Oenology Anonymous: Identifying Temporal Changes in Wine Microbial Communities Throughout a Fermentation Process and in Response to Biological and Chemical Additives

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Project Summary:

The earliest evidence of wine fermentation dates to 8000 BCE (Daley 2017). Historically, wines were fermented using the bacteria and yeast naturally present on grapes and in the winemaking environment. In recent years, a growing demand for wine makers to produce a consistent, stable product has pushed producers to further rely on these chemical additives such as potassium metabisulfite and potassium sorbate to stabilize biological and chemical changes in their wine. While the effects of potassium metabisulfite and potassium sorbate on certain spoilage organisms are well studied, less is known about the effects of these and other additives such as pH adjustment agents and alternative plant extracts on the entire microbial community of wine (Takahashi 2014).

Our first objective is to identify the microbial communities of wine and grapes at several time points throughout the fermentation process of Chill Switch wines in Cedaredge, Colorado. We will obtain samples of the common grape vine, *Vitis vinifera*, samples of wine must before and after cultured yeast was added, and samples of aged wine before and after small amounts of potassium metabisulfite are added from the Chill Switch Winery. Each sample will be subject to DNA extraction and sent to the Wyoming Public Health Laboratories for sequencing. The DNA sequencing will allow us to identify microbial communities present at each timepoint without the need to culture organisms. We expect inoculated microbes to outcompete others natural microbiota and we also anticipate a decrease in the microbial biodiversity as the fermentation process progresses. In order to test the effects of additives, we will replica-plate microbial communities from wine onto plates with the following standard and alternative additives: potassium metabisulfite, potassium sorbate, citric acid, tartaric acid, and thyme oil. Additionally, we hypothesize that each additive will inhibit growth of some organisms while permitting growth of others where, in general, lactic acid bacteria (LAB) and acetic acid bacteria will be more resistant than yeast to the pH adjustment agents. Regardless of whether our hypotheses are supported, the results of this study will contribute to a greater understanding of microbial dynamics in fermenting grape must. There may be additives that are more effective at killing spoilage organisms while allowing for survival of beneficial organisms than the chemicals currently in use. This understanding will open doors to production of higher quality wine with greater benefit to the human microbiome. We hope our findings will encourage winemakers to explore alternative additives and methods for wine making. Future studies could test these additives on the sensorial qualities of wine to determine which additive(s) contribute to a final product of wine that is more desirable to wine consumers.

Problem and Significance:

The United States Food and Drug Administration (FDA) requires any food product to include an extensive label listing any ingredient it may contain. However, unlike food products, alcoholic beverages, such as wine, are exempt from listing any ingredients or additives beyond the percent alcohol by volume. As such, many winemakers include a long list of additives to their product that remain unknown to the consumer. Many consumers believe some additives increase the severity of the symptoms of the so-called “hangover” or “red wine headache” and some experience severe sensitivities to common additives such as sulfites (Costanigro et al. 2014). As such, some small-scale wine makers such as Dave Aschwanden of Chill Switch Wines in Cedaredge, Colorado, prefer not to use additives and rely primarily on wild yeasts, bacteria, and nutrients naturally present in and on grapes. The proposed research aims to characterize the microorganisms present throughout the fermentation process of Chill Switch wines.

Additionally, we will test the in-vitro effects of common and alternative wine additives on the microorganisms found in Chill Switch wines. This research has many implications for the wine industry as the microbes present throughout the fermentation process define the taste and quality of the final product. Additionally, the discovered effects of these additives on the microbial diversity of wine may promote future research as to their effects on the human microbiome as the human microbiome is highly influenced by food and drink consumed.

Introduction:

Relevant literature:

Historically, wines made throughout the world were fermented using wild yeast and bacteria naturally present on grapes. The microbial communities of grapes from many different regions around the world have been characterized and found to contribute significantly to the concept of *terroir*, the subtle differences between wines from different regions (Belda et al. 2017). Diverse microorganisms have the ability to change many factors, such as flavor, texture, or acidity, that affect the quality of the final wine product (Medina 2013). These microorganisms, such as yeast of the genus *Saccharomyces* and lactic acid bacteria, contribute favorably to the final product by increasing the alcohol content and converting malic acid to the more mild and favorable lactic acid (Lonvaud-Funel 2010). However, other naturally occurring organisms, including acetic acid bacteria (AAB), such as *Acetobacter* and *Gluconobacter* species, contribute to spoilage and "off flavors" in wine (du Toit et al. 2005). Due to the growing demand for wine makers to produce a stable, consistent product, many rely on chemical additives such as potassium metabisulfite and potassium sorbate to serve as antimicrobial and anti-oxidative agents to stabilize biological and chemical changes in their wine. Of these agents, potassium metabisulfite is the most extensively studied. Studies have found that, although this additive is used to kill undesirable microorganisms and generally reduces their growth, some spoilage organisms such as *Acetobacter pasteurans* show resistance to potassium metabisulfite (du Toit et al. 2005) while some desirable organisms are more sensitive (Bokulich et al. 2015). This is problematic, because if resistant organisms are being selected for, then these additives may one day no longer function to inhibit unwanted organisms, such as wine spoiling bacteria, causing faster spoilage and a shorter shelf life.

Previous research has focused on the effects of sulfites on the growth of spoilage organisms commonly found in wine (Takahashi 2014). Minimal research has been done on the effects of alternative antimicrobials on select microbes found in wine. Further research is necessary to elucidate these effects in the scope of the whole microbial community of wine (Sabel et al. 2017). When it comes to naturally fermented wines, the incredible microbial biodiversity and unique microbial communities of wine and grapes from different regions must be considered when analyzing the effects of wine additives. This research aims to provide a broader understanding of the effects of biological and chemical additives on the entire microbial community of Chill Switch naturally fermented wine. As consumers become increasingly concerned about chemical additives in foods and beverages, it is critical to better understand the role that additives, both chemical and biological, play in the winemaking process. Additionally, an understanding of the effects of pH adjusting agents will become increasingly important in our changing climate as warmer weather produces grapes with a higher sugar content and lower acidity (Mayer 2013). Chill Switch is already noticing these changes.

Preliminary Data:

Our community partner, Chill Switch Winery from Cedaredge, Colorado, strives to make wine that is as natural as possible with little to no additives. We will strive to provide useful information regarding the microbiome of Chill Switch wine and its sensitivity to various additives of interest. Therefore, an understanding of the methods Chill Switch uses is critical to an understanding of our proposed research. The winemaker, Dave Aschwanden, uses only Colorado-grown grapes, primarily from vineyards located near Cedaredge and Palisade, CO. When he receives grapes, usually in September or October, he crushes them to remove the stems and allows them to ferment in an open vat to ferment with only the wild yeast and bacteria naturally present on the grapes and in the environment for approximately one week. When he "can no longer stand the smell," he uses another batch of wine further along in the fermentation process to inoculate the newer batch and allows the mixture to continue fermenting in the open vat for about another month. Dave maintains a batch of Sauvignon Blanc to which he has pitched an unknown strain of *S. cerevisiae* to inoculate other batches of wine, thus the microbial profile of this inoculum is likely somewhat diverse rather than a monoculture of *S. cerevisiae*. However, he may, for example, inoculate a batch of Cabernet Franc using this Sauvignon Blanc but use the inoculated Cabernet Franc to inoculate a future batch of Petite Verdot. When Dave estimates that almost all the sugar present in the grapes has been converted to alcohol, he presses the grapes, removing the skins and stems, and transfers the wine to oak barrels. At this point he inoculates the wine with wine containing *S. cerevisiae* EC-1118, champagne yeast very tolerant of high alcohol, low sugar environments. The wine is left in the barrels to age for 30 months before being bottled. Dave adds a small amount of sulfite when bottling the wine and uses a sulfite solution to store his barrels, but otherwise uses no chemical additives. However, he rarely observes spoilage or acetic acid fermentation in his wine. He primarily relies on taste and intuition more than objective measurements such as pH.

Conceptual Model:

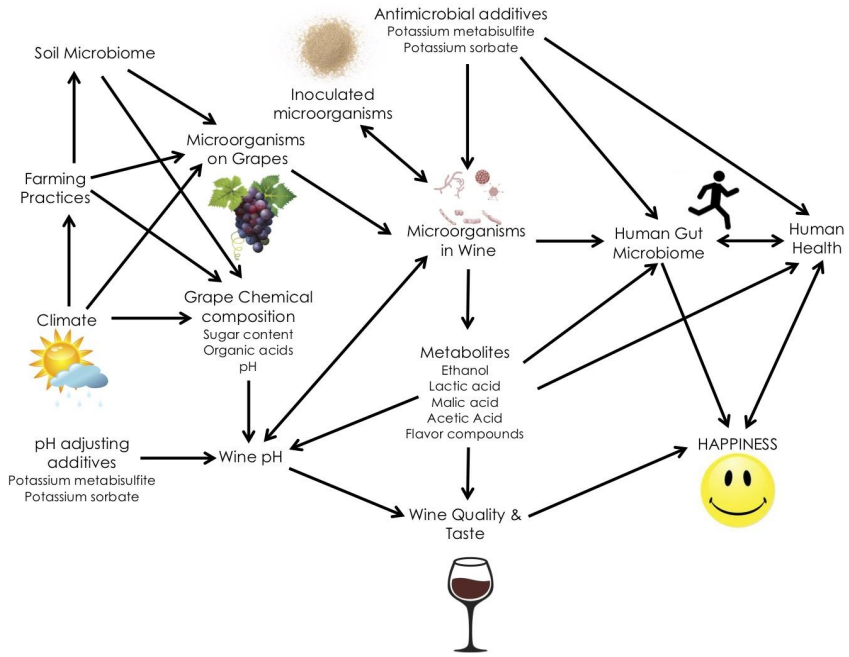

Figure 1: A conceptual model of the broad significance of our research.

Justification of methods:

We will use both culture dependent and culture-independent methods in this study. Because most organisms are difficult to culture or cannot be cultured at all, DNA sequencing methods are becoming the gold standard for identification of microbes. 16s rRNA gene regions of many bacteria and ITS regions of fungal rRNA genes have been sequenced and extensive databases exist to identify organisms based on these sequences (Clemmenson et al. 2016). Next-generation sequencing methods to sequence the V4/V5 regions of the bacterial 16s rRNA gene and fungal ITS2 region will be used to identify organisms present in our samples and how these communities change throughout the fermentation process and in response to additives. Culture-based replica plating will be used to test the effects of additives on microorganisms present during the fermentation process. Furthermore, a wide variety of both complete and selective media will allow us to culture much of the wine microbial community as well as roughly identify the organisms grown. Yeast peptone dextrose media (YPD) and trypticase soy agar (TSA) are both rich, complete media allowing for the growth of most yeast and bacterial species respectively. Lactobacilli MRS media allows for the growth of *Lactobacillus* species and other LAB which may not grow on TSA. We will use Lin's Wild Yeast Media (LWYM) and Lin's Cupric Sulfate Media (LCSM) to select for wild *Saccharomyces* yeast and non-*Saccharomyces* yeast respectively while inhibiting the growth of cultivated *S. cerevisiae* (Zainasheff 2010). Finally, Wallerstein Laboratories differential (WLD) media will allow us to select for LAB and AAB while inhibiting the growth of yeast (Zainasheff 2010).

Objectives:

Objective 1a: To gather data and quantify the microbial biodiversity throughout each step of the Chill Switch winemaking process from grapes to bottled wine.
Objective 1b: To understand the effect that pitched or inoculated yeasts, such as *Saccharomyces cerevisiae*, have on the naturally occurring microbes isolated from samples of grapes, must, and wine.
Objective 2: To understand and provide data on the effects of standard and alternative additives on microbial communities that could potentially be used to control the microbiome during the wine fermentation process.

Hypotheses:

Hypothesis 1: The microbial diversity of the wine will be observed to decrease throughout the wine production process as the less prevalent microbes are outcompeted by the innoculated species, primarily *Saccharomyces cerevisiae*.
Hypothesis 2a: Yeast species, especially wild ones, will show reduced growth in the presence of antimicrobial, antioxidant, and pH adjusting agents relative to lactic and acetic acid bacteria such as those from the genera *Lactobacillus, Acetobacter, and Gluconobacter*. This selective effect will be more pronounced at lower concentrations of additives.
Hypothesis 2b: The antimicrobial and antioxidant agents potassium metabisulfite potassium sorbate) will reduce growth of all organisms more than pH adjusting agents (citric and tartaric acids).

Specific Aims:

Specific Aim 1: We will characterize the microbial community using bacterial 16S and fungal ITS2 gene sequencing of Chill Switch Gewurztraminer and Petite Verdot grapes and wines at the following time points during the fermentation processes:

a. Freshly picked grapes
b. Grape must fermented by wild yeasts for one week (just prior to inoculation)
c. Grapes fermented for 3 days after inoculation
d. Wine aged for 12 months (2017)
e. Wine aged for 24 months (2016)
f. Wine aged for 30 months and bottled with sulfites (2015)

Specific Aim 2: We will characterize the sensitivity of microorganisms isolated from both types of grape must fermented by wild yeasts for one week to several commonly used and alternative wine additives: We will use replica plating techniques with varying concentrations of each of the following additives:

a. Common antimicrobial and antioxidant agents:
    i. Potassium metabisulfite
    ii. Potassium sorbate
b. pH adjustment agents:
    i. Citric acid
    ii. Tartaric acid
c. Alternative additives:
    i. Thyme oil

Materials and Methods:
Overview:

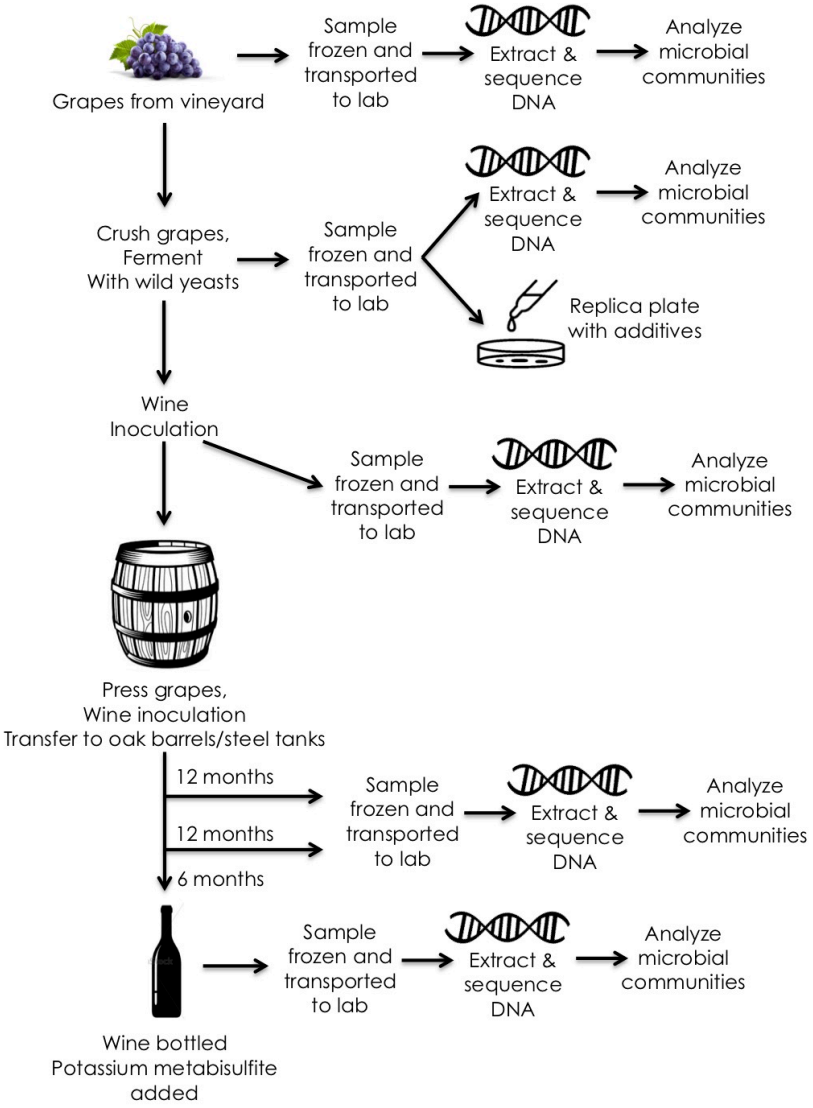

Figure 2. Overview of the Chill Switch wine making process and the sampling and tests to be done at each stage.

Table 1. The methods for this experiment and how they correspond to our hypotheses.

	Hypothesis 1	Hypothesis 2a	Hypothesis 2b
Sequencing	X		
Replica plating		X	X

Materials and Methods:

Sample collections:

We will collect samples of fresh wine grapes *Vitis vinifera* of the Gewurztraminer and Petite Verdot varieties in sterile Whirl-Paks for extraction of microbial DNA. We will sample liquid from grape must fermenting with wild yeast for 3-7 days (time point 1) and must that has fermented for 1-3 days following inoculation (time point 2). Additionally, we will sample the wine(s) used for the inoculation. Samples will also be collected from wines made from the same grapes from the same vineyards in 2017 and 2016, along with bottled wine with added sulfites made in 2015. These wines will have aged for 12, 24, and 30 months, respectively. For each sample, we will collect approximately 35ml of liquid for DNA extraction in 50ml falcon tubes. Additionally, 1ml of grape must from time point 1 will be combined with 250μl glycerol to create a freezer stock for future culturing use. All the above samples will be frozen on dry ice on site and transported back to the lab in Laramie, WY.

Replica Plating:

Freezer stocks will be used to inoculate appropriate 5mL broth tubes of the following media: yeast peptone dextrose media (YPD), trypticase soy broth (TSB), lactobacilli MRS broth, Lin's Wild Yeast Media (LWYM), Lin's Cupric Sulfate Medium (LCSM), and Wallerstein Laboratories differential (WLD) Media. These broth tubes will be incubated at 30^{o}C for 48 hours and broth cultures will be plated onto a corresponding agar plate (e.g. TSB broth cultures will be plated onto TSA). A dilution scheme using a phosphate buffered saline (PBS) solution may be needed to achieve isolated colonies on agar plates. Plates with isolated colonies will be used as template plates to replica-plate colonies onto corresponding agar containing the following concentrations of wine additives.

Table 2: Wine Additives

Additive	Concentration (g/L)		
	Low	Mid	High
Potassium metabisulfite	.014	0.029	0.057
Potassium sorbate	0.100	0.250	0.500
Tartaric acid	0.98	1.95	3.91
Citric acid	0.90	1.80	3.60
Thyme oil	0.01	0.10	1.00

Colonies on original plates will be numbered and accounted for on replica plates. These colonies will be identified as sensitive or resistant to the additive. Colonies of interest based on susceptibility to additives may be chosen to undergo further biochemical testing and identification. We will also make freezer stocks of these colonies of interest.

DNA extraction and sequencing:

The following procedure has been adapted from Wei et al. 2017. We will suspend frozen grapes in 25ml PBS buffer in 50 ml falcon tubes and vortex for 15 minutes on maximum speed. Tubes will be centrifuged at 9000xG for 10 minutes. The supernatant will be discarded and 250mg of the pellet transferred to DNA extraction bead tube. We will extract DNA from the pellet using an OMEGA Bio-Tek E.Z.N.A. Soil DNA kit according to the manufacturer's protocols. DNA extractions will be tested using PCR and Nanodrop to ensure sufficient yield. We will amplify the ITS2 of the fungal rRNA gene region from 50μl from each DNA extraction using the gITS7 and ITS4 primers (Clemmeson et al. 2016). From the other 50μl of extracted DNA, we will amplify the V4/V5 region of the bacterial 16s rRNA gene using 518F and 926R primers. Amplicon-prepped DNAs will be sent to the Wyoming Public Health Laboratories for sequencing by our collaborator Noah Hull.

Data Collection and Analysis:

For the replica plating procedure, data will be collected by enumerating the number of colonies before and after addition of additive. Colonies will be identified as sensitive or resistant to the additive. These data will be analyzed for statistical significance using an ANOVA test to determine if there were any significant differences between the number of colonies on each plate. These statistics will inform us of the effect that each additive had on the growth of the colonies and allow us to determine if the microbes were resistant or sensitive to any of the additives. This data will be kept on a shared online spreadsheet for easy access to all group members.

DNA sequencing results in the form of fast-q files from the Wyoming Public Health Laboratories will be stored on the University of Wyoming's TETON computing cluster and we will analyze sequence data via the R package DADA2. We will compare alpha and beta diversity between samples as well as comparing the presence and absence of specific organisms with known oenological functions.

Expected Results:

Sequencing Results:

We anticipate that the alpha diversity will be highest for fresh grapes and will decrease throughout the fermentation process, especially following inoculation. An unexpected result is that the microbial diversity in the fermenting must sample is greater than in the grape sample. This may indicate that other sources of microorganisms (e.g. the hands of those picking the grapes, crushing equipment, and other surfaces in the winery) significantly contribute to the organisms present during fermentation. Increasing microbial diversity at later stages in the process may indicate contamination in either Dave's process our own methods We also anticipate that communities found on fresh grapes will be similar to those found in fermenting grape must prior to inoculation and that these communities will differ slightly between grape varieties. Differing microbial communities between varieties will support the notion that microbial communities contribute to subtle differences between wine types and regions. Following inoculation, we anticipate the microbial communities of both wine varieties to

converge and resemble that of the wine used for inoculation, containing a significant proportion of *S. cerevisiae* and very few wild yeast and bacterial species. These results will indicate that the inoculation method currently employed by Chill Switch is an effective means of controlling growth of wild microbial species that may be spoilage organisms. Results other than those described above may indicate that these inoculation methods are not successful and other methods should be considered and researched.

Replica Plating Results:

We expect to culture and isolate both wild and cultivated yeasts along with LAB, AAB and other bacteria. In general, we expect to find fewer colonies on plates with additives than on the original plates. If there was no significant reduction or a higher number of colonies was observed between the original plate and the replica plate with additives, then we will look at other factors influencing our results, such as contamination. We anticipate that wild yeast will be the most susceptible to additives (showing the least resistances), while we expect to find some LAB and AAB that show resistance to many additives, especially those used to adjust pH. This will result in selection for LAB and AAB, especially at low additive concentrations and low pH. These results will indicate a need to reconsider use of low potassium metabisulfite concentrations, as used by Chill Switch. If such a selective effect is not found, our results will support this practice. At higher concentrations of the additives potassium metabisulfite and potassium sorbate, growth of most organisms will be inhibited. The growth of most organisms will also be inhibited in the presence of thyme oil. These results will support the use of thyme oil in wine making and present a need for future research into the effects of other plant extracts on the wine microbiome as well as their effects on other aspects of wine quality such as taste.

Timeline:

Table 3: Projected Timeline

Task	Week 0	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6
Collect Samples	X						
Prepare media		X					
Culture and Isolate		X	X				
Replica Plating			X	X	X		
Genetic extraction and prep		X	X				
Send DNA to be sequenced				X			
Analysis of data						X	X

References:

Belda, I., Zarraonaindia, I., Perisin, M., Antonio Palacios, A., Acedo, A., "From Vineyard Soil to Wine Fermentation: Microbiome Approximations to Explain the "Terroir" Concept", Frontiers in Microbiology, 8 (2017), doi: 10.3389/fmicb.2017.00821.

Accessed via the University of Wyoming Web of Science database. This article supports that the unique microbiome of different grapes and wine is important to the taste and quality of the final product (so very applicable to a project involving the microbial diversity of natural wine). It is a very new article, and cited 5 times in its year of existence.

Bokulich, N., Swadener, M., Sakamoto, K.,Mills, D., Bisson, L., "Sulfur Dioxide Treatment Alters Wine Microbial Diversity and Fermentation Progression in a Dose-Dependent Fashion", American Journal of Enology and Viticulture, 66 (2015), 73-79.

Accessed via the University of Wyoming Web of Science database. This newer article supports some of the findings of the above but used some more sequencing-based methods to look at effects of SO2 on microbial community diversity more generally (rather than particular organisms with known influences on wine). This article could be useful for methods as well. Cited 16 times in the last ~3 years.

Clemmensen, E., Ihrmark, K., Durling, M., Lindahl, B., "Sample Preparation for Fungal Community Analysis by High-Throughput Sequencing of Barcode Amplicons", Methods in Molecular Biology 1399 (2016), 61-88.

Accessed via the University of Wyoming Web of Science database. We used this article to aid us in establishing methods for whole community sequencing analysis for fungi.

Costanigro, M., Appleby, C., Menke, S., "The wine headache: Consumer perceptions of sulfites and willingness to pay for non-sulfited wines", Food Quality and Preference 31 (2014) 81–89.

Accessed via the University of Wyoming Web of Science database. This article was used to provide background information on consumer perspectives on additives in wine to contribute to the significance of our project.

Daley, Jason., "Oldest Evidence of Wine Making Found in Georgia," Smithsonian.com, (2017).

This article is important to establish history and credit in our introduction and to draw readers in.

du Toit, W.J., Pretorius, I.S., Lonvaud-Funel, A., "The effect of sulphur dioxide and oxygen on the viability and culturability of a strain of Acetobacter pasteurianus and a strain of Brettanomyces bruxellensis isolated from wine", Journal of Applied Microbiology, 98 (2005), 862-871.

Accessed via the University of Wyoming Web of Science database. Also a little old but the finding that "undesirable" organisms may be somewhat resistant to SO2 treatment is critical to informing this project.

Isci, B., Yildirim, H. K., and Altindisli, A., "Evaluation of Methods for DNA Extraction from Must and Wine," Institute of Brewing and Distilling., 120(2014)238-243.

This article was accessed through the Web of Science database using the phrase 'wine and must DNA extraction.' This article is for the DNA extraction method referenced in the methods.

Laegreid, I. R., "Genotypic and Phenotypic Characterization of Norweigan Farmhouse Ale Yeast Cultures: A Domestication-Driven Evolution," Norwegian University of Science and Technology., (2017).

This study described certain media that were beneficial for isolating certain yeast species from beer and was relevant to our work with isolating certain yeast from wine. This article was accessed through the University of Wyoming databases using the phrase 'media for isolating yeast cultures.

Lonvaud-Funel, A., Managing Wine Quality, Woodhead Publishing, Cambridge, UK (2010) Chapter 3.

Accessed via the University of Wyoming Web of Science database. Lonvaud-Funel has published many papers on various aspects of winemaking that I looked at, mostly focusing on lactic acid fermentation. This chapter of a book summarizes a lot of his research very nicely. It's a little old but in the grand scheme of how long humans have been making wine, not old at all.

Lopez, I., Ruiz-Larrea, F., Cocolin, L., Orr, E., Phister, T., Marshall, M., VanderGheynst, J., and Mills, D. A., "Design and Evaluation of PCR Primers for Analysis of Bacterial Populations in Wine by Denaturing Gradient Gel Electrophoresis," Applied and Environmental Microbiology., 69(2003)6801-6807.

This article was accessed through the Web of Knowledge database using the phrase 'primer for bacterial population in wine.' It provides good information about what primers to use and regions to amplify to sequence wine bacteria and yeast.

Mayer, A, "Climate Change Already Challenging Agriculture", Bioscience , 63 (2013) 781-781.

Access via the University of Wyoming Web of Science database, this article was used to verify Dave's observations of effects of warmer, drying climate on sugar and acid content of grapes.

Mazziotti, M., Henry, S., Laval-Gilly, P., Bonnefoy, A., and Falla, J., "Comparison of Two Bacterial DNA Extraction Methods from Non-Polluted and Polluted Soils," Folia Microbial., 63(2018)85-92.

This is the source for the bead beating procedure referenced in the methods. It was accessed through the Web of Science database using the keywords 'beat beating' and 'DNA extraction.'

Medina, K, Boido, E, Farina, L, Gioia, O, Gomez, M.E., Barquet, M, *et al.* "Increased flavour diversity of Chardonnay wines by spontaneous fermentation and co-fermentation with *Hanseniaspora vineae,"* Food Chemistry., 141(2013)2513-2521

We used the University of Wyoming's Web of Knowledge database to access this article. It has been cited in other publishing 65 times since its 2013 publication, according to ScienceDirect.com. Our interest in this article stems from its novel methods wherein the

researchers made wines with three different yeast community inoculations. The findings were that co-fermentation with a strain of *Heniaspora vineae* followed by the standard *Saccharomyces vineae* led to the product wine with the highest chemical and sensory complexity. Many of their methods could be adopted for our study including the use of WL nutrient medium to differentiate wild yeast strains and the use of a panel of professional wine-tasters to identify the prominence of different flavors between the wines.

Sabel, A., Bredefeld, S., Schlander, M., and Claus, H., "Wine Phenolic Compounds: Antimicrobial Properties against Yeasts, Lactic Acid and Acetic Acid Bacteria," Microbiology and Wine Research., 3(2017).

This article is important to our work as it showed the potential of certain natural antimicrobials to be used as wine additives, while stating the need for further research. This article was accessed through the University of Wyoming databases using the keywords 'antimicrobial properties against bacteria and yeast in wine.'

Takahashi, M., Ohta, T, Masaki, K, Mizuno, A, Goto-Yamamoto, N, "Evaluation of microbial diversity in sulfite-added and sulfite-free wine by culture-dependent and-independent methods" J. Biosci. Bioeng., 117 (2014), pp. 569-575

We discovered this article on Pubmed.org. Though it is cited only 9 times since its 2014 publication, it is one of few articles looking at the entire microbiome of wine with and without the addition of sulfites. Though their culture independent identification method of gel electrophoresis is not the approach we will be taking for DNA identification, the media used for the detection of Lactic Acid Bacteria is pertinent to our study. Since we want to identify as many microbes as possible within our wine samples, we need a diverse set of media to culture the diverse microbiota of the wine and must.

Wei, Y., Wu, Y., Yan, Y., Zou, Y., Xue, Y., Zou, Y., Xue, Y., Ma, W., Wang, W., Tian, G., Wang, L., "High-throughput sequencing of microbial community diversity in soil, grapes, leaves, grape juice and wine of grapevine from China", PLoS ONE 13(3), doi: 10.1371/journal.pone.0193097.

Accessed via the University of Wyoming Web of Science database. This article was used to develop methods for preparing grape and wine must samples for DND extraction.

Zainasheff, W. "Yeast: The Practical Guide to Beer Fermentation", Brewer's Publications (2010).

This book was used to identify selective and differential media to use in our replica-plating technique.