, musts, and wines from Apulian grape cultivars through time and space. PLoS ONE 11:e0157383.

Medina K, Boido E, Fariña L, Gioia O, Gomez ME, Barquet M, Gaggero C, Dellacassa E and Carrau F. 2013. Increased flavour diversity of Chardonnay wines by spontaneous fermentation and co-fermentation with *Hanseniaspora vineae*. Food Chem 141:2513-2521.

Mendoza LM, Neef A, Vignolo G and Belloch C. 2017. Yeast diversity during the fermentation of Andean chicha: A comparison of high-throughput sequencing and culture-dependent approaches. Food Microbiol 67:1-10.

Mezzasalma V, Sandionigi A, Bruni I, Bruno A, Lovicu G, Casiraghi M and Labra M. 2017. Grape microbiome as a reliable and persistent signature of field origin and environmental conditions in Cannonau wine production. PLoS ONE 12:e0184615.

Mezzasalma V, Sandionigi A, Guzzetti L, Galimberti A, Grando MS, Tardaguila J and Labra M. 2018. Geographical and cultivar features differentiate grape microbiota in Northern Italy and Spain vineyards. Front Microbiol 9:946.

Morgan HH, du Toit M and Setati ME. 2017. The grapevine and wine microbiome: Insights from high-throughput amplicon sequencing. Front Microbiol 8:820.

Morgan SC, Haggerty JJ, Johnston B, Jiranek V and Durall DM. 2019a. Response to sulfur dioxide addition by two commercial *Saccharomyces cerevisiae* strains. Fermentation 5:69.

Morgan SC, Tantikachornkiat M, Scholl CM, Benson NL, Cliff MA and Durall DM. 2019b. 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 290:1-14.

Morgan SC, Haggerty JJ, Jiranek V and Durall DM. 2020. Competition between *Saccharomyces cerevisiae* and *Saccharomyces uvarum* in controlled Chardonnay wine fermentations. Am J Enol Vitic 71:198-207.

Morrison-Whittle P, Lee SA and Goddard MR. 2017. Fungal communities are differentially affected by conventional and biodynamic agricultural management approaches in vineyard ecosystems. Agric Ecosyst Environ 246:306-313.

Nadai C, Vendramini C, Carlot M, Andrighetto C, Giacomini A and Corich V. 2019. Dynamics of *Saccharomyces cerevisiae* strains isolated from vine bark in vineyard: Influence of plant age and strain presence during grape must spontaneous fermentations. Fermentation 5:62.

Pinto C, Pinho D, Sousa S, Pinheiro M, Egas C and Gomes AC. 2014. Unravelling the diversity of grapevine microbiome. PLoS ONE 9: e85622.

Pinto C, Pinho D, Cardoso R, Custódio V, Fernandes J, Sousa S, Pinheiro M, Egas C and Gomes AC. 2015. Wine fermentation microbiome: A landscape from different Portuguese wine appellations. Front Microbiol 6.

Portillo M del C and Mas A. 2016. Analysis of microbial diversity and dynamics during wine fermentation of Grenache grape variety by high-throughput barcoding sequencing. LWT - Food Sci Technol 72:317-321.

Pretorius IS. 2000. Tailoring wine yeast for the new millennium: Novel approaches to the ancient art of winemaking. Yeast 16:675-729.

Querol A, Barrio E, Huerta T and Ramon D. 1992. Molecular monitoring of wine fermentations conducted by active dry yeast strains. Appl Environ Microbiol 58:2948-2953.

Quirós M, Rojas V, Gonzalez R and Morales P. 2014. Selection of non-*Saccharomyces* yeast strains for reducing alcohol levels in wine by sugar respiration. Int J Food Microbiol 181:85-91.

Raymond Eder ML, Conti F and Rosa AL. 2018. Differences between indigenous yeast populations in spontaneously fermenting musts from *V. vinifera* L. and *V. labrusca* L. grapes harvested in the same geographic location. Front Microbiol 9:1320.

Renouf V and Lonvaud-Funel A. 2004. Racking are key stages for the microbial stabilization of wines. OENO One 38:219-224.

Renouf V, Miot-Sertier C, Strehaiano P and Lonvaud-Funel A. 2006. The wine microbial consortium: A real terroir characteristic. J Int Sci Vigne Vin 40:209-216.

Roudil L, Russo P, Berbegal C, Albertin W, Spano G and Capozzi V. 2020. Non-*Saccharomyces* commercial starter cultures: Scientific trends, recent patents and innovation in the wine sector. Recent Pat Food Nutr Agric 11:27-39.

Ruiz J et al. 2019. Occurrence and enological properties of two new non-conventional yeasts (*Nakazawaea ishiwadae* and *Lodderomyces elongisporus*) in wine fermentations. Int J Food Microbiol 305:108255.

Tristezza M, Tufariello M, Capozzi V, Spano G, Mita G and Grieco F. 2016. The oenological potential of *Hanseniaspora uvarum* in simultaneous and sequential co-fermentation with *Saccharomyces cerevisiae* for industrial wine production. Front Microbiol 7:670.

Umiker NL, Descenzo RA, Lee J and Edwards CG. 2013. Removal of *Brettanomyces bruxellensis* from red wine using membrane filtration. J Food Process Pres 37:799-805.

van Breda V, Jolly N and van Wyk J. 2013. Characterisation of commercial and natural *Torulaspora delbrueckii* wine yeast strains. Int J Food Microbiol 163:80-88.

Waterhouse AL, Sacks GL and Jeffery DW. 2016. Understanding Wine Chemistry. John Wiley & Sons, Hoboken, New Jersey.

Wilker KL, Dharmadhikari MR and Goin JC. 2004. Effect of sweetening treatments on white wine aroma and composition. Am J Enol Vitic 55:168-173.

Zhang Y, Chang SKC, Stringer SJ and Zhang Y. 2017. Characterization of titratable acids, phenolic compounds, and antioxidant activities of wines made from eight Mississippi-grown Muscadine varieties during fermentation. LWT - Food Sci Technol 86:302-311.