Controlling Microbes at Crush with Chitosan in Cabernet Franc

Blenheim Vineyards

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Summary

The 2018 vintage in Virginia was marked with heavy rainfall leading to damaged fruit and potentially heavier microbial loads on the fruit. Though many winemakers used ample SO_2 at crush, wines produced from this vintage show higher than normal levels of volatile acidity. One option for combatting microbial spoilage during fermentation is chitosan. Chitosan is a naturally occurring molecule that has been used at nearly every stage of winemaking to bind to microbial cell walls and cause disruption, leakage and sedimentation. In this experiment, chitosan was used to treat one half of a lot of Cabernet Franc while the other remained untreated. There were no notable differences in volatile acidity nor microbial load between treatments in the finished wine. However, overall microbial load was lower in 2019 than 2018. Chitosan may be an effective companion to SO_2 in high microbe years.

Introduction

In 2018, Blenheim Vineyards noticed surprisingly high levels of microbial counts in finished wine, despite using 50 mg/L SO_2 at crush. High microbial load can be the source of volatile acidity and other spoilage during aging. One likely source of microbial diversity are the grapes themselves.

Grapes enter the winery covered in microbes from the vineyard. To date, 52 different species of yeast from 22 different genera have been identified on grapes including *Hanseniospora (AKA Klockera), Candida, Pichia, Hansenula, Metschnikowia, Sporoblomyses, Cryptococcus, Thodotorula, and Aurobasidium*[1]. The cast of characters changes as grapes ripen, with the greatest abundance of microbes present in the last few weeks[1]. Healthy grapes are generally inhospitable environments for any microbe because they are covered in plates of wax that form a cuticle to hold in nutrition and repel water. Microbes cluster around the stomata or next to cracks in the cuticle where seepage from the openings provides both water and nutrients. The overall microbial load on grapes depends on environmental factors such as climate, altitude, variety, age of grapes, disease pressure and vineyard practices. Fog, rain, and fruit damage (like that seen in wet vintages like 2018) quickly transform the microbial desert of grape skins into a microbial oasis. Cells that are present in small numbers quickly multiply when given the chance[1]. For example, *Botrytis* infection can increase the overall abundance of microbes by 1000x. Grapes with sour rot have significantly higher microbial diversity and abundance[2]. Insect pressure will also increase abundance due to increased vectoring from diverse environments[3]. The overall inoculant of non-*Saccharomyces* yeast and bacteria coming

into the winery from the vineyard on the grapes is often larger than the inoculant of selected *Saccharomyces* yeast added at the beginning of fermentation[1].

Non-*Saccharomyces* yeast have several impacts on the wine, both positive and negative. *Klockera apiculata* (aka *Hanseniaspora uvarum*) is a common member of the non-*Saccharomyces* yeast community found on grapes[1,4]. This yeast strain is easily identified under a microscope by its lemon shaped cells. It is tolerant to up to 100 mg/L SO_2, can grow at low temperature (such as that found during cold soak), and can produce both acetic acid and ethyl acetate (which smells like nail polish remover) under aerobic conditions[3]. Other offenders in the non-*Saccharomyces* yeast community include *Pichia guilliemondii*, a film forming yeast prevalent in warm conditions when fermentation is delayed. This yeast can form spores that become resident in barrels and produce ethyl acetate and 4 ethyl phenol (which can smell like band-aid, wet dog, horse sweat)[1–3].

In addition to yeast, many spoilage bacteria also come into the winery on grapes. Sour rot and *Botrytis* increase the prevalence of *Acetobacter*, *Gluconobacter* and *Gluconacetobacter*, all of which produce acetic acid. Several *Lactobacillus* species (*hilgardii*, *plantarum*, *casei)* and *Pediococcus* (*damnosus*) are also residents of mature grapes. These can produce acetic acid, mousy flavor and biogenic amines (which have names like putrescine and cadaverine…). They may also produce polysaccharides that lead to ropy texture[1,3,5].

High levels of native flora may also cause nutrient depletion early in fermentations, limiting nutrient availability for *Saccharomyces*, potentially leading to stuck fermentations[6,7]. In a study of nutrient depletion by non-*Saccharomyces* yeast species, Medina et al (2012)[6] found that *Metchnikowia*, a non-*Saccharomyces* yeast strain present in potentially high numbers on grapes[8], consumed YAN quickly in fermentation. Mimicking what may be occurring in fermentations with cold soaking or later inoculation, sequential inoculation with *Metchnikowia* followed by *Saccharomyces* led to stuck fermentations that could be resolved with nutrient addition. In the same study, *Hanseniaspora*, another prevalent member of the grape microbiota, did not show large YAN depletion (90% of the YAN remained 3 days after inoculation with this species), however it did deplete thiamine, an essential vitamin for *Saccharomyces*. Excessive use of SO_2, as would occur in vintages with high microbial load, also leads to reduction in thiamine, further increasing the potential for stuck fermentations.

Despite the risks, there are also some benefits to having a rich microbial community early in fermentation. Several non-*Saccharomyces* yeast species have been shown to produce positive compounds that add complexity to wine aroma such as esters, higher alcohols, glycerol, succinic acid and thiols. Proteases produced by non-*Saccharomyces* yeast have been shown to break down cells and add nutrients, ultimately making a more protein stable wine. Some produce glycosidases that help unmask aromas compounds that are bound to sugar molecules. Others produce enzymes to break down polysaccharides that would otherwise inhibit clarification and filtration. *Lachanacea thermotolerans* has been shown to consume

acetic acid, reducing volatile acidity[1,8,9]. It is likely these are some of the mechanisms that occasionally lead winemakers to employ ambient fermentations.

Many of our winemaking decisions affect the abundance and diversity of the microbial community present at the beginning of fermentation. Mechanical harvesting and long transport times, especially at warm temperatures, can lead to high microbial load[8,10]. As soon as the grapes are crushed, nutrients are released to feed the organisms that are present. *Klockera* (*Hanseniaspora*) is often the most abundant species on the grapes , and remains prevalent until alcohol levels rise above 4-7% and oxygen is used up[3,10]. The low pH environment of the juice, rising alcohol, rising temperatures, and presence of phenolics also tends to inhibit spoilage organisms in early fermentation. Harvesting of wet grapes, prolonged cold soak, cool fermentation conditions, low inoculant of yeast, and lack of clarification (for white wines) can all lead to higher counts of yeast and bacteria in the fermentation[3,11]

In wet vintages such as 2018, the prevalence of damaged berries and wet grapes likely increased the inoculant of non-*Saccharomyces* microbes in fermentation and may have contributed to overall higher volatile acidity in wines that year. One approach to microbial management is to use higher than normal levels of SO_2. Though SO_2 has efficacy against some microbial spoilage, many of these microbes (such as *Hanseniaspora*) have high tolerance to it. Much of the SO_2 added at crush is lost as it binds to grape solids that are prevalent in red wine fermentations, making it less effective. High SO_2 additions can also bind thiamine and slow down or halt fermentation, and may even select for SO_2 tolerant microbes that will cause spoilage during aging[12,13]. Still, fermentations that have some SO_2 added at crush do tend to have faster onset of fermentation and steadier kinetics (Egli et al 1998).

When SO_2 isn't enough, or when you want to limit SO_2 for other reasons, another option for combatting microbial spoilage during fermentation is chitosan. Chitosan is a naturally occurring molecule that can also be produced by the de-acetylation of chitin using NaOH or chitinase enzymes[14]. Chitin is the second-most common polymer found in nature (after cellulose), making up the cell walls of fungi and shells of crustaceans and thus readily available as a renewable resource[5]. In winemaking applications, chitin from *Aspergillus niger* is used as the source for chitosan. The effectiveness of any given formulation of chitosan depends on its molecular weight, deacetylation degree and the pH of the medium[15]. Lower molecular weight, higher degree of deacetylation is the favored formulation for antimicrobial purposes[16]. At juice and wine pH, chitosan is very positively charged, which increases efficacy[16].

Different microbes bind chitosan to different degrees. Chitosan binding to cell walls is driven by chemical properties of the cell wall itself, with high degree of correlation to the hydrophilicity of the wall[17]. Gram negative bacteria are more susceptible to binding than gram positive[17]. Chitosan has been shown to have some efficacy against a wide range of grapevine and wine microbes including downy mildew[18], powdery mildew[19], *Phomopsis*[20], *Lactobacillus*, *Oenococcus*, and *Brettanomyces*[17]. Due to its versatility as an antimicrobial agent, chitosan in

various forms has been used worldwide at nearly every stage of wine production including vineyard applications, on grapes during transport and storage, at crush, after fermentation and during the aging of wine[16,21,22].

Many different mechanisms of antimicrobial action have been proposed for chitosan in wine. Chitosan has been shown to physically bind to the cell walls of microbes[15,17]. Binding may aid in sedimentation, leading to reduction in overall cell number with racking. Binding of the positively charged molecular of chitosan may also disrupt cell membranes, leading to leakage of ATP, potassium, and proteins, all essential components of cell function[15,17]. Leakage may therefore result in semi-viable cells or cell death. Other proposed mechanisms include the physical blocking of cell permeability by chitosan binding, chelation of survival factors such as copper, penetration of the cell membrane and binding to DNA[15].

Regardless of the mechanism, chitosan has been shown to be quite effective in treating microbial infection of wine. When treating wine already inoculated with *Brettanomyces bruxellensis*, Taillander et al (2014) found that 85% of the population of *Brettanomyces* cells were dead within 20 hours of treatment. There was a dose effect in the rate of cell death, with 0.4 g/L treatment killing cells faster than 0.04 g/L. They also found differences in susceptibility based on the strain of *Brettanomyces* used, presumably due to genetic differences in cell wall components. Many cells initially compromised by chitosan treatment recovered, as evidenced by growth in the *Brettanomyces* population after 7 days. Other researchers tested the effect of 0.04 g/L chitosan on aging wine and found that, even at low dose, aging of wine on chitosan helped prevent infection by *Brettanomyces*[23].

Most chitosan products are recommended for use in finished wine. Stab Micro M (Enartis) is a chitosan-based product specially formulated for use on juice and must to reduce the activity of a wide range of microbes, leading to reduction in volatile acidity, sulfide defects, volatile phenols and production of other off-flavors. It can be used in conjunction with SO_2 or as a replacement, depending on the condition of grapes and preference of the winemaker[16]. In this experiment, a single harvest lot of Cabernet Franc was split so that half received treatment with chitosan while the other half did not. Both bins received SO_2. Resulting chemistry and microbial load were assessed post fermentation and after aging.

Methods

At the time of harvest, fruit was divided evenly and randomly among four TBins (two each received treatment and control). Cabernet Franc was made according to the winery's standard protocol. Grapes were picked, chilled overnight, sorted, destemmed, and loaded into TBins with 50 mg/L SO_2. There were two TBins per treatment. All bins received SO_2. Stab Micro M was sprinkled in layers into the treatment at the de-stemmer at alternative times with SO_2. Tbins were moved to a temperature-controlled warehouse and inoculated at a rate of 15g/hL of EC1118 yeast the following day. Tartaric acid (1.5 g/L) was added after the end of lag phase (on

day 4 after destemming). Fermentations were monitored for Brix and temperature and punched down twice daily throughout the fermentation until Brix were below -1.5. Both Tbins per treatment were pressed together 10 days after processing, after the completion of alcoholic fermentation, with free run and press fractions combined. Pressed wine was allowed to settle, racked, inoculated with 1 g/hL ML One (Enartis), then transferred to barrel. Malic acid depletion was checked after three weeks and confirmed dry with enzymatic analysis. Tartaric acid (2 g/L) and SO_2 (75 ppm) were added without racking. Two TBins of each treatment were pressed together. Each treatment had three finished barrels of the same cooperage, age, and forest after pressing, which were maintained separately until the time of sampling.

Results

Fruit was harvested at 22.9°Bx with pH of 4.07. Fermentation proceeded normally with no noticeable differences among TBins (data not shown). After completion of primary fermentation, there were no differences in pH or malic acid (Table 1). After completion of malolactic fermentation, all malic acid was <0.10 g/L and glucose/fructose was <1.0 g/L. General chemistry was similar for all barrels in both lots, with no difference in volatile acidity (Table 2), color (Figure 1) or microbiology (Table 3).

Preliminary Conclusions

This experiment was initiated to address higher than acceptable microbial loads found in 2018 wines. The rationale was that SO_2 may not be enough to control microbial growth and additional measures were needed. The overall microbial load on grapes varies with vintage, variety, and region, an effect seen here.

When compared with overall microbial loads from 2018, both treatment and control lots in 2019 had notably lower levels of spoilage organisms (Table 3). Lower levels of acetic acid bacteria, *Brettanomyces*, and lactic acid bacteria can significantly reduce risk of spoilage during longer aging. In 2019, the winery's normal dose of SO_2 (50 ppm at crush) was sufficient to control populations of spoilage organisms. However, in 2018 it was not. Though this experiment showed few differences in 2019, chitosan may still be a valuable tool to augment SO_2 in wet or otherwise compromised vintages.

Table 1: Wine chemistry following primary fermentation for two treatments of Cabernet Franc (in-house lab)

Treatment	pH	Malic Acid (g/L)
Control	3.9	1.03
Stab Micro	3.89	0.98

Table 2: Wine chemistry after malolactic fermentation and aging for two treatments of Cabernet Franc (ICV labs)

Treatment	VA (g/L)	pH	TA (g/L)	Lactic Acid (g/L)	Alcohol (%)
Control	0.51	3.56	5.22	1.45	13.52
Control	0.53	3.59	5.16	1.5	13.66
Control	0.51	3.54	5.23	1.43	13.47
Stab Micro	0.53	3.57	5.1	1.5	13.25
Stab Micro	0.53	3.64	4.99	1.62	13.5
Stab Micro	0.58	3.56	5.14	1.48	13.3

Figure 1: Color intensity for two treatments of Cabernet Franc (ICV Labs)

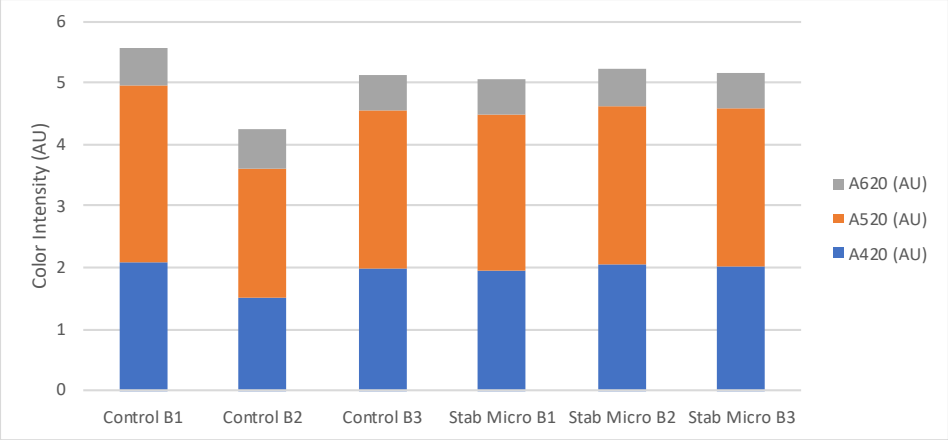

Table 3: Microbiology after aging for two treatments of Cabernet Franc (ETS Labs)

	2019		2018	
Microbe (Cells/mL)	Control	Stab Micro M	2018 Merlot	2018 Petit Verdot
Saccharomyces cerevisiae	$3x10^4$	$4.5x10^4$	460	$3.67x10^4$
Oenococcus oeni	$>1x10^7$	$>1x10^7$	2.9×10^6	$>1x10^7$
Acetic Acid Bacteria	$1x10^5$	$2x10^5$	$2x10^6$	$8.2x10^6$
L. brevis/hilgardii/fermentum	0	0	830	$2.4x10^4$
Lactobacillus plantarum/casei/mali	0	10		
Lactobacillus kunkeei	0	0		
Pediococcus species	0	0	140	$1.39x10^5$
Brettanomyces bruxellensis	0	0	$2x10^6$	$3.4x10^4$
Zygosaccharomyces species	10	0	390	3230

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