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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