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The effect of sulfur dioxide addition at crush on the fungal and bacterial communities and the sensory attributes of Pinot gris wines

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ABSTRACT

Modern day winemaking often involves the addition of sulfur dioxide (SO_2) at crush to act as both an antioxidant and an antimicrobial agent. While the effects of SO_2 on microbial communities and particularly on spoilage microorganisms has been well-studied, the advent of culture-independent molecular technologies, such as Illumina sequencing, allows the subject to be re-visited in a new context. High-throughput amplicon sequencing allows for a more thorough evaluation of microbial communities, as thousands of microbial sequences per sample can be identified and even rare microorganisms can be studied. This research investigated whether the addition of different levels of SO_2 at crush (0, 20, or 40 mg/L) would affect the composition of fungal and bacterial communities, as well as the sensory attributes of the resulting wines. Samples were taken from uninoculated fermentations of Pinot gris and analyzed via high-throughput amplicon sequencing using the Illumina MiSeq platform. Yeast relative abundance and overall fungal community composition differed among the SO_2 additions. Notably, a *Hanseniaspora* yeast appeared in all treatments and persisted until the end of alcoholic fermentation, although its relative abundance was significantly higher in the fermentations to which low or no SO_2 had been added. Two key wine sensory attributes (citrus aroma and pome fruit flavor) differed among the SO_2 treatments. This research provides an in-depth look into the fungal and bacterial communities during alcoholic fermentation and gives a better understanding of the microbial community response to SO_2 additions during the crush period.

1. Introduction

Sulfur dioxide (SO_2) has been used in winemaking for centuries, acting as both an antioxidant and an antimicrobial agent. It is often added at crush, prior to the start of alcoholic fermentation, to prevent the growth of unwanted microorganisms that enter the grape must from the vineyard or winery equipment. SO_2 is almost always added post-fermentation as well as at bottling to act as a preservative agent. Using excessive amounts of SO_2 in winemaking can be undesirable from both a health standpoint and from an enological perspective, where the addition of too much SO_2 can negatively impact the sensory attributes of a wine (Guerrero and Cantos-Villar, 2015; Yang and Purchase, 1985). Because of these reasons, there has been a consumer-driven push in recent years for SO_2 alternatives in winemaking; however, SO_2 remains the most effective antioxidant and preservative available (Falguera et al., 2013; Guerrero and Cantos-Villar, 2015; Izquierdo-Canas et al., 2012).

Saccharomyces cerevisiae, the dominant yeast in winemaking, tends to be more resistant to SO_2 addition than bacteria and non-*Saccharomyces* yeasts (Bokulich et al., 2014; Constantí et al., 1998; Henick-Kling et al., 1998). *S. cerevisiae* is found in very low numbers on healthy grapes (Mortimer and Polsinelli, 1999), and therefore non-*Saccharomyces* yeasts dominate the must stage before the onset of alcoholic fermentation (cold-settling). The predominance of these yeasts as well as bacteria is generally not favored and winemakers can therefore choose two methods to prematurely remove them: the addition of sufficiently high levels of SO_2 at crush, and/or the inoculation of the must with a commercial *S. cerevisiae* strain, which will usually outcompete the vineyard yeasts. While non-*Saccharomyces* yeasts were originally thought to be exclusively spoilage organisms, a substantial and growing body of evidence has pointed to the ability of non-*Saccharomyces* yeasts to play important roles in the expression of varietal

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aromas, as well as the production of unique sensory-active secondary by-products that can increase the complexity of a wine and the expression of terroir (Ciani et al., 2010; Fleet, 2003; Jolly et al., 2014; Romano et al., 2003; Viana et al., 2008). For these reasons, many winemakers are opting to add less or no SO_2 at crush, or to let their musts ferment uninoculated (spontaneously). However, more research needs to be conducted in order to fully understand the implications of these decisions to allow winemakers to make informed decisions in the context of uninoculated and low-SO_2 winemaking.

Previous research has investigated these topics (Constantí et al., 1998; Egli et al., 1998; Henick-Kling et al., 1998; Suzzi and Romano, 1982; Takahashi et al., 2014), but the introduction of new molecular technologies, that allow for a more accurate and thorough evaluation of the microorganisms involved in winemaking, necessitates further research into this area. Next-generation sequencing technologies such as Illumina MiSeq, among others, have enabled the detection of microorganisms in wine fermentations that were previously undetectable using culture-dependent techniques. It was previously thought that non-*Saccharomyces* yeasts were unable to survive in conditions exceeding 3–4% (v/v) ethanol, but culture-independent molecular identification has shown that non-*Saccharomyces* yeasts and bacteria may survive until the end of alcoholic fermentation and in turn may be contributing significantly to the aroma and flavor profile of the wine (Bokulich et al., 2014; Kioroglou et al., 2018; Stefanini et al., 2016). These microorganisms may be present in too low an abundance to be identified through culture-dependent methods, they may be unable to grow on the media most commonly used for yeast or bacterial isolation, or they may be present in the fermentation in a viable but nonculturable (VBNC) state (Agnolucci et al., 2010; Divol et al., 2012). New research suggests that *S. cerevisiae* may produce metabolites that decrease the culturability of non-*Saccharomyces* yeasts (Wang et al., 2016), necessitating the use of culture-independent techniques to accurately identify the full yeast communities present in fermentations. Due to these reasons, using culture-dependent analysis when attempting to evaluate the entire microbial community in a wine sample may underestimate microbial diversity and overestimate the importance of a few species or genera (Serpaggi et al., 2012).

The closed-system conditions of winemaking mean that as alcoholic fermentation progresses, the availability of nutrients decreases concomitantly with an increase in alcohol content and creates a progressively inhospitable environment for the microorganisms present. Towards the end of fermentation, the amount of dead yeasts and bacteria that can no longer contribute to the fermentation accumulate significantly (Branco et al., 2012). To prevent these organisms from misrepresenting the viable microbial community during analysis, DNA-binding dyes such as propidium monoazide (PMA) can be added to samples prior to DNA extraction to prevent the amplification of DNA from dead cells (Andorrà et al., 2010; Tantikachornkiat et al., 2016). When microbes die in fermentation, the integrity of their cell membranes becomes compromised, allowing PMA to enter dead cells and bind to genomic DNA. When exposed to light, PMA binds irreversibly to the DNA, preventing it from being amplified during polymerase chain reaction (PCR). This current study is the first of its kind to evaluate the living microbial communities (via the use of PMA) of commercial wine fermentations with respect to SO_2 addition at crush. To our knowledge, only one other study has used high-throughput amplicon sequencing to evaluate the effects of SO_2 addition on fungal and bacterial communities during alcoholic fermentation (Bokulich et al., 2014).

This current study builds upon the design and results of seven important and relevant studies, three published in 1998, one published in 2008, and three published in 2014 (Andorrà et al., 2008; Bokulich et al., 2014; Constantí et al., 1998; Egli et al., 1998; Henick-Kling et al., 1998; Pateraki et al., 2014; Takahashi et al., 2014). While these studies form the basis of our understanding of uninoculated and/or sulfite-free fermentations, our research attempts to fill some of the gaps of these studies and to update knowledge of the topic using current molecular technologies. Constantí et al. (1998) investigated the combined effects of SO_2 addition and commercial yeast inoculation but neither included biological replicates in their experimental design nor evaluated the effects of these treatments on the sensory attributes of the resulting wines. Both Henick-Kling et al. (1998) and Egli et al. (1998) also investigated the combined effects of SO_2 addition and commercial yeast inoculation. These two studies evaluated the sensory attributes of the wines produced, but only Henick-Kling et al. (1998) compared sulfited and unsulfited wines during sensory analysis. Neither study included enough biological replicates to allow for the use of inferential statistics. Furthermore, all three experiments published in 1998 were scaled down to between 80 L and 12 L fermentations and were conducted away from commercial wineries, thus limiting their direct applicability to the commercial winemaking process. These studies used a combination of culture-based methods to identify yeasts to the species and sometimes to the strain level, and while some of these techniques are still used today, the advent of culture-independent analysis such as high-throughput amplicon sequencing has allowed for the identification of rare and VBNC yeasts and bacteria in fermentations. Takahashi et al. (2014) and Pateraki et al. (2014) compared culture-dependent and culture-independent methods of evaluating microbial diversity, using denaturing gradient gel electrophoresis (DGGE) as the culture-independent method, but did not conduct a sensory evaluation of the wines. Andorrà et al. (2008) used both DGGE and quantitative PCR (qPCR) to identify the fungal and bacterial communities in sulfited and unsulfited wines, but did not replicate treatments. Bokulich et al. (2014) used Illumina MiSeq to evaluate the fungal and bacterial communities in fermentations to which a range of SO_2 concentrations were added, and observed changes in the bacterial, but not the fungal, community in response so SO_2 addition. Each treatment was replicated in triplicate, but a sensory evaluation of the wines was not performed. All three studies from 2014 were also conducted at experimental scales (< 1 L, 14 L, and 19 L, respectively), and Takahashi et al. (2014) evaluated only inoculated fermentations.

The objectives of this study were to: i) determine the effect of different levels of SO_2 addition at crush (0, 20, and 40 mg/L SO_2) on the relative abundance and the composition of fungal and bacterial communities present throughout uninoculated (spontaneous) fermentations and ii) evaluate the effect of SO_2 addition on the wine sensory attributes of Pinot gris wines fermented at a commercial winery in British Columbia, Canada. Each treatment was replicated in triplicate in new 225 L oak barrels, and the fungal and bacterial communities were determined using Illumina MiSeq sequencing; samples were treated with PMA addition to identify only the living community. We expected that the diversity and composition of the fungal and bacterial communities would differ among the three SO_2 treatments, and that the resulting wines would differ in their sensory attributes.

2. Materials and methods

2.1. Experimental design and sampling

This study was conducted during the 2014 vintage at Cedar Creek Estate Winery, a medium-sized commercial winery located on the east side of Okanagan Lake in British Columbia, Canada. This winery produces 30,000–40,000 cases (270,000–360,000 L) of wine annually, and conducts both inoculated and uninoculated (spontaneous) fermentations of many grape varietals.

In this study, uninoculated fermentations of Pinot gris were evaluated. Grapes were sourced from a single vineyard associated with the winery, and were harvested and crushed/pressed according to standard viticultural practices in British Columbia, Canada. The grape must was first crushed and pressed into a large stainless steel tank, and then transferred into nine new 225 L French oak medium-toast barrels (Alain Fouquet & Associates Inc., Napa, CA, USA), which were steam-cleaned prior to the addition of the grape must. SO_2 was added in three

Table 1
Pinot gris grape must parameters at Cold-settling, ± the standard error of the mean (SEM). Three different levels of sulfur dioxide (SO_2) were added to the must immediately prior to sampling ($n = 3$ per treatment). Yeast assimilable nitrogen (YAN) was measured as the sum of alpha amino nitrogen (mg/L) and ammonia (mg/L) concentrations.

	Chemical composition ± SEM		
Parameter	0 mg/L SO_2	20 mg/L SO_2	40 mg/L SO_2
Temperature (°C)	14.0 ± 0.07	13.9 ± 0.03	13.9 ± 0.03
pH	3.25 ± 0.006	3.26 ± 0.003	3.27 ± 0.003
Molecular SO_2 (mg/L)	N/A	0.21 ± 0.04	0.54 ± 0.01
Residual sugar (°Brix)	22.5 ± 0.0	22.6 ± 0.03	22.6 ± 0.0
YAN (mg/L)	242.6 ± 6.5	234.5 ± 11	215.5 ± 7.2
Volatile acidity (g/L)	0.10 ± 0.003	0.10 ± 0.009	0.10 ± 0.003
Total acidity (g/L)	7.3 ± 0.03	7.3 ± 0.07	7.2 ± 0.03
Malic acid (g/L)	2.7 ± 0.06	2.7 ± 0.07	2.6 ± 0.0

concentrations: 0, 20, and 40 mg/L SO_2 ($n = 3$ per treatment) in the form of potassium metabisulfite (KMS). Alcoholic fermentation progressed uninoculated (spontaneously) without the addition of any commercial yeast strains. Fermentations were conducted at cellar temperature, beginning at 13.9 °C ± 0.03 and rising to 20.8 °C ± 0.09 by the middle of fermentation.

2.2. Chemical analysis

Samples for chemical analysis were taken from the grape must during the cold-settling stage (Table 1) and again at the end of alcoholic fermentation (Table 2). Residual sugar levels, measured as °Brix, were evaluated daily throughout alcoholic fermentation. Depending on the pH of the wine, SO_2 can be free or can be bound to various compounds present in the grape must (Divol et al., 2012). Both free and total SO_2 levels were determined at four stages of fermentation: Cold-settling (> 22°Brix), Early (14–19°Brix), Mid (7–12°Brix), and Late (< 2°Brix). Samples were collected aseptically in sterile 50 mL plastic centrifuge tubes and were immediately transported to the laboratory on ice for processing.

Temperature and residual sugar levels were measured using a portable Anton Paar© density meter (Saint Laurent, QC, Canada). All other chemical parameters (excluding SO_2 determination) were measured using an OenoFoss™ wine analyzer (Foss, Hilleroed, Denmark) following manufacturer protocols. SO_2 levels were determined using an aeration, oxidation, distillation, and titration procedure described by Zoecklein et al. (1995). Briefly, SO_2 in the wine samples was distilled using aspiration from an acidified solution (20 mL fermentation sample mixed with 10 mL 25% phosphoric acid) into a hydrogen peroxide trap (10 mL hydrogen peroxide, three drops of an indicator solution (Zoecklein et al., 1995), and one drop of 0.01 N sodium hydroxide). For free SO_2 determination, the acidified wine sample was kept in an ice bath so only the volatile SO_2 in the sample would be distilled. For total SO_2 determination, the sample was heated in a water bath to release bound SO_2. Samples were aerated for 15 min, and then the hydrogen peroxide sample was titrated by hand using 0.01 N sodium hydroxide.

Table 2
Pinot gris wine parameters at the end of alcoholic fermentation (Late stage) ± the standard error of the mean (SEM). Three levels of sulfur dioxide (SO_2) were added to the grape must at the Cold-settling stage.

	Chemical composition ± SEM		
Parameter	0 mg/L SO_2	20 mg/L SO_2	40 mg/L SO_2
Temperature (°C)	18.4 ± 0.1	18.7 ± 0.06	18.9 ± 0.09
pH	3.03 ± 0.006	2.97 ± 0.03	2.98 ± 0.03
Molecular SO_2 (mg/L)	0.019 ± 0.01	0.025 ± 0.02	0.015 ± 0.008
Residual sugar (°Brix)	−1.0 ± 0.1	−0.2 ± 0.7	−0.2 ± 0.1
Volatile acidity (g/L)	0.25 ± 0.009	0.26 ± 0.01	0.21 ± 0.02
Total acidity (g/L)	7.7 ± 0.06	8.1 ± 0.2	8.0 ± 0.2
Malic acid (g/L)	1.6 ± 0.09	1.8 ± 0.09	1.8 ± 0.1
Ethanol content (%)	12.1 ± 0.03	11.7 ± 0.3	12.0 ± 0.3

2.3. Sample treatment with propidium monoazide (PMA)

Samples for microbial analysis were taken at four stages of fermentation: Cold-settling (> 22°Brix), Early (14–19°Brix), Mid (7–12°Brix), and Late (< 2°Brix). Samples were collected aseptically in sterile 50 mL plastic centrifuge tubes and were immediately transported to the laboratory on ice for processing. Aliquots of 10 mL from each sample were transferred to a sterile 15 mL plastic centrifuge tube. Samples were centrifuged at 4000 rpm for 5 min, and the supernatant was discarded. The pellet was then re-suspended in 10 mL molecular-grade water and centrifuged at 4000 rpm for 3 min before discarding the supernatant. A cell membrane recovery step was added for samples taken at the Early, Mid, and Late stages of fermentation to ensure the presence of alcohol would not decrease the membrane integrity of living cells in these samples (Goldstein, 1986). In this step, the pellet was re-suspended in 10 mL freshly made, autoclaved YEPD broth (20% yeast extract, 10% peptone, 10% dextrose) and placed horizontally on ice for 2 h. After 2 h, the samples were centrifuged at 4000 rpm for 3 min and the supernatant was discarded. The pellet was washed with molecular-grade water, centrifuged, and the supernatant was discarded. The remaining steps of the described protocol were then completed for all samples. Next, the pellet was re-suspended in 2.25 mL molecular-grade water by pipetting up and down 10 times. A 1.1 µL of 20 mM PMA aliquot was added to each sample to achieve a total concentration of approximately 6–7 mM (Tantikachornkiat et al., 2016). Samples were placed horizontally on ice and left in the dark for 10 min before being exposed to light (550 W halogen lamp) for 8 min on an oscillating platform. Exposure to light is what allows the PMA to bind irreversibly to any exposed DNA in the sample, and results in bound DNA being excluded from amplification during PCR. Samples were centrifuged, and the pellets were transferred to 2.0 mL microcentrifuge tubes. Samples were washed with 1.5 mL molecular-grade water twice and then stored at −20 °C until further processing.

2.4. DNA extraction and Illumina MiSeq library preparation

Total DNA from PMA-treated fermentation samples was extracted using an Omega E.Z.N.A.® Stool DNA Kit (Omega Biotek, Norcross, GA, USA), following the manufacturer protocol with the following modifications: 200 mg of 0.1 mm glass disruptor beads (Scientific Industries, Inc., Bohemia, NY, USA) were used in place of the provided beads; samples were homogenized using a TissueLyser II mechanical bead beater (Qiagen®, Hilden, Germany). Extracted DNA samples were stored at −20 °C until further processing. Sample library preparation for Illumina MiSeq sequencing was conducted by a two-step PCR process consisting of 'amplicon' and 'index' PCR reactions, described below.

2.4.1. Amplicon PCR

For bacteria, the V3/V4 region of the 16S ribosomal ribonucleic acid (rRNA) gene was amplified using F341 and R805 primers (Herlemann et al., 2011) with CS1 and CS2 linker sequences on the forward and reverse primers, respectively. These overhanging linker sequences are necessary for the second PCR reaction, 'index' PCR, which attaches the Illumina MiSeq adapter sequence and unique 8 nucleotide barcode to each sample. Amplicon PCR was prepared using the following reaction mix: 6.8 µL molecular-grade water; 3.0 µL GoTaq reaction mix (5× buffer); 0.3 µL dNTP (10 mM each); 0.24 µL BSA (10 mg/mL); 0.45 µL forward and reverse primers each (10 µM); 1.5 µL $MgCl_2$ (25 mM); 0.16 µL GoTaq DNA Polymerase (5 units/µL); 2.1 µL DNA template, for a total reaction volume of 15 µL per well. Amplicon PCR for bacteria

was performed using the following program: 95 °C for 3 min (1 cycle); 95 °C for 40 s, 53 °C for 40 s, 72 °C for 1 min (32 cycles); 72 °C for 7 min (1 cycle). All PCR reactions were performed on an Applied Biosystems Veriti 96-Well Fast Thermal Cycler (Foster City, CA, USA).

For fungi, the ITS1 region of the rRNA gene was amplified using BITS and B58S3 primers (Bokulich and Mills, 2013) with CS1 and CS2 linker sequences on the forward and reverse primers, respectively. Amplicon PCR was prepared using the following reaction mix: 4.67 μL molecular-grade water; 2.5 μL GoTaq Reaction Mix (5× buffer); 0.25 μL dNTP (10 mM each); 0.2 μL BSA (10 mg/mL); 0.25 μL forward and reverse primer each (10 μM); 1.25 μL $MgCl_2$ (25 mM); 0.13 μL GoTaq DNA Polymerase (5 units/μL); 3.0 μL DNA template, for a total reaction volume of 12.5 μL per well. Amplicon PCR for fungi was performed using the following program: 95 °C for 2 min (1 cycle); 95 °C for 40 s, 55 °C for 40 s, 68 °C for 1 min (32 cycles); 68 °C for 5 min (1 cycle).

2.4.2. Index PCR

For both bacteria and fungi, a Gel Logic 400 Imaging System (Mandel, Rochester, NY, USA) was used to visualize and confirm PCR amplification in a 1.5% agarose gel containing SYBR™ Safe DNA gel stain (Life Technologies, Carlsbad, CA, USA). Depending on the strength of the band visualized on the gel, the amplicon products were diluted using molecular-grade water before Index PCR was performed. The Index PCR primers contained the CS1/CS2 linker sequence, an 8 nucleotide barcode sequence, and the P5/P7 Illumina adapter sequence. Each sample received a unique combination of forward and reverse primer barcodes. Index PCR was prepared using the following reaction mix: 20.5 μL molecular-grade water; 10 μL GoTaq Reaction Mix (5× buffer); 1 μL dNTP (10 mM each); 1.5 μL BSA (10 mg/mL); 2.5 μL forward and reverse primers each (2 μM); 9 μL $MgCl_2$ (25 mM); 0.5 μL GoTaq DNA Polymerase (5 units/μL); 2.5 μL DNA template, for a total reaction volume of 50 μL per well. Index PCR was performed using the following program: 95 °C for 1 min (1 cycle); 95 °C for 30 s, 62 °C for 30 s, 68 °C for 1.5 min (12 cycles); 68 °C for 5 min (1 cycle). The PCR products were visualized in a 1.5% agarose gel containing SYBR™ Safe DNA gel stain. Index PCR was deemed successful when the amplicon length was extended by 69 bp (Illumina MiSeq P5/P7 adapter + barcode length) as compared to the Amplicon PCR product length.

Samples were submitted to the IBEST Genomics Resources Core facility at the University of Idaho (Moscow, ID, USA) for quantification, normalization, pooling, and sequencing. Paired-end sequencing (300 bp length) was performed on an Illumina MiSeq Desktop Sequencer (Illumina® Inc., San Diego, CA, USA).

2.5. Illumina MiSeq data processing

The open-source bioinformatics pipeline Quantitative Insights Into Microbial Ecology (QIIME1 and QIIME2) was used for identifying the microbial communities using gene sequencing data (Caporaso et al., 2010). Forward and reverse barcode files were combined using the "extract_barcodes.py" function in QIIME1 (version 1.8). All other processing of bacterial and fungal sequences was performed in QIIME2 q2cli version 2017.12.

For bacterial sequence data, "demux" was used to demultiplex samples (https://github.com/qiime2/q2-demux). DADA2 (using the "data2 denoise-single" command) was used to de-noise and correct Illumina-sequenced amplicon errors in forward-read sequences only (Callahan et al., 2016). Sequences were truncated at 260 bp. Paired-end assembly was not conducted because reverse sequence reads were determined to be low quality based on observations from the "demux summarize" command. The "feature-table" plug-in was used to create a feature table and a table of representative sequences (McDonald et al., 2012). A Naïve Bayes classifier was trained on the target region of the amplicon primer sets using Greengenes software (version 13.5) (DeSantis et al., 2006; McDonald et al., 2012) using the "q2-feature-classifier" plugin (https://github.com/qiime2/q2-feature-classifier). Taxonomy was assigned to the genus level. MAFFT-aligned sequences (Katoh and Standley, 2013) were used to produce a phylogenetic tree using FastTree software (version 2.1.7) (Price et al., 2009). The "q2-taxa" plug-in (https://github.com/qiime2/q2-taxa) was used to filter out mitochondrial and chloroplast contamination, and to remove sequence variants that could not be identified to the order level or lower as well as those that appeared with a total frequency of < 100. Samples were then rarefied to a sampling depth of 10,000 sequences (based on the sample with the lowest number of sequences) before being exported from QIIME2 for further analysis.

For the fungal sequence data, a similar protocol was used as the one described above, with the following changes. Paired-end assembly of fungal sequences was performed; forward reads were truncated at 206 bp and reverse reads were truncated at 180 bp when performing the de-noising step using DADA2 (Callahan et al., 2016). Sequences were classified to the species-level using a 99% threshold classifier created using UNITE software (version 7.2) (Kõljalg et al., 2013). The "q2-taxa" plug-in (https://github.com/qiime2/q2-taxa) was used to remove sequence variants that could not be identified to the order level or lower as well as those that appeared with a total frequency of < 100. Samples were then rarefied to a sampling depth of 6000 sequences (based on the sample with the lowest number of sequences) before being exported from QIIME2 for further analysis. In one instance, the name of the identified fungal organism was changed from the classification given by UNITE; sequences identified as *Saccharomyces bayanus* are termed *Saccharomyces uvarum* in this study. According to the UNITE database (version 7.2), *S. bayanus* and *S. uvarum* are synonymous, but in recent years *S. uvarum* has been re-instated as a separate species (Nguyen and Gaillardin, 2005). We are confident in the assessment that sequences from this research belong to *S. uvarum* based on culture-dependent sequencing data from these fermentations (data not included here). All software packages, versions, and parameters used for fungal and bacterial analysis can be viewed under the "provenance" tab of the QIIME2 artifact files available here: https://osf.io/y4xba/. These files can be viewed on https://view.qiime2.org.

2.6. Wine bottling and sensory analysis

Post-alcoholic fermentation, 6 L of wine was transferred from each barrel into 2 L glass bottles with screw caps (three bottles per barrel). The bottles were purchased new and cleaned with a 5% citric acid and KMS solution. Long plastic tubing was cleaned with the same solution and was used to rack the wine from the barrels to the bottles. Sulfur dioxide (60 mg/L SO_2, added as 120 mg/L KMS) was added to each bottle to act as a preservative. The glass bottles were filled to the top with wine and a carbon dioxide/nitrogen (CO_2/N_2) gas mixture was added to the headspace before the lid was screwed on to remove any oxygen. These bottles were kept in the dark in a cooled cellar for approximately two months, with racking after one month, before being transferred to clean 750 mL wine bottles with screw caps supplied by the winery. The three 2 L glass bottles from each wine barrel were combined into a 19 L glass carboy cleaned with a 5% citric acid and KMS solution. From the carboy, six 750 mL wine bottles were filled from each barrel replicate (for a total of 54 wine bottles from nine barrels). Wine was filled to the neck of the bottle and N_2 gas was added to the headspace to remove any oxygen. Free and total SO_2 were measured at this stage and it was determined that each bottle had between 7.4 and 14.3 mg/L free SO_2 at the time of bottling.

Sensory evaluations of the wines were conducted in the spring of 2015 at the Summerland Research and Development Centre (Summerland RDC) in Summerland, British Columbia, Canada, adhering to standard sensory evaluation protocols and using a panel of 10 industry wine experts (Cliff and Dever, 1996; Guinard and Cliff, 1987; King et al., 2013). Each wine was evaluated in duplicate in a blind study. The wines were labeled with three-digit random numbers, and served in random order using a William's design, to control for first-

Table 3
Sensory attributes of wines evaluated by an expert panel of 10 judges.

Aroma attributes	Flavor attributes	Other attributes
Citrus aroma	Citrus flavor	Acidity
Pome aroma	Pome flavor	Astringency
Tropical fruit aroma	Tropical fruit flavor	Body
Spice aroma	Spice flavor	Length of aftertaste
Vanilla aroma	Vanilla flavor	
Toasty/smoky aroma	Toasty/smoky flavor	
Wood aroma	Wood flavor	

order bias and carryover effects (Williams and Arnold, 1991). A short break of 5–10 min was taken between tasting sessions. Wine was served in 210 mL International Standards Organization (ISO) wine glasses in 30 mL aliquots. The judges first evaluated the perceived aroma of the wines, followed by the flavor and mouthfeel attributes, using sensory characteristics defined during a prior roundtable discussion (Table 3). Sensory standards for panelist reference were created for each of the attributes listed in Table 3, the composition of which can be found in Table A.1. All wine samples were expectorated and panelists were instructed to rinse their mouths with water between wines. The intensity of each attribute was evaluated on an unstructured 100 unit line scale, using Compusense *five*© sensory evaluation software (Compusense, Inc., Guelph, ON, Canada). Evaluations were conducted individually in separate booths lit by red lighting.

Since a preliminary bench-testing revealed that no color differences were present among the wines, color assessments were not conducted. The use of human subjects in this study was approved by the Agriculture and Agri-Food Canada (AAFC) Human Research Ethics Committee (Certificate of Approval 2015D004) and the UBC Okanagan Research Ethics Board.

2.7. Statistical analysis

Wine chemical parameters were analyzed with RStudio software (version 3.4.4) and reported ± the standard error of the mean (SEM). SO_2 measurements at crush were compared by performing a one-way analysis of variance (ANOVA) on total SO_2 measurements using the "aov" function. Normality was assessed visually, and homogeneity of variance was assessed using the "leveneTest" function from the car package (version 3.0–0), which indicated no significant differences among treatments ($F(1,4) = 0.36$, $P = 0.58$). Fermentation progression (°Brix throughout fermentation) was compared among the three SO_2 treatments by performing a repeated-measures one-way ANOVA on rank-transformed data using the "aov" function.

Fungal and bacterial diversity were analyzed separately in RStudio (version 3.4.4). Simpson's Index of Diversity was calculated using the "diversity" function in the vegan package (version 2.5–1) and reported ± the SEM. Diversity was also evaluated for among treatments (Cold-settling, Early, Mid, Late) for each of the SO_2 treatments, by performing a repeated-measures one-way ANOVA using the "aov" function. Normality was assessed visually and Levene's test indicated no violation of the assumption of heterogeneity in variance for either fungal diversity ($F(2,6) = 2.60$, $P = 0.09$) or bacterial diversity ($F(2,6) = 2.40$, $P = 0.10$). When appropriate, a Tukey post-hoc test, adjusted for multiple comparisons using the Holm method, was performed to evaluate differences among treatments using the "lme" and "glht" functions in the nmle (version 3.1–137) and multcomp (version 1.4–8) packages, respectively (Hothorn et al., 2008). Fungal and bacterial relative abundance was visualized by creating stacked bar charts using GraphPad Prism® software (version 7) (La Jolla, CA, USA). Fungi or bacteria that represented fewer than 100 sequences in every sample were grouped together and termed "minor fungi" or "minor bacteria."

Fungal and bacterial composition data were analyzed separately in RStudio (version 3.4.4). A Bray-Curtis dissimilarity index was calculated from untransformed fungal/bacterial abundance for each sample. Multivariate homogeneity of group dispersions (PERMDISP) was analyzed utilizing the "betadisper" and "permutest" functions (vegan package) using Bray-Curtis dissimilarity and calculating deviation from centroid. Permutational analysis of variance (PERMANOVA) tests, using Bray-Curtis dissimilarity, were performed on fungal and bacterial composition data to test for differences in composition among treatments, using the "adonis" function (vegan package). When appropriate, pairwise post-hoc tests were performed, adjusted for multiple comparisons with the Holm method and using the "pairwise.adonis" function in the pairwiseAdonis package (version 0.0.1).

When analyzing fungal community composition, the PERMDISP test indicated significant differences among treatments ($F(2,24) = 3.50$, $P = 0.04$). However, PERMANOVA tests are robust to unequal variances among treatments (Anderson and Walsh, 2013), so no data transformations were made prior to performing the PERMANOVA test. Fungal community composition was initially analyzed using data from all four stages of fermentation, but the communities of the Cold-settling stage were extremely similar among all treatments and were also dramatically different from the communities present during alcoholic fermentation (Early, Mid, and Late stages). Therefore, this stage was removed from the analysis to more accurately assess the effect of SO_2 addition during fermentation. For the bacterial community composition, a PERMDISP test indicated no significant differences among treatments ($F(2,33) = 0.68$, $P = 0.50$) with regards to multivariate dispersion. Because the bacterial communities were consistent throughout fermentation and did not change from the Cold-settling stage to the alcoholic fermentation stages (as the fungal communities did), samples from all four stages were included in the analysis of bacterial community composition.

Test statistics (F values for PERMDISP and Pseudo-F values for PERMANOVA) were calculated based on 999 permutations of raw data. The design of this study involved repeated measures, and therefore not all data were independent of one another, potentially leading to an overestimation of treatment differences as a result of the PERMANOVA tests. Therefore, principal coordinates analysis (PCoA) ordinations were used to visualize distances between samples of different treatments (Knight et al., 2015; Lorion and Kennedy, 2009). These PCoA ordinations were created using the "wcmdscale" and "ordihull" functions using Bray-Curtis dissimilarity (vegan package).

Statistical analysis of sensory evaluation data was performed in RStudio (version 3.4.4). The "panelperf" function in the SensoMineR package (version 1.23) was used to evaluate the sensory panel's performance in its ability to discriminate among products (wines), using three-way ANOVA. A principal component analysis (PCA) was performed using the "averagetable" and "pca" functions in order to visualize the relationship between the sensory attributes and wines from different SO_2 treatments. A radar plot was generated in Excel 2016 to visualize sensory differences among the wines from different SO_2 treatments; it was converted to a high-resolution image using IrfanView software (version 4.51). Values for the radar plot were standardized for each sensory attribute grouping in Excel using the "STANDARDIZE" function, based on the mean and standard deviation of a set of data. Standardizing involves re-scaling each variable to have a mean of zero and a standard deviation of one. This can improve the visualization of differences among treatments for each attribute, and enable comparisons of data.

3. Results and discussion

3.1. Sulfur dioxide and wine chemistry

The chemical composition of the Pinot gris must at the Cold-settling stage (Table 1) and at the end of alcoholic fermentation (Table 2) was similar for all treatments and was within an expected range for cool-climate Pinot gris wines. Alcoholic fermentation progressed at a steady

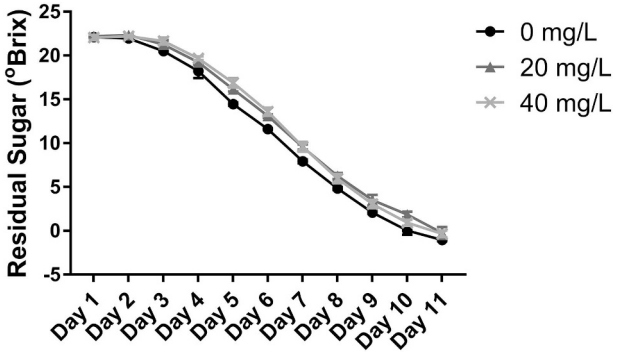

Fig. 1. Residual sugar levels (°Brix) ± the standard error of the mean (SEM) measured throughout alcoholic fermentation of Pinot gris wines to which three different levels of sulfur dioxide had been added at crush (n = 3 per treatment). Fermentations from all treatments were complete within 11 days. A one-way repeated measures ANOVA was performed, and no significant differences among treatments were found (F(2,6) = 3.90, P = 0.08).

rate for all treatments, and although the fermentations to which 0 mg/L SO_2 was added at crush completed one day before the other two treatments, the difference in fermentation rate among treatments was non-significant (F(2,6) = 3.90, P = 0.08) (Fig. 1). This result is in accordance with the findings of Henick-Kling et al. (1998), who also found no differences in fermentation rate in response to SO_2 addition.

The pH of the grape must was an average of 3.26 across all treatments at the Cold-settling stage (Table 1). By the end of alcoholic fermentation, the average pH of the wines had dropped to just under 3.0 (Table 2). The grapes used for this study were grown in a cool-climate wine region towards the northern-most limit for wine grape production, and the pH observed here is not unexpected for white wine grapes from such regions (Knoll et al., 2012). However, we note that low pH musts can mitigate the risk of contamination by spoilage yeasts and bacteria, especially when conducting uninoculated and sulfite-free fermentations, which are at an inherently higher risk for stuck or spoiled fermentations. Bacteria, including lactic acid and acetic acid bacteria, have trouble growing in musts with low pH (Bartowsky, 2009). High pH can promote the growth of bacteria such as *Pediococcus* spp. and can also volatilize nitrogen-heterocyclic compounds, produced by heterofermentative lactic acid bacteria, which can give wines a mousy character (Bartowsky, 2009; Costello et al., 2001; Costello and Henschke, 2002). When dealing with musts of higher pH, wineries utilizing low-intervention winemaking techniques such as uninoculated or sulfite-free fermentations may experience an increased risk of microbial spoilage.

Both free and total SO_2 were measured throughout alcoholic fermentation (Fig. 2). Interestingly, the initial total SO_2 measurements made for the 20 mg/L SO_2 and 40 mg/L SO_2 treatments were slightly lower than expected based on the level of addition; however, no significant difference was found between the two treatments in terms of expected versus observed total SO_2 (one-way ANOVA, F(1,4) = 0.36, P = 0.58), indicating that both treatments had total SO_2 levels that were lower than expected by the same amount. Therefore, it was likely that natural SO_2 loss and not SO_2 addition or sampling error led to this discrepancy. Between the time of sampling and the time of SO_2 determination, several hours had elapsed in the case of some samples, which could have contributed to SO_2 loss. Samples were also collected in plastic centrifuge tubes, which could have absorbed some volatile compounds, including free SO_2.

While total SO_2 is useful for confirming the initial concentration of SO_2 added, free SO_2 is a more useful measurement when studying the relationship between SO_2 addition and microbiological composition. Free SO_2, in combination with pH measurements, can be used to determine the molecular SO_2 concentration using the Henderson-Hasselbalch equation (Divol et al., 2012; Zoecklein et al., 1995), which

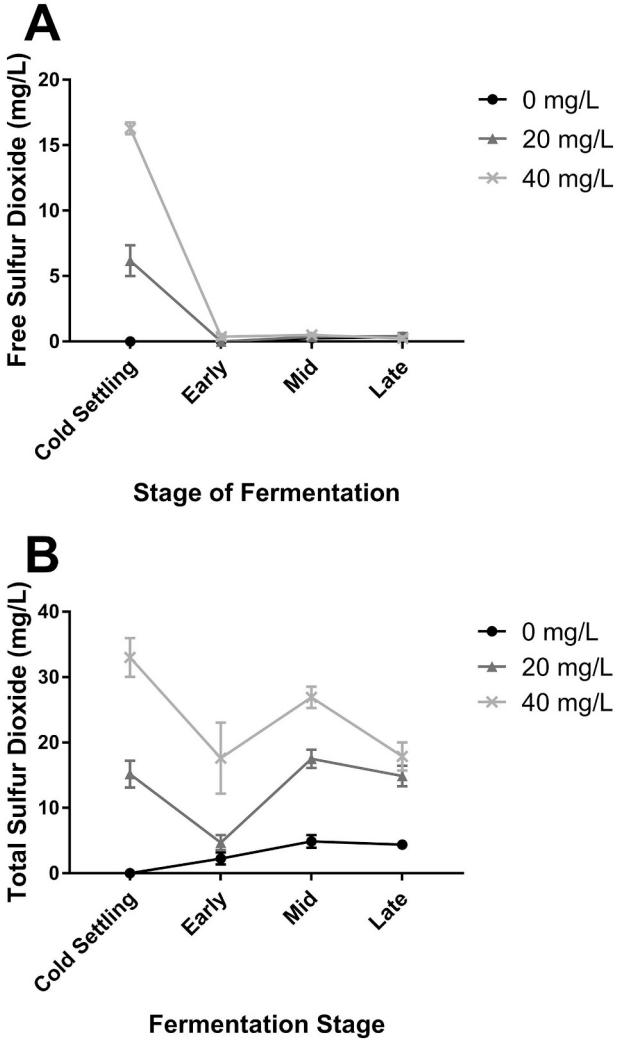

Fig. 2. Free (A) and total (B) sulfur dioxide (SO_2) levels ± the standard error of the mean (SEM) measured throughout the alcoholic fermentation of Pinot gris wines to which three different levels of total SO_2 had been added at crush (n = 3 per treatment). SO_2 was added in the form of potassium metabisulfite (KMS) immediately prior to the sample taken at the Cold-settling stage of fermentation.

is the most effective form of SO_2 in terms of its antimicrobial activity (Divol et al., 2012). An effective concentration of molecular SO_2 for antimicrobial use has been estimated at between 0.2 mg/L and 0.8 mg/L, depending on the publication and the susceptibility of each particular must to spoilage based on chemical profiles such as pH, temperature and microbial load (Howe et al., 2018; King et al., 1981; Margalit and Crum, 2004). Both sulfited treatments (20 mg/L SO_2 and 40 mg/L SO_2) achieved a minimum effective concentration at the Cold-settling stage (Table 1). The concentration of free SO_2 at cold-settling was approximately half the concentration of total SO_2 for the two treatments to which SO_2 was added (Fig. 2). However, by the Early stage of fermentation, free SO_2 dropped to negligible amounts in all treatments and remained very low throughout the rest of fermentation. Total SO_2, however, dropped sharply after cold-settling in the two sulfited treatments (due to the loss of free SO_2), but then increased between the Early and Mid stages of fermentation in all three treatments. This was likely due to the production of SO_2 by *S. cerevisiae* yeasts during fermentation, which primarily produce bound SO_2 (Andorrà et al., 2017; Suzzi et al., 1985). Total yeast counts, based on culture methods, were highest in the 40 mg/L SO_2 treatment and lowest in the 0 mg/L SO_2 treatment (data not shown), which may explain the different amounts

of bound SO_2 produced among the treatments during alcoholic fermentation.

SO_2 is added prior to the onset of alcoholic fermentation (and prior to the addition of commercial yeasts in inoculated fermentations) for the removal of potential spoilage microorganisms coming in from the vineyard (Divol et al., 2012). Bacteria are particularly sensitive to SO_2, and previous research has noted that non-*Saccharomyces* yeasts are more SO_2-sensitive than *S. cerevisiae* yeasts (Constantí et al., 1998; Divol et al., 2012; Henick-Kling et al., 1998). Therefore, SO_2 resistance is likely to play a much larger role in the ability of vineyard microorganisms to persist during fermentation than it is for strains of winery-resident yeasts such as *S. cerevisiae*. Because free (and molecular) SO_2 decreases to a very low level soon after its addition, *S. cerevisiae* strains that subsequently enter a fermentation, whether intentionally or not, are likely less affected by the additions of SO_2. For these winery-resident yeast strains, it is likely to be other factors, such as growth rate, range of nutrient consumption, and other competitive abilities such as the production of killer toxins that determine whether these yeasts are able to persist in a fermentation. We previously published an investigation of the *S. cerevisiae* strains present in the fermentations from this study, and identified no patterns between SO_2 resistance and yeast strain dominance in any SO_2 treatment (Morgan et al., 2017).

3.2. Fungal and bacterial communities

3.2.1. Fungal and bacterial diversity during fermentation

Fungal community diversity throughout fermentation was significantly lower in the 40 mg/L SO_2 treatment than in the 0 and 20 mg/L SO_2 treatments (Table 4). At the Cold-settling stage, all treatments contained a similar fungal diversity, as was expected given the samples were taken directly after the SO_2 treatment had been applied, leaving little time for the SO_2 addition to alter the fungal community. Diversity decreased in all treatments throughout fermentation, but most drastically decreased in the 40 mg/L SO_2 treatment. This was likely due to the higher level of SO_2 addition at crush that removed all but the most resistant vineyard yeasts. Alcohol tolerance of the vineyard yeasts likely did not play a role in the observed decrease in diversity in the 40 mg/L SO_2 treatment, because the wines from all three treatments finished alcoholic fermentation (< 0°Brix at the time of the last sample), and reached a similar ethanol concentration (Table 2). While limited research is available on the resistance of non-*Saccharomyces* yeasts to increasing ethanol concentrations using current identification technologies (however see Wang et al., 2015), the previous literature emphasizes *S. cerevisiae* as the most important yeast in winemaking at least in part due to its high ethanol tolerance (Bisson and Joseph, 2009).

Bacterial community diversity throughout fermentation seemed to be unaffected by either the SO_2 treatment applied at crush or the stage of alcoholic fermentation (Table 4). Because PMA was added to the fermentation samples before DNA extraction, the fungi and bacteria identified represented those that were living at the time of sampling. Therefore, this precluded the possibility that the bacterial diversity remained unchanged throughout fermentation due to only dead bacteria being present in the sample (either due to SO_2 addition, being outcompeted, or susceptibility to alcohol). This result is in contrast with the results obtained by Bokulich et al. (2014), who found decreased bacterial alpha-diversity in uninoculated, sulfite-free fermentations as compared to uninoculated, sulfited fermentations, likely caused by the overgrowth of a few dominant bacterial species. Sun et al. (2016) also noted a decrease in bacterial abundance in response to increasing SO_2 concentrations in strawberry wine fermentations, although this study involved much higher levels of SO_2 addition and used culture-dependent identification techniques.

Table 4
Fungal and bacterial diversity, measured as Simpson's Index of Diversity, of Pinot gris wines to which different levels of sulfur dioxide (SO_2) were added at crush (n = 3 per treatment). Diversity was measured at four stages of alcoholic fermentation (Cold-settling, Early, Mid, Late) and reported ± the standard error of the mean (SEM). Fungal and bacterial data were analyzed separately, and treatments were compared across all fermentation stages by performing repeated measures one-way ANOVA and subsequent post-hoc tests when appropriate. An asterisk next to either fungal or bacterial diversity indicates significance at $\alpha = 0.05$; treatments marked with different superscript letters had significantly different overall diversity across all stages of fermentation ($\alpha = 0.05$).

	Simpson's Index of Diversity ± SEM		
Fungal diversity*	0 mg/L SO_2[a]	20 mg/L SO_2[a]	40 mg/L SO_2[b]
Cold-settling	0.59 ± 0.02	0.57 ± 0.005	0.60 ± 0.01
Early	0.42 ± 0.03	0.48 ± 0.03	0.30 ± 0.08
Mid	0.48 ± 0.02	0.21 ± 0.02	0.11 ± 0.03
Late	0.43 ± 0.03	0.35 ± 0.05	0.16 ± 0.03
Bacterial diversity	0 mg/L SO_2	20 mg/L SO_2	40 mg/L SO_2
Cold-settling	0.46 ± 0.04	0.52 ± 0.06	0.46 ± 0.07
Early	0.52 ± 0.05	0.48 ± 0.03	0.52 ± 0.05
Mid	0.47 ± 0.04	0.50 ± 0.05	0.44 ± 0.04
Late	0.53 ± 0.05	0.47 ± 0.03	0.50 ± 0.06

3.2.2. Fungal and bacterial abundance during fermentation

At the Cold-settling stage, all SO_2 treatments contained a variety of different fungi (Fig. 3; Table 5), most notably *Aureobasidium pullulans*, *Cladosporium* sp., and a small amount of *Hanseniaspora* sp. *A. pullulans* is a ubiquitous yeast-like fungus that is commonly associated with vineyard and winery environments, and has been found throughout the winemaking process (Takahashi et al., 2014; Varela and Borneman, 2017). An attempt was made to identify all fungal taxa to the species-level using the UNITE database (version 7.2), although this was not always possible. In cases where species-level identification could not be obtained, the most specific taxonomic classification available was assigned.

We note the presence in all treatments of low levels of certain fungi with the potential to produce mycotoxins: namely, *Aspergillus flavus* and *Penicillium* spp. Both genera of fungi are commonly found on wine grapes and other food crops (Freire et al., 2017). Mycotoxins produced by these fungi include aflatoxin B_1 and ochratoxin A, among others, which can present a health risk to consumers if present in a food product in high enough concentrations (Inoue et al., 2013; Mateo et al., 2007). Low levels of ochratoxin A (< 4.5 ng/mL) are common in commercially produced wines (Mateo et al., 2007). However, Canadian wines have been shown to contain a lower occurrence of ochratoxin A, along with lower levels of contamination, than imported wines sold in Canada (Soleas et al., 2001). In the study conducted by Soleas et al.

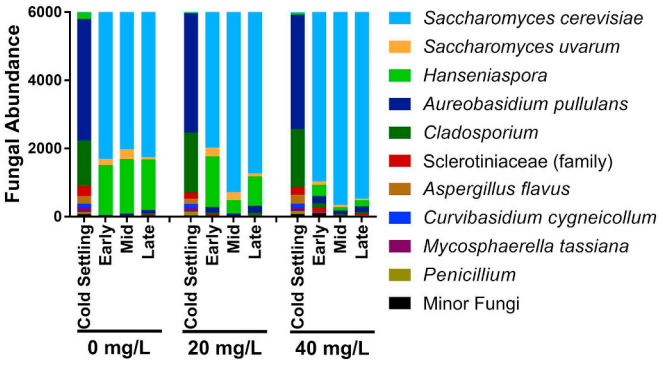

Fig. 3. Relative abundance of fungi (based on 6000 sequences per sample) present in wines fermented with three levels of sulfur dioxide (SO_2) added at crush (n = 3 per treatment). Samples were taken at four stages of alcoholic fermentation. For variation among samples of the same treatment and for the identities of minor fungi, please refer to Table 5.

Table 5
Fungal community composition (based on 6000 sequences per sample) of wines fermented with different levels of sulfur dioxide (SO_2) added at crush (0, 20, or 40 mg/L SO_2), reported ± the standard error of the mean (SEM). Three replicate fermentations were conducted for each treatment for a total of nine barrels. Samples were taken from each barrel at four stages of fermentation (Cold-settling, Early, Mid, Late).

	Fungal community composition ± SEM											
	0 mg/L SO_2				20 mg/L SO_2				40 mg/L SO_2			
Fungi	Cold-settling	Early	Mid	Late	Cold-settling	Early	Mid	Late	Cold-settling	Early	Mid	Late
Saccharomyces cerevisiae	13 ± 1	4310 ± 192	4030 ± 129	4258 ± 231	9 ± 2	3983 ± 275	5285 ± 80	4735 ± 201	26 ± 17	4970 ± 294	5654 ± 95	5478 ± 98
Aureobasidium pullulans	3552 ± 143	30 ± 9	62 ± 3	95 ± 11	3496 ± 54	158 ± 6	79 ± 16	198 ± 32	3355 ± 99	219 ± 34	108 ± 8	163 ± 20
Hanseniaspora	192 ± 22	1461 ± 179	1583 ± 105	1482 ± 226	23 ± 12	1490 ± 289	392 ± 46	860 ± 157	44 ± 9	327 ± 140	100 ± 60	180 ± 65
Cladosporium	1338 ± 122	7 ± 4	13 ± 8	43 ± 5	1765 ± 69	55 ± 8	6 ± 3	67 ± 19	1700 ± 121	138 ± 17	23 ± 8	61 ± 4
Saccharomyces uvarum	–	176 ± 19	288 ± 60	62 ± 11	–	247 ± 41	233 ± 73	90 ± 26	2 ± 2	93 ± 15	67 ± 17	38 ± 5
Sclerotiniaceae (family)	299 ± 15	7 ± 4	15 ± 8	34 ± 5	181 ± 3	36 ± 18	–	17 ± 11	232 ± 30	110 ± 10	28 ± 1	42 ± 7
Aspergillus flavus	224 ± 86	9 ± 8	10 ± 6	9 ± 3	153 ± 58	21 ± 11	1 ± 1	12 ± 9	258 ± 88	12 ± 9	6 ± 3	8 ± 4
Curvibasidium cygneicollum	130 ± 5	–	–	13 ± 3	126 ± 18	4 ± 4	3 ± 3	10 ± 6	131 ± 11	–	4 ± 3	17 ± 4
Mycosphaerella tassiana	118 ± 28	–	–	3 ± 3	101 ± 13	6 ± 3	–	9 ± 5	88 ± 10	35 ± 5	–	10 ± 4
Penicillium	58 ± 6	–	–	–	105 ± 23	–	–	1 ± 1	88 ± 23	–	–	2 ± 1
Hanseniaspora vineae	–	–	–	–	–	–	–	–	–	96 ± 77	9 ± 7	1 ± 1
Udeniomyces puniceus	20 ± 2	–	–	–	9 ± 1	–	–	1 ± 1	15 ± 5	–	–	–
Penicillium spinulosum	16 ± 3	–	–	1 ± 1	11 ± 6	–	–	–	12 ± 8	–	–	–
Rhodosporidiobolus colostri	21 ± 11	–	–	–	9 ± 9	–	–	–	9 ± 9	–	–	–
Rhizopogon abietis	9 ± 3	–	–	–	12 ± 5	–	–	–	13 ± 6	–	–	–
Mucor circinelloides	9 ± 5	–	–	–	–	–	–	–	10 ± 8	–	1 ± 1	–
Alternaria	2 ± 2	–	–	–	–	–	–	–	16 ± 16	–	–	–

(2001), which included 96 Canadian wines, only three red wines and none of the white wines contained > 0.05 μg/L ochratoxin A. The process of fermentation itself has been shown to reduce the presence of mycotoxins in wine, either through conversion of the mycotoxin to a less toxic form (Inoue et al., 2013) or possibly through their adsorption to yeasts/lees and eventual removal from the wine (Petruzzi et al., 2014). In total, *Aspergillus* and *Penicillium* spp. made up approximately 5% of the total fungal community at the Cold-settling stage, and < 0.2% of the total fungal community during alcoholic fermentation (Table 5). The distribution of these fungi were even across all treatments, indicating that the lack of SO_2 added to the 0 mg/L SO_2 treatments did not contribute to the presence or abundance of these fungi. Although mycotoxin levels were not measured for this study, we do not anticipate mycotoxin production to be of concern here.

After Cold-settling, the three stages of alcoholic fermentation (Early, Mid, and Late) contained a consistent assemblage of fungi within each treatment, although the relative abundance of major yeasts differed markedly among SO_2 treatments (Fig. 3). In all treatments, *S. cerevisiae* dominated the fermentations. This was an expected result, as even in uninoculated fermentations, winery-resident *S. cerevisiae* strains are known to dominate in most commercial settings, especially in the Okanagan winemaking region of Canada (Hall et al., 2011; Morgan et al., 2017; Scholl et al., 2016). The quick succession from vineyard yeasts during the Cold-settling stage to the dominance of *S. cerevisiae* from the Early stage of fermentation onwards suggests that an abundance of winery-resident strains were present in the environment and were able to quickly enter the musts and outcompete the remaining vineyard yeasts. Indeed, at the winery in question where this research was conducted, most of the fermentation vessels are inoculated with commercial yeast strains, and > 40 different commercial strains had been used over the five years leading up to this study (Morgan et al., 2017). An analysis of the strain-level composition of *S. cerevisiae* strains in these fermentations was previously published (Morgan et al., 2017), and found that close to 98% of the strains identified were of commercial origin, regardless of SO_2 addition level at crush.

Saccharomyces uvarum was also observed in all treatments in low concentrations. This yeast is not typically used as a commercial starter, and only one commercial strain, Lallemand Velluto BMV58™, was available at the time of publishing. However, *S. uvarum* has been identified in wineries around the globe, generally in cool-climate winemaking regions; even when musts are uninoculated, this yeast can sometimes dominate fermentations and out-compete winery-resident *S. cerevisiae* (Demuyter et al., 2004; Naumov et al., 2000, 2002). The third yeast of note is *Hanseniaspora* sp. (not identified to the species-level), which appeared in all fermentations but with striking differences in relative abundance between treatments. In the 0 mg/L SO_2 treatment, *Hanseniaspora* represented up to 20% of the total fungal community, and maintained this abundance through the end of fermentation. The abundance of *Hanseniaspora* decreased with increasing initial SO_2 addition, and in the 40 mg/L SO_2 treatment it represented no > 5% of the total fungal community. As mentioned above, the yeasts and fungi identified represent those that were living at the time of sampling. The *Hanseniaspora* yeasts identified at the Late stage of fermentation (Fig. 3) were therefore living, even at approximately 12% (v/v) alcohol. *Hanseniaspora* yeasts are uncommonly identified at these late stages of fermentation using culture-dependent identification techniques such as plating and incubating (Combina et al., 2005; Constantí et al., 1997; Ocón et al., 2010). Before the advent of culture-independent molecular technologies such as Illumina sequencing (among others), it was assumed that these vineyard yeasts could not tolerate high levels of alcohol. Our results, however, are in accordance with results from other studies conducted using next-generation sequencing technologies, which have identified vineyard yeasts such as *Hanseniaspora* spp. at late stages of alcoholic fermentation (Bokulich et al., 2014; Pateraki et al., 2014; Stefanini et al., 2016). These yeasts may be in a viable but nonculturable (VBNC) state, potentially induced into this state by

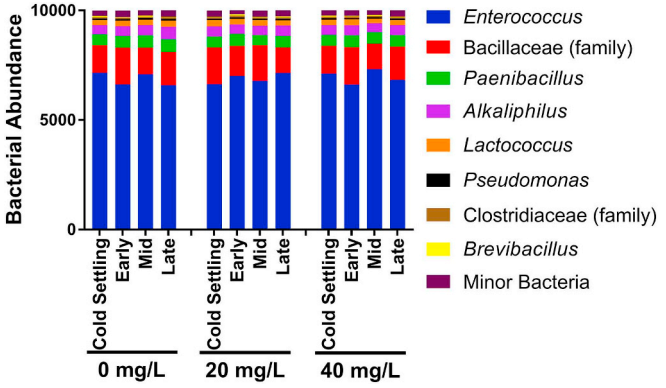

Fig. 4. Relative abundance of bacteria (based on 10,000 sequences per sample) present in wines fermented with three levels of sulfur dioxide (SO_2) added at crush ($n = 3$ per treatment). Samples were taken at four stages of alcoholic fermentation. For variation among samples of the same treatment and for the identities of minor bacteria, please refer to Table 6.

metabolites produced by *S. cerevisiae*, which is why they are not identified using plating and culturing methods (Wang et al., 2015, 2016). There is evidence that other yeasts such as *Brettanomyces bruxellensis* can be induced into a VBNC state by SO_2 (Capozzi et al., 2016), but within the context of our results, it seems that SO_2 at higher levels was effective in eliminating much of the *Hanseniaspora* population (Fig. 3). Even in a VBNC state, these yeasts are metabolically active and able to contribute to the final chemical and sensory profiles of the wines.

The bacterial community remained consistent throughout alcoholic fermentation, and was unaffected by either SO_2 addition level or the stage of fermentation. *Enterococcus* was the most commonly identified genus, representing approximately 70% of the bacterial community, followed by a member of the Bacillaceae family, representing approximately 15% of the bacterial community (Fig. 4; Table 6). Both *Enterococcus* and *Bacillus* spp. bacteria produce lactic acid and have been previously identified in wine fermentations, although *Enterococcus* spp. have only been previously identified during malolactic fermentation in red wines (Bokulich et al., 2012; Capozzi et al., 2011; Dündar, 2016; Pérez-Martín et al., 2014). Enterococci in particular are noted to be resistant to a wide range of environmental stresses, including elevated ethanol concentration (Capozzi et al., 2011; Giraffa, 2002). Some strains of *Enterococcus faecium* isolated from red wine have been shown to produce bacteriocins, toxins produced by one bacterium to inhibit the growth of closely related bacteria (Dündar, 2016). Enterococci are potential spoilage bacteria due to their ability to produce tyramine, a vasoactive amine responsible for headaches in some people, and likely enter fermentations due to contamination via harvest or winery equipment (Dündar, 2016). Although traditionally associated with the mammalian gastrointestinal tract, these bacteria are quite ubiquitous and have been isolated from soil, water, and fermented foods, as well as on plants, vegetables, and fruits (Giraffa, 2002). It should be noted that food-borne *Enterococcus* spp. have not been implicated in any clinical *Enterococcus* infections (Giraffa, 2002; Pérez-Martín et al., 2014).

A notable absence from the bacterial communities of these wines are common members of the lactic acid bacteria group (Table 6). Specifically, sequences identified to the genera *Lactobacillus* and *Leuconostoc* as well as the order Lactobacillales were present in these fermentations, but due to their extremely low abundance and/or lack of specificity in taxonomic identification, they were filtered out before analysis (see Materials and methods section 2.5). *Lactobacillus* spp. do not grow well at low pH, and the addition of SO_2 can delay their growth (Edwards et al., 1993); both of these factors may have contributed to their low abundance and eventual removal from this study.

3.2.3. Fungal and bacterial community composition

Fungal community composition was affected by SO_2 addition levels (Table 7), with each treatment having a significantly different composition ($P \leq 0.03$ for all pairwise comparisons). Samples from the 40 mg/L SO_2 treatment contained unique assemblages of fungi, while the 0 mg/L SO_2 and 20 mg/L SO_2 treatments contained some composition overlap among samples (Fig. 5). Composition takes into account not only the relative abundance but also the identity of different organisms. Therefore, samples that contain similar fungal species but with different relative abundance appear as separate points on the PCoA ordination, and the more dissimilar the identity and relative abundance of these fungi, the more distant two points will be from each other. A similar result was found when assessing the *S. cerevisiae* strain composition of these treatments, where each SO_2 treatment contained a unique assemblage of yeast strains and significantly different population composition (Morgan et al., 2017). Henick-Kling et al. (1998) and Takahashi et al. (2014) noted differences in yeast community composition in response to SO_2 addition levels, which is in accordance with the results reported here.

No significant differences in bacterial community composition were found between treatments (Table 7, $F(2,33) = 0.02$, $P = 0.99$), and there was significant overlap between treatments in the PCoA ordination (Fig. 6). Interestingly, the only other study to evaluate fungal and bacterial communities using the same Illumina sequencing platform noted differences in the bacterial communities, but not in the fungal communities, in response to a gradient of SO_2 concentrations (Bokulich et al., 2014). This discrepancy is possibly due to the fact that these fermentations were conducted outside of a commercial winery environment, and had different environmental factors influencing the progression of fermentation. It is likely that the use of PMA in this study also contributed to these observed differences. Because this research evaluated only relative abundance and not total abundance of bacteria and fungi, it is possible that the bacterial communities in these fermentations were present in low abundance and had low metabolic activity during fermentation. It is also known that yeast activity can affect the behavior of bacteria during both alcoholic and malolactic fermentation (Reguant et al., 2005). Therefore, it is possible that the differences in yeast communities between this study and that of Bokulich et al. (2014) resulted in different interactions with the bacterial communities, leading to the observed differences in bacterial community performance.

3.3. Wine sensory attributes

A sensory panel of 10 wine experts found significant differences among treatments in two of the 18 sensory attributes evaluated – citrus aroma and pome fruit flavor (Table A.2); these two attributes are among the most commonly identified attributes in cool-climate white wines, including Pinot gris. Wines from the 0 mg/L SO_2 treatment were noted to be higher in citrus aroma than the wines from the 20 mg/L SO_2 and 40 mg/L SO_2 treatments (Fig. 7). Pome fruit flavor was associated with both the 0 mg/L SO_2 and 20 mg/L SO_2 treatments (and especially with the 20 mg/L SO_2 treatment) but not with the 40 mg/L SO_2 treatment. Although not significant, there was a trend towards a more fruit-forward expression (citrus, pome, and tropical fruits) in the wines from the 0 mg/L SO_2 treatment, with fruity attributes being less dominant in the 40 mg/L SO_2 wines, and somewhere in between for the 20 mg/L SO_2 wines (Figs. 7 & 8). In this study, no wine defects (sensorial or visual) were detected in wines from any treatment.

Because wine yeasts are known to differ in their production of secondary by-products, it is likely that the differences in fungal community composition contributed to the sensory differences observed among treatments, including those attributes that trended towards differences but were not significantly different in this study. In the Cold-settling stage, the dominant fungi were *Aureobasidium pullulans* and a member of the genus *Cladosporium*. A *Hanseniaspora* yeast appeared in

Table 6
Bacterial community composition (based on 10,000 sequences per sample) of wines fermented with different levels of sulfur dioxide (SO_2) added at crush (0, 20, or 40 mg/L SO_2). Three replicate fermentations were conducted for each treatment for a total of nine barrels. Samples were taken from each barrel at four stages of fermentation (Cold Settling, Early, Mid, Late).

	Fungal community composition ± SEM											
	0 mg/L SO_2				20 mg/L SO_2				40 mg/L SO_2			
Fungi	Cold-settling	Early	Mid	Late	Cold-settling	Early	Mid	Late	Cold-settling	Early	Mid	Late
Enterococcus	7159 ± 391	6626 ± 484	7088 ± 399	6589 ± 412	6637 ± 530	7019 ± 302	6790 ± 446	7146 ± 282	7125 ± 582	6621 ± 447	7321 ± 371	6841 ± 503
Bacillaceae (family)	1251 ± 378	1675 ± 419	1220 ± 358	1513 ± 339	1677 ± 427	1362 ± 373	1624 ± 420	1164 ± 333	1260 ± 421	1698 ± 433	1165 ± 362	1511 ± 388
Paenibacillus	504 ± 17	536 ± 28	564 ± 42	584 ± 14	502 ± 35	548 ± 19	464 ± 8	529 ± 39	505 ± 51	552 ± 29	515 ± 10	526 ± 34
Alkaliphilus	413 ± 1	439 ± 35	459 ± 16	569 ± 36	458 ± 18	437 ± 29	423 ± 37	473 ± 27	436 ± 46	453 ± 5	421 ± 7	454 ± 22
Lactococcus	240 ± 27	258 ± 36	249 ± 21	270 ± 32	281 ± 44	244 ± 22	261 ± 41	235 ± 24	252 ± 41	271 ± 13	201 ± 13	228 ± 34
Pseudomonas	69 ± 7	71 ± 11	76 ± 11	87 ± 8	69 ± 7	70 ± 16	62 ± 11	71 ± 10	68 ± 11	53 ± 27	61 ± 15	76 ± 12
Clostridiaceae (family)	61 ± 7	58 ± 8	61 ± 10	75 ± 8	62 ± 10	88 ± 23	59 ± 14	60 ± 8	74 ± 16	79 ± 17	60 ± 10	57 ± 2
Brevibacillus	51 ± 24	30 ± 21	50 ± 19	28 ± 18	28 ± 20	57 ± 29	29 ± 17	52 ± 23	51 ± 24	32 ± 21	46 ± 19	25 ± 20
Virgibacillus	36 ± 5	28 ± 6	34 ± 8	39 ± 8	27 ± 10	38 ± 11	36 ± 8	48 ± 10	32 ± 4	38 ± 6	41 ± 13	28 ± 5
Arthrobacter	31 ± 3	39 ± 12	41 ± 17	49 ± 15	47 ± 7	27 ± 1	36 ± 7	23 ± 4	30 ± 11	39 ± 5	22 ± 2	41 ± 12
Clostridiales (order)	36 ± 4	52 ± 9	37 ± 3	44 ± 6	39 ± 14	22 ± 15	30 ± 21	38 ± 8	20 ± 9	31 ± 16	24 ± 12	40 ± 8
Bacillus	40 ± 5	36 ± 2	23 ± 12	26 ± 3	34 ± 2	17 ± 17	32 ± 8	32 ± 6	37 ± 6	14 ± 7	22 ± 11	28 ± 4
Lysinibacillus	23 ± 4	33 ± 5	29 ± 3	27 ± 5	25 ± 3	18 ± 9	38 ± 6	29 ± 6	21 ± 3	33 ± 6	32 ± 3	31 ± 10
Psychrobacter	24 ± 6	24 ± 6	29 ± 7	30 ± 4	25 ± 3	26 ± 6	24 ± 2	30 ± 9	23 ± 1	28 ± 5	18 ± 2	19 ± 5
Enterococcaceae (family)	13 ± 13	18 ± 18	–	27 ± 27	7 ± 7	22 ± 22	22 ± 22	17 ± 17	11 ± 11	–	26 ± 26	34 ± 18
Paenibacillaceae (family)	13 ± 13	33 ± 18	–	–	32 ± 13	–	24 ± 24	21 ± 21	15 ± 9	33 ± 33	–	24 ± 24
Leuconostoc	6 ± 4	11 ± 1	13 ± 3	12 ± 1	12 ± 4	–	13 ± 4	12 ± 4	8 ± 4	8 ± 4	7 ± 4	11 ± 2
Enterobacteriaceae (family)	7 ± 7	21 ± 8	4 ± 4	8 ± 5	6 ± 6	2 ± 2	8 ± 2	–	5 ± 4	8 ± 8	4 ± 4	5 ± 3
Bacillaceae (family)	5 ± 5	–	6 ± 6	4 ± 4	5 ± 5	–	17 ± 7	5 ± 5	1 ± 1	–	6 ± 6	6 ± 3
Sporosarcina	3 ± 3	5 ± 5	–	2 ± 2	7 ± 4	–	4 ± 4	–	3 ± 3	2 ± 2	–	4 ± 4
Clostridium	3 ± 2	2 ± 2	6 ± 3	2 ± 1	3 ± 1	–	3 ± 2	3 ± 1	3 ± 1	1 ± 1	2 ± 2	2 ± 2
Carnobacterium	4 ± 4	0 ± 0	3 ± 3	4 ± 4	6 ± 3	–	–	4 ± 4	4 ± 4	–	–	3 ± 3
Myroides	1 ± 1	2 ± 2	2 ± 1	–	3 ± 1	1 ± 1	2 ± 1	1 ± 1	2 ± 1	2 ± 1	3 ± 2	1 ± 1
Streptococcus	5 ± 1	1 ± 1	2 ± 1	–	3 ± 2	–	1 ± 1	3 ± 1	3 ± 1	–	1 ± 1	1 ± 1
Planococcaceae (family)	1 ± 1	–	–	10 ± 5	2 ± 2	–	–	2 ± 2	4 ± 4	1 ±	–	–
Bacillales (order)	1 ± 1	2 ± 2	5 ± 5	–	4 ± 2	–	–	–	2 ± 1	–	–	3 ± 1
Enhydrobacter	1 ± 1	1 ± 1	–	–	–	1 ± 1	–	–	2 ± 2	2 ± 2	1 ± 1	–

Table 7
Results of one-way PERMANOVA tests evaluating the effects of sulfur dioxide (SO_2) addition level at crush (0, 20, or 40 mg/L SO_2, n = 3 per treatment) on fungal and bacterial composition throughout fermentations of uninoculated Pinot gris wines. Fungal composition was compared using three stages of fermentation (Early, Mid, Late) and bacterial composition was compared using four stages of fermentation (Cold-settling, Early, Mid, Late). Results are based on 999 unrestricted permutations of raw data. Statistical analysis was conducted separately for fungi and bacteria. Results marked with an asterisk are significant at $\alpha = 0.05$.

Fungal composition	df	SS	MS	Pseudo-*F*	*P*
SO_2 treatment	2	0.23807	0.119033	16.298*	0.001*
Residual	24	0.17529	0.007304		
Total	26	0.41335			
Bacterial composition	df	SS	MS	Pseudo-*F*	*P*
SO_2 treatment	2	0.00020	0.0000999	0.015358	0.992
Residual	33	0.21457	0.0065022		
Total	35	0.21477			

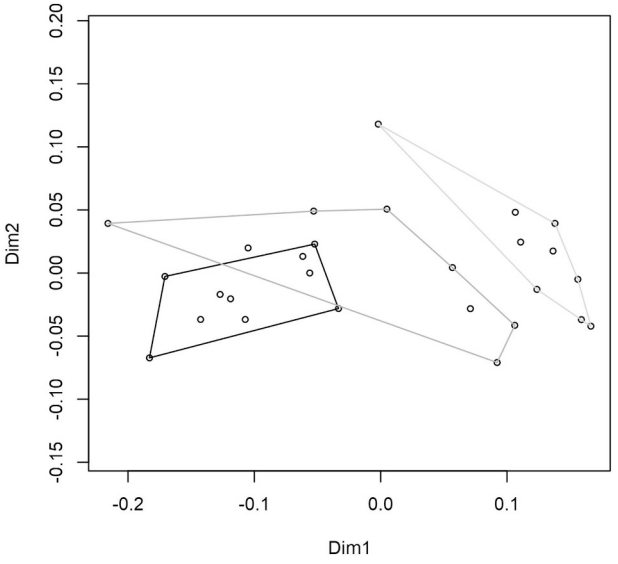

Fig. 5. Principal coordinates analysis (PCoA) ordination representing the fungal community composition of wines fermented with three levels of sulfur dioxide (SO_2) added at crush: 0 mg/L SO_2 (black), 20 mg/L SO_2 (dark grey), or 40 mg/L SO_2 (light grey) (n = 3 per treatment). Dimension 1 (Dim 1) explains 49.3% of total variation and Dimension 2 (Dim 2) explains 6.0% of variation. Samples were taken at three stages of alcoholic fermentation, for a total of nine samples per treatment. One-way PERMANOVA tests indicated significant differences in fungal community composition among treatments ($P \leq 0.03$ for all comparisons).

all treatments and remained in the fermentations until the end of alcoholic fermentation, although its relative abundance was much higher in the fermentations to which low or no SO_2 had been added (Fig. 3). Non-*Saccharomyces* yeasts including *Hanseniaspora* and *A. pullulans* are able to influence grape varietal aromas by producing several different enzymes such as β-glucosidases and proteases that can react with grape precursor compounds (Varela and Borneman, 2017). *Hanseniaspora* spp., including *H. uvarum*, *H. guilliermondii*, *H. vinae*, and *H. osmophila*, are known to contribute significantly to a wine's sensory profile, producing elevated levels of acetoin and ethyl acetate and decreased levels of higher alcohols (Jolly et al., 2014; Romano et al., 2003). Acetoin produces a soft, buttery aroma at sufficient concentrations, and ethyl acetate can range from a pleasant and fruity aroma at lower concentrations to a solvent aroma when concentrations exceed its preference threshold (Liu et al., 2015). *S. uvarum* was present in all treatments in low abundance, and was able to survive until the end of fermentation. *S. uvarum* is a competent fermenter itself, and is

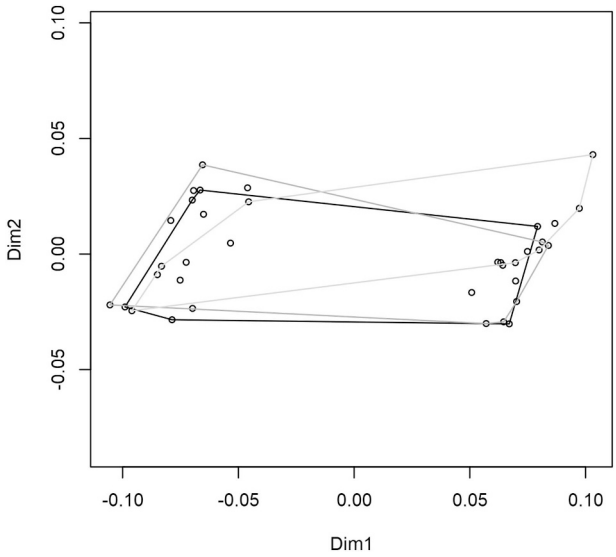

Fig. 6. Principal coordinates analysis (PCoA) ordination representing the bacterial community composition of wines fermented with three levels of sulfur dioxide (SO_2) added at crush: 0 mg/L SO_2 (black), 20 mg/L SO_2 (dark grey), or 40 mg/L SO_2 (light grey) (n = 3 per treatment). Dimension 1 (Dim 1) explains 57.7% of total variation and Dimension 2 (Dim 2) explains 4.2% of variation. Samples were taken at four stages of alcoholic fermentation, for a total of 12 samples per treatment. A one-way PERMANOVA test indicated no significant differences in bacterial community composition among treatments (F (2,33) = 0.02, P = 0.99).

characterized by its cryotolerance and its ability to produce elevated levels of glycerol, which can improve the mouthfeel of a wine (Magyar and Tóth, 2011). *S. cerevisiae* dominated the fermentations from all SO_2 treatments, likely making the most significant contributions to the wine sensory profiles. Morgan et al. (2017) previously showed that the *S. cerevisiae* strains in these fermentations were almost exclusively commercial strains used previously by the winery, and that different commercial strains were able to dominate the fermentations of different SO_2 treatments. Other previous studies have also shown that there is significant variability in the ability of different *S. cerevisiae* strains to produce compounds such as isobutanol, acetaldehyde, *n*-propanol, and isoamyl alcohol (Fleet, 2003; Romano et al., 2003). Both *H. uvarum* and *S. cerevisiae* strains are also highly variable in their production of acetic acid, an undesirable by-product (Romano et al., 2003).

Our results correspond with those of other studies that evaluated sulfited and sulfite-free wines. Studies investigating SO_2 alternatives such as ozone, gallic tannin and ascorbic acid, or hydroxytysosol have also noted increased fruitiness in wines where no SO_2 has been added, as compared with sulfited wines (Bellincontro et al., 2017; Boroski et al., 2017; Raposo et al., 2016). It should be noted that the comparisons made in these studies were between SO_2 and SO_2 alternatives, rather than simply SO_2 addition at different levels, as was investigated in this study. Henick-Kling et al. (1998) found a decrease in fruitiness and an increase in undesirable off-flavors when wines were unsulfited, but this result was confounded by the influence of inoculated wine treatments, which scored highest in attributes such as sweaty, oxidized, and paper. When only uninoculated treatments were considered, the results more closely resembled those from this study, with increased fruitiness and overall flavor intensity corresponding to lower SO_2 addition levels. It has been suggested that this increase in fruitiness could be a result of the production of compounds such as ethyl acetate, ethyl decanoate, ethyl dodecanoate, or ethyl octanoate/ethyl hexanoate by wine yeasts (Liu et al., 2016), or simply a result of the ability of SO_2 to neutralize aromas (Guerrero and Cantos-Villar, 2015; Ribereau-Gayon et al., 2006).

We suggest that future studies be conducted using a higher number

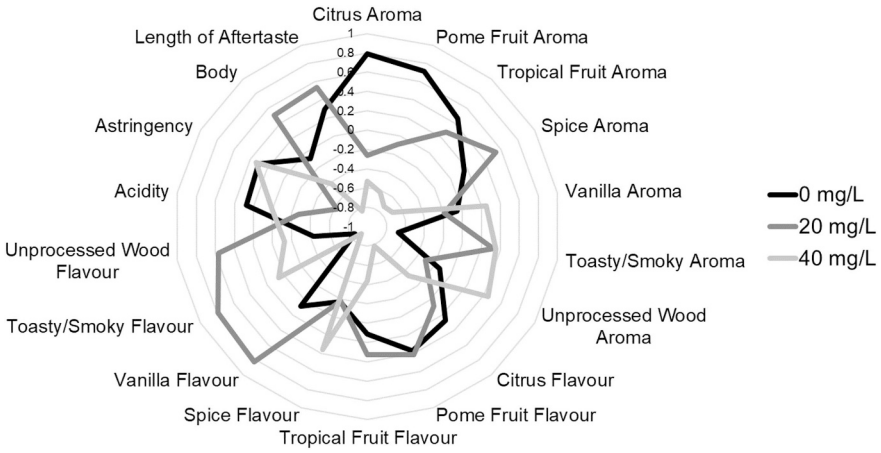

Fig. 7. Radar plot depicting the normalized relative intensity of sensory attributes of Pinot gris wines fermented with three different levels of sulfur dioxide (SO_2) added at crush. Radar plots were created using the average of three replicate wines per SO_2 treatment, evaluated in duplicate by a panel of 10 wine experts. Values were standardized separately for each sensory attribute. For visual clarity, variation among treatments for each attribute was not included.

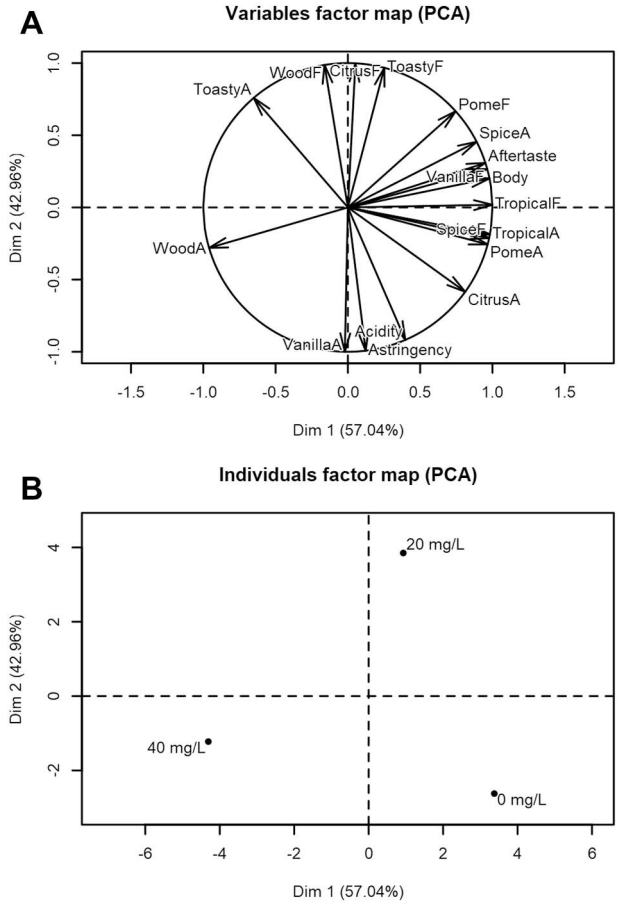

Fig. 8. Principal component analysis (PCA) ordination of the sensory profiles of wines fermented with three different levels of sulfur dioxide (SO_2) added at crush (n = 3 per treatment). Plots depict: (A) the variables factor map, and (B) the individuals factor map. Sensory attributes were analyzed by a panel of 10 wine experts. Attributes ending with "A" indicate aroma attributes, and those ending in "F" indicate flavor attributes.

of biological replicates and/or a more neutral fermentation vessel, which would allow for better elucidation of sensorial differences among wines. Previous studies have shown that when using wooden barrels in winemaking, microbes from previous fermentations can survive in the pores of wood even after cleaning and can contaminate future fermentations (González-Arenzana et al., 2013). For this reason, new oak barrels were utilized in this study to ensure that the microbial communities that were identified throughout fermentation were a true reflection of the SO_2 treatments being applied and not confounded by yeasts and bacteria harbored in the barrels from previous vintages. This resulted in all wines scoring very high in oak-related attributes such as toasty/smoky, unprocessed wood, spice, and vanilla attributes. It is therefore likely that some sensory differences attributed to differences in SO_2 addition level may have been masked by the intensity of the oak-related attributes. To maintain both microbiological and sensorial integrity, future research of this kind, where oak barrel fermentation/ageing is not a factor, should be conducted using stainless steel barrels or tanks.

4. Conclusion

This research used high-throughput amplicon sequencing (Illumina MiSeq), combined with the addition of propidium monoazide (PMA), to accurately capture the living fungal and bacterial communities present throughout uninoculated fermentations of Pinot gris wines to which different levels of sulfites (0, 20, or 40 mg/L SO_2) had been added at crush. SO_2 addition at crush significantly altered the fungal communities, with lower levels of SO_2 resulting in fermentations with higher diversity and a greater abundance of vineyard-associated yeasts. Bacterial community composition was unaffected by either SO_2 addition or fermentation stage. A sensory evaluation of the finished wines found significant differences in two key attributes (citrus aroma and pome fruit flavor), with treatments that received lower levels of SO_2 displaying increased fruitiness. Wines from all treatments were of acceptable quality and no undesirable sensory traits (off-flavors or aromas) were identified. These results are of interest to both the academic winemaking communities.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2018.09.020.

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