Washington State Grape and Wine Research Program

FINAL REPORT
2017-2020 Project Duration

Project Title: **Impact of pH on the wine microbial ecology and wine quality**

PI: Hailan Piao, Co-PIs: Thomas Henick-Kling, Thomas Collins, James Harbertson

Summary

Wine is a very complex alcoholic beverage as it contains a large number of components and its quality is affected by many environmental factors and diverse microorganisms. The growing environment and harvest maturity are key factors determining must and wine pH and acidity. The grape must pH in turn is a key determinant for the development of the yeast and bacteria populations during wine fermentation. The high pH and low grape must acidity affects many aspects of wine, such as, flavor and mouthfeel, color, and microbial stability. Typical Washington State red grape varieties have a high pH and low amount of malic acid. In high pH grape must, potential spoilage bacteria such as *Lactobacillus* sp. and P*ediococcus* sp. easily grow. L-malic acid and tartaric acid are the main organic acids of grape and wine and both acids play a dominant role in the wine acidity. Grapes grewn in hot climates often show low acidity with high pH because most of the malic acid has been metabolized during grape ripening. Must and wine pH a critical physiological factor which significantly influences the yeast and bacterial populations and their metabolic activities in wine. Optimizing must pH to enhance the activity of beneficial wine microorganisms while to suppress growth of spoilage microorganisms are of vital importance to winemakers. Yeast and bacteria starter cultures are valuable tools in managing the microbial populations in high pH grape musts. In this project we evaluate the impact of acid additions such as a high acid wine, malic and tartaric acid, as well as the addition of yeast and bacteria starter cultures on fermentation rates, microbial populations, the metabolites and sensory profiles. We evaluated various acid and starter culture additions in two vintages, 2017 and 2018.

Yeast and bacteria population analysis by plating showed that native yeast and bacteria population from grapes were between 10^3-10^5, which population effectively reduced 10-100 fold with SO2 (30 -50 mg/L) addition. Alcohol fermentation (AF) was completed within 7 days after yeast starter culture addition. Spontaneous AF was also completed in 7 days when native yeast population reached to similar numbers of inoculated starter yeast. AF rates were neither affected by different targeting pH (4.0, 3.75, and 3.5) nor different acid adjustments (acid wine, tartaric acid, and DL-malic acid). Malolactic fermentation (MLF) was finished in about 10 days after adding ML starter culture in the coinoculated fermentation, while it was needed 5-7 weeks to completer MLF with native ML bacteria in the spontaneous and yeast only fermentations. MLF rates were not affected by different pH, however, MLF rate was slower in the tartaric acid adjusted musts in coinoculated fermentation.

With high-throughput sequencing technique, we were able to identify 72 bacteria genera including well known lactic acid bacteria (LAB), i.e. *Enterococcus, Lactobacillus, Oenococcus*,

Streptococcus in 2017 wine musts. We also identified 38 fungi species including wine related yeasts, such as *Pichia* sp., *Saccharomyces* sp., *Zygosaccharomyces* sp., *Hanseniaspora* spp. Insufficient quantity and quality of DNA material resulted in a lack of data during the progress of MLF for the non-ML inoculated wines. For 2018, we were able to extract higher quantity and quality of DNA material and completed DNA sequencing at more fermentation time points than 2017. This will allow us evaluate more detailed microbial profiles as affected by acid adjustment and starter culture addition and native fermentation.

Metabolites were analyzed in wines from both years by LC-MS (non-volatile compounds) and additionally by GC-MS (volatile compounds) in the 2018 wines. The treatments for the 2017 wines produced few differences in the metabolite compositions as measurable by the selected LC-MS method. It is possible this is due to the fact that the starting must pH was 3.8 in 2017, and that as a result, relatively small acid adjustments were required, with low consequence for must composition. These findings in the 2017s differ with those of the 2018s, in which differences were attributed to both the Target Initial pH and the Inoculation Method. This disparity may lie inherent to the differing chemistries of a must harvested at pH 3.8 (2017) and one harvested at pH 4.3. A 2-way ANOVA of the 2018 GC-MS metabolite data showed concentrations of several volatile compounds were correlated with Target Initial pH. There were also differences in the GC-MS metabolites resulting from coinoculation versus inoculation with yeast only. These differences in the volatile profile were reflected in the sensory analysis, in which slight but significant differences in some aroma attributes were correlated with Target Initial pH.

These research findings suggest that the practical ways in which high pH musts are managed have the potential to influence the aroma and chemical profiles in measurably varied and nuanced ways. In the 2018 wines, there were trends in both metabolite and sensory that differed with the initial adjusted pH, the inoculation method, and whether tartaric acid or acid wine were used. More research is required to understand how the character of the starting must influences the potential for these changes, as fewer differences were observed in the 2017 wines, which required less adjustment. There were no singular compounds discovered to be driving the differences between these treatments, rather a broad scope of changes in the sensory and chemical composition resulting from the conjoint effects of pH, inoculation method, and acidification method. Regardless of the inoculation method, all treatments completed alcoholic and malolactic fermentation. The qualitative nature of these measured changes was not evaluated in this work, and further research should be done to evaluate how these factors influence preference.

These research findings show practical ways to manage high pH musts. The relatively small pH adjustments compared to the target pHs nevertheless resulted in significant sensory differences in the wines and equally significant differences in the metabolites (aroma compounds). This shows that pH adjustment is a powerful tool to affect the wine sensory profile. This project demonstrated that the addition of yeast and bacteria starter is a very effective tool to manage native yeast and bacteria populations and to avoid spoilage problems in high pH musts. The added starter cultures strongly dominated the native yeast and bacteria all through the fermentation. These diverse yeast and bacteria populations, inoculated vs spontaneous, and different populations based on pH produced clear significant differences in the wine composition

as analyzed by LC (non-volatile compounds) and by GC (volatile compounds). The sensory analysis showed a similar separation of the wines based on starting pH and use of starter culture or fermentation with native microorganisms.

For high pH musts (pH 3.6-4.3) acid adjustments and use of yeast and/or bacteria starter cultures have significant impact on the metabolites formed during fermentation and on the perceived flavors. This was seen even in the wines of this study where the pH adjustments were rather small. The results indicate that it would be beneficial to explore these winemaking tools more to help differentiate our wines. Yeast and bacteria starter cultures are a great tool to work with high pH musts if a winemaker does not or cannot make large acid adjustments using tartaric and/or malic acid or acid wine.

The detailed microbial genomic data and metabolic data are providing deep insight into the fermentations pathways as affected by different must pH and starter culture additions. We were able to see that specific microbial populations form specific metabolite profiles. Additional research is needed to identify and quantify specific flavor impact compounds and the metabolic links of specific microorganisms and to major flavor impact compounds.

Washington State Grape and Wine Research Program

FINAL REPORT
2020-21 FUNDING CYCLE

Abbreviations:
AF: alcohol fermentation
MLF: malolactic fermentation
LAB: lactic acid bacteria
MLB: malolactic bacteria
16S rRNA DNA: 16S ribosomal ribonucleic acid DNA
ITS DNA: internal transcribed spacer
OTU: operational taxonomic unit
PCA: Principle Coordinates Analysis

1. **Summary**: On a separate page.

2. **Final Report**

3. **Project Title**: **Impact of pH on the wine microbial ecology and wine quality**
(**Project Duration: 2017-2020)**

4. **Principal Investigator/Cooperator(s)**: Name, institutional affiliation, address, phone number and e-mail.

PI Name:	Hailan Piao
Organization	Washington State University
Address	359 University Drive, Richland, WA 99354
Telephone	509-372-7665
Email	Hailan.piao@wsu.edu

CO-PI Name:	Thomas Henick-Kling	**CO-PI Name:**	Thomas Collins
Organization	Washington State University	Organization	Washington State University
Address	359 University Drive, Richland, WA 99354	Address	359 University Drive, Richland, WA 99354
Telephone	509-372-7292	Telephone	509-372-7515
Email	thk@wsu.edu	Email	tom.collins@wsu.edu
CO-PI Name:	James Harbertson	**CO-PI Name:**	
Organization	Washington State University	Organization	
Address	359 University Drive, Richland, WA 99354	Address	

Telephone	509-372-7506	Telephone	
Email	jfharbertson@wsu.edu	Email	

5. **Objective(s) and Experiments Conducted to Meet Stated Objective(s)**: The report objectives should match the objectives in the original proposal.

Objective 1: Winemaking and Sample Collection

We proposed to select red grapes with a target pH of 4 and titratable acidity of 4 g/L or lower. In 2017, we had unusual weather conditions in which our selected grapes did not reach pH 4 even by late October. In 2018, we were able to harvest the grapes at high pH. In that year they came in a bit higher at pH 4.3. We used Merlot grapes in both years. Winemaking, microbial and chemical analysis were carried out at the Ste Michelle Wine Estates WSU Wine Science Center. When the wines approximately 1 year old were evaluated sensory differences.

The grapes were crushed into 200L fermentation tanks, added SO_2 (50 mg/L for 2017 and 30 mg/L for 2018), and left soaking overnight before measuring pH and titratable acid. Our original plan for the control wines was no acid addition. Since in 2017, the grape pH was 3.8, we had to modify research plan for the control wines. We added 25 g of potassium bicarbonate and raised pH to the 4.0 for the control must. In 2018, we left the control wines at the initial pH of 4.3. Details of the acid additions are listed in the **Table 1**. After the acid additions and SO2 additions made on day 2, the wines, were inoculated on day 3 with the yeast starter culture (D254) and ML starter culture (VP41) as listed in the **Table 2**. For the yeast and bacteria co-inoculation, the ML starter culture was be added 24h after the yeast addition. For the sequential inoculation, the ML culture was added after completion of AF. All fermentations were temperature controlled with hot and cold water run through cooling jackets on each tank. Each fermentation was set to follow the same temperature profile and the same pump over protocol. Cap and must temperature, fermentation rate (Brix) and pump overs were automatically recorded. Samples were collected from each ferment as following: every day for the first 4 days (Day 1, 2, 3, 4), then every two or three days until day 20 (Day 6, 8 10, 13, 15, 18, 20), after that every 5 days until completion of MLF (Day 25, 30, 35 and end of MLF). **This objective has been completed.**

Objective 2: Wine Components Analysis

For both 2017 and 2018 wines, pH and titratable acidity (TA) were measured with a pH meter and an automated titrator. Glucose, fructose, tartaric acid, L-malic acid, L-lactic acid, and acetic acid were measured with enzymatic assay kits (Admeo, Hollister, CA) on the Admeo Y15 analyzer (Admeo, Hollister, CA). **This objective has been completed.**

Objective 3: Analyze Yeast and Bacteria Populations by Plating

We used semi-selective nutrient media to plate viable *Saccharomyces* and non-saccharomyces yeast and lactic acid bacteria over the course of the fermentations. **This objective has been completed.**

Objective 4: Analyze Yeast and Bacteria Populations by Sequencing

4a) DNA extraction and microbial community sequencing

We also applied a culture independent method which allows us to identify viable but non-culturable yeast and bacteria.
Due to the viable but non-culturable nature of many wine microorganisms, the conventional plating methods miss out large numbers of microorganisms. To get a complete picture of the diversity of bacteria and yeast total populations, we applied advanced next-generation sequencing techniques. First, total microbial genomic DNA was extracted from each wine sample (10-50 mL) using a FastDNA SPIN Kit for Soil (MP Biomedical, Solon, OH) according to the manufacturer's instructions. Extracted DNA were quantified with Qubit 2.0 fluorometer (Invitrogen, Carlsbad, CA). For identification of bacteria genus and species, the hypervariable V4 region of the 16S rRNA gene were amplified from the genomic DNA using the primer set (16S Amplicon PCR Forward Primer =5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGCCAGCMGCCGCGGTAAA and 16S Amplicon PCR Reverse Primer =5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGGACTACHVGGGTWTCTAAT). For identification of yeast genus and species, the ITS1 region were amplified with primer set (ITS1-Fw =5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGACCTGCGGARGGATCA and ITS2-Rv = 5'GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGAGATCCRTTGYTRAAAGTT) The 16S rRNA and ITS1 PCR amplicons were run on an Illumina Miseq instrument. The Illumina Miseq 16S and ITS sequencing were processed at AKESOgen, Inc (Peachtree Corners, GA). **This objective has been completed.**

4b) Data analysis and construction of microbial profiles

For the 2017 wines, raw Illumina Miseq data were analyzed using Mothur open-source software. The two sets of reads for each sample (forward and reverse) were combined using (make.contigs) command. Sequences were removed if they had homopolymeric regions of more than 8 nt and were larger than 275 nt. Duplicated sequences were merged using the (unique.seqs) command. SILVA database was customized to the V4 region of 16S rRNA gene using the (pcr.seqs) command. Unique sequences were aligned in SILVA customized reference database using the (align.seqs) command. Any column that only contains gap characters was ignored without losing information using (filter.seqs) command. Then the sequences were preclustered using (pre.cluster) command which allowed 2 differences between sequences. Chimeras were detected using UCHIME algorithm using (chimera.uchime) command and removed chimera sequences using (remove.seqs) command. The Quality filtered sequences were assigned into operational taxonomic units (OTUs) at 97% similarity cut-off and taxlevel of 4 which corresponded to order level using (cluster.split) command. Next, the number of sequences in each OTU was determined using (make.shared) command. Then, the taxonomy for each OTU was obtained using (classify.otu) command. To normalize the data set for the comparison of reads numbers across the samples, subsampling was conducted using (sub.sample) command. Rarefaction curves, describing the number of OTUs observed as a function of sampling effort, were generated using (rarefaction.single) command. The number of observed OTUs, Chao1 richness indices, coverage richness estimator, and inverse Simpson diversity indices were calculated. A summary of all the calculated values was generated using (summary.single) command. The similarity of the community membership and structure among the different samples were calculated with Jaccard and Thetayc calculators using (dist.shared) command. The

relationships among the samples were measured using Principle Coordinates Analysis (PCoA). **Data analysis for the 2017 wines has been completed and is shown in this report. The samples for the 2018 wines have been submitted for sequencing. Data analysis will be completed by August 2020.**

Objective 5: Metabolomic Analysis

For the 2017 and 2018 wines we analyzed the non-volatile composition using an Agilent 1290 Infinity II UHPLC coupled with an Agilent 6545 QTOF-MS system and the Data were analyzed using Agilent's MassHunter and Mass Profiler Professional software packages. For 2018 wines we also carried out an analysis of volatile compounds using using GC-MS method described in this report. **This objective has been expanded and completed.**

Objective 6: Sensory Analysis

In November 2019, a trained panel performed a descriptive analysis on thirty of the 2018 experiment wines listed in table A. Fifteen panelists representing students, employees, and former associates of WSU Tri Cities were recruited. This research was approved under WSU IRB #12546-011. Panelists completed six hours of training to develop the list of aroma attributes for the descriptive analysis (Table C). Taste attributes were sweet, sour, hot, bitter, and astringent. Formal evaluations were held over nine hour-long sessions. Ten wines were evaluated in each session: two sets of 5 with 75 second breaks between samples, and a mandatory 5-minute break between sets. Twelve panelists completed the evaluation. **This objective has been completed.**

6. Summary of Major Research Accomplishments and Results by Objective

6.1 Procedures to Accomplish Objectives

6.1.A. Winemaking – acid adjustments

For this project, we made wines in two seasons, 2017 and 2018, using Merlot grapes. In 2017, the Merlot grapes from the Bacchus Vineyard (Sagemoor Vineyards, Pasco, WA). In 2018, we received Merlot grapes from Klipsun Vineyard (Benton City, WA). The grapes were processed in the research winery at the Ste Michelle Wine Estates WSU Wine Science Center, Richland, WA. In 2017 and 2018, forty-five and thirty-nine lots of wine were made in jacketed tanks (200 L capacity, 130 L fermentation volume). The grapes were fermented with the same pumpover regime (5 min per PO, 6x per day), temperature profile, and yeast and bacteria culture additions as specified in the research protocols.

In 2017, we had unusual weather conditions during the growing season resulting our research grape block not reaching the expected pH of 4 and the low L-malic acid content of less than 1 g/L. Instead the pH of the Merlot grapes harvested in late October was surprisingly still 3.8 and L-malic acid content was still approximately 2.5 g/L. This forced us to modify the original research plan. We used potassium bicarbonate additions to increase the must pH to 4 and we added only small amounts of tartaric acid, DL-malic acid, and acid wine to lower the pH to 3.75 and 3.5 in the other lots (**Table 1**). For acidification of the must with acid wine, we prepared a high acid wine by early harvesting Merlot (WSU vineyard, Roza Road, Prosser, WA) grapes at 15.1 Brix, pH 3.06, TA 15.5 g/L. The resulting wine (Brix 0.7, pH 2.9, TA 15.1 g/L, tartaric acid 3.17 g/L and malic acid 4.9 g/L) was used to acidulate one set of musts to pH 3.75 and pH 3.5. This 'acid' wine was made with high SO2 additions (60 mg/L at crush and 40 mg/L

at end of AF, with the resulting wine containing total 82 mg/L total SO2 and 30 mg/L free SO2) to prevent malolactic fermentation (MLF). After completion of (AF), the wine was kept cold until it was used for must acidification. This wine was fermented and stored in stainless steel containers. In 2017, with the relatively high concentration of L-malic acid (~ 2.5 g/L) in the grapes we were not able to follow the impact of L-malic acid concentration on growth and metabolism of lactic acid bacteria (LAB) as planned. We had anticipated that the grapes at such late harvest date would contain 1 g/L of L-malic acid or less. With the addition of DL-malic acid for adjustment of the grape must pH, we would have increased the L-malic acid content to 2 or more than 2 g/L. We are planning to study this impact in separate future experiments in small scale laboratory trials and in commercial trials with collaborating wineries.

In 2018, the fruit contained a large amount of sugar (~28 Brix) which would have resulted in wines with approximately 16% alcohol. We removed as much juice as possible (16 L per 130 L volume) and added back the volume of water to reduce initial sugar content to 24 Brix. For 2018 we made the following acid adjustments: no acid addition (as control, target pH 4.0, but actual pH 4.3), acid wine addition (adjusted musts pH to 3.75 and 3.5) and tartaric acid addition (adjusted musts to pH to 3.75 and 3.5). For a summary of acid adjustments see **Table 1**.

Table 1. pH adjustments

2017 acid adjustments*					
Initial must pH	3.8				
Must Volume (L)	140.0	127.0	127.0	127.0	127.0
Acid addition	Control	Acid wine	Acid wine	Tartaric acid	DL-malic acid
Target pH	4.0	3.75	3.5	3.75	3.5
Amount of acid added	25.0 g (potassium bicarbonate)	1.0 L	4.0 L	87.4 g	80.0 g
2018 acid adjustments*					
Initial must pH	4.25 - 4.33				
Must Volume (L)	132.6	142.5	150.0	133.3	134.0
Acid addition	Control**	Acid wine	Acid wine	Tartaric acid	Tartaric acid
Target pH	4.0	3.75	3.5	3.75	3.5
Amount of acid added	0.0 L	9.5 L	17.0 L	120.0 g	200.0 g

* All treatments were made in triplicates.
** In the 2018 musts the actual pH was 4.3. For the Control wines this pH was not changed.

6.1.B. Winemaking – different inoculation methods

To allow equilibration of acid and sugar in the must, the grapes were cold soaked (5 °C) in the tank for 24 hours before any adjustments with acids or acid wine were made. After acid adjustments the temperature in the tanks was raised to 18 °C for yeast and bacteria inoculations. For a summary of starter culture additions in 2017 and 2018, see Table 2. In 2017, we applied three different fermentation methods, i.e. yeast starter culture only, co-inoculation (yeast and bacteria starter culture 24h apart), and sequential inoculation (yeast starter culture with bacteria

starter culture at completion of AF). Details see Table 2. In 2018, we used spontaneous fermentation (with no yeast and no bacteria culture addition), yeast only inoculation and co-inoculation. We switched the malic acid addition treatment (in 2017) in favor of a spontaneous fermentation (no yeast and no bacteria addition) in the control (no acid adjustment) wine and in the acid wine adjusted wines of pH 3.75 and pH 3.5. Unfortunately, we were not able to also include the treatment of no yeast addition but addition of ML bacteria as recommendation made by the Wine Research Advisory Committee. We would not be able to prepare these many fermentations at once due to personal and equipment limitations. It would be nice to evaluate the treatment of yeast starter and no ML bacteria starter against no yeast and plus bacteria starter another time. After AF, each tank was drained into stainless steel kegs and allowed to undergo MLF. In both years, we used D254 (yeast starter culture) strain and VP41 (ML starter culture) strain in the inoculated fermentations. Diammonium phosphate (DAP) and Fermaid K were added to raise the yeast assimilable nitrogen (YAN) in the must to 225 mg/L.

Table 2. Yeast and MLB starter culture additions

	2017 Fermentation Treatments														
Acid addition	No Addition			Acid Wine Addition						Tartaric Acid Addition			DL-Malic Acid Addition		
	(Control 4.0)			(AW3.75)			(AW3.5)			(TA3.5)			(MA3.5)		
SO_2 addition (mg/L)	50	50	50	50	50	50	50	50	50	50	50	50	50	50	50
Yeast start culture*	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes
MLB start culture*	no	yes	yes	no	yes	yes	no	yes	yes	no	yes	yes	no	yes	yes
Fermentation **	Y	CI	SI	Y	CI	SI	Y	CI	SI	Y	CI	SI	Y	CI	SI
2018 Fermentation Treatments															
Acid addition	No Addition			Acid Wine Addition						Tartaric Acid Addition					
	(Control 4.0)			(AW3.75)			(AW3.5)			(TA3.75)		(TA3.5)			
SO_2 addition (mg/L)	30	30	30	30	30	30	30	30	30	30	30	30	30		
Yeast start culture*	no	yes	yes	no	yes	yes	no	yes	yes	yes	yes	yes	yes		
MLB start culture*	no	no	yes	no	no	yes	no	no	yes	no	yes	no	yes		

Fermentation**	S	Y	CI	S	Y	CI	S	Y	CI	Y	CI	Y	CI	

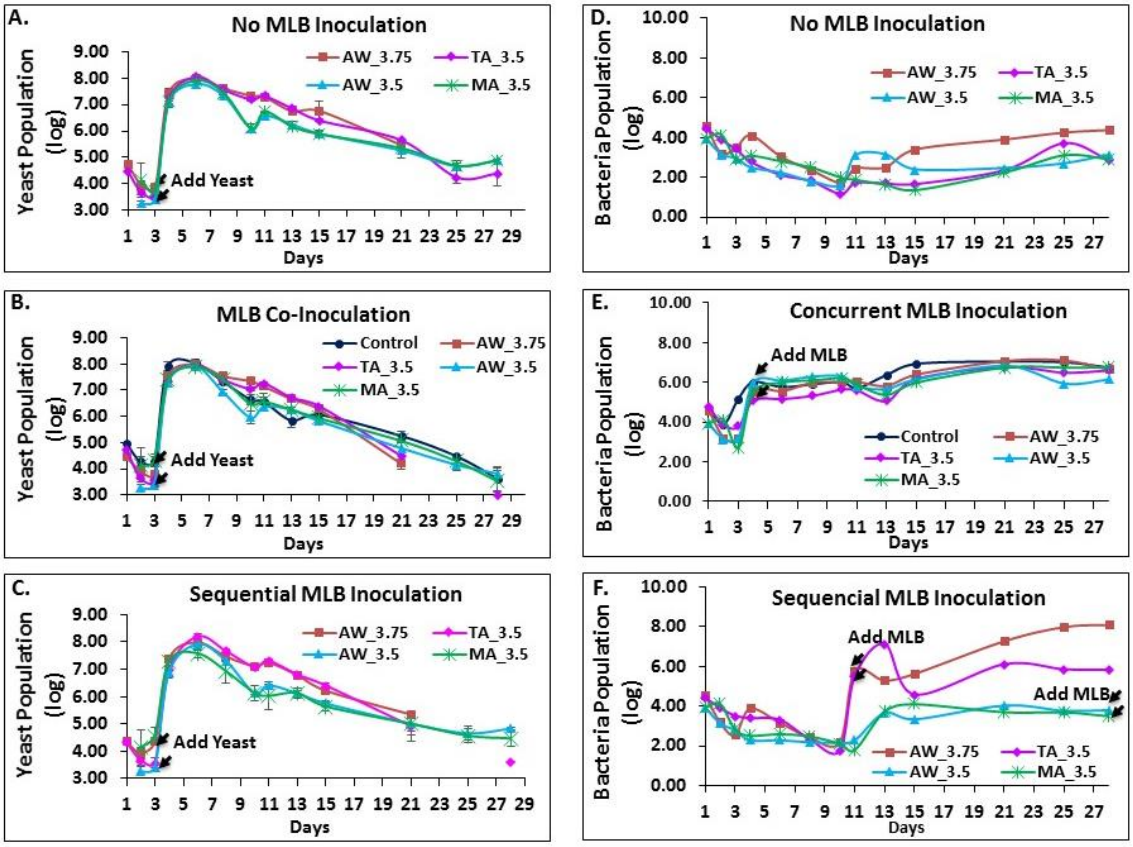
* "no" indicates no bacteria or yeast start culture was added; "yes" indicates bacteria or yeast start culture was added.
** Fermentation method: "S" indicates spontaneous fermentation (no yeast or bacteria inoculation), "Y" indicates only yeast inoculation, "CI" indicates co-inoculation (MLB starter culture added 24 h after yeast addition), "SI" indicates sequential inoculation (MLB starter culture added after completion of alcoholic fermentation).

6.2 Results for 2017 wines

6.2.A. Yeast and bacteria populations by plating

We analyzed total yeast and bacteria populations by plating on semi-selective nutrient media., The native yeast population coming from the grapes was between 10^4-10^5 CFU/mL of must (**Figure 1, A-C, Day1**). With SO_2 addition (50 mg/L), the native yeast population decreased to about 10^3 CFU/mL of must (day 2). Yeast starter culture was added on day 3 and the yeast population reached its peak on day 6. Thereafter the overall yeast population declined progressively throughout fermentation. In the 2017 wines, we observed about 10^4 native bacteria per milliliter of must. These native bacteria populations also dropped with the SO_2 addition (**Figure 1, D-F, Day2**). These declines in total yeast and bacteria populations are expected. We also expect to see differences in the composition of these populations (different species of yeast and bacteria with and without SO2 addition). Without ML starter culture inoculation, the native bacteria population was decreased during primary fermentation, after completion of AF the native bacteria population increased again slightly (**Figure 1, D**). This decrease is due to antagonistic behavior of the dominant yeast (nutrient competition and production of inhibitors such as SO2). In the co-inoculated wines, overall MLB starter culture population remained steady during AF (**Figure 1, E**). In 2017, we also used sequential inoculation in the acid wine adjusted to pH 3.75 wine an, tartaric acid adjusted pH 3.5 wines. We also evaluated sequential ML inoculation (ML starter added right after completion of AF). In the acid wine adjusted pH 3.5 wine and in the DL-malic acid adjusted pH 3.5 wine, the ML bacteria were inoculated 17 days after completion of AF (**Figure 1, F**). In these wines the ML bacteria inoculation had been forgotten by mistake, we added the starter culture when we realized the mistake.

Figure 1. Yeast and bacteria population changes in 2017 wines.



6.2.B. Evaluation of wine chemical components (metabolites) during fermentation

We observed rapid AF between day 4 and day 6 (Figure 2). Alcoholic fermentation rates were not affected neither by acid adjustment nor by ML inoculation methods – co-inoculation or sequential inoculation. Malolactic fermentation (MLF) completed in 7-10 weeks with native bacteria (Figure 3_A) compared to MLF completing in 11-16 days with co-inoculation (Figure 3_B), and in 20 to 25 days with sequential inoculation (Figure 3_C). ML fermentation rates, once started, were similar in the co-inoculated MLF and the sequential MLF (Figs 3_B and 3_C). Across all the fermentations, acetic acid was quickly produced after yeast inoculation. Overall acetic acid content stayed below 0.4 g/L (Figure 4). In 2017, the grape pH did not reach the expected pH of 4.0. Therefore we added potassium bicarbonate to raise pH to 4.0 in the control must. However, the pH dropped back to 3.8 by the next day. We made not further attempt to increase the pH because the time delay would have impacted the other fermentation treatments as well. In the 2017 must (initial pH 3.8), we found acid wine additions were effective in making pH adjustments (Figure 5). Only very small amounts of acid wine were needed. In all fermentations, we observed a temporary decrease in pH drop three days after yeast inoculation (same results observed in the 2018 project). Within about 5 days the pH moved back up. This pH drop was observed in all fermentations and seemed to be related to the yeast metabolism during AF.

Figure 2. Glucose and fructose concentrations in 2017 wines.

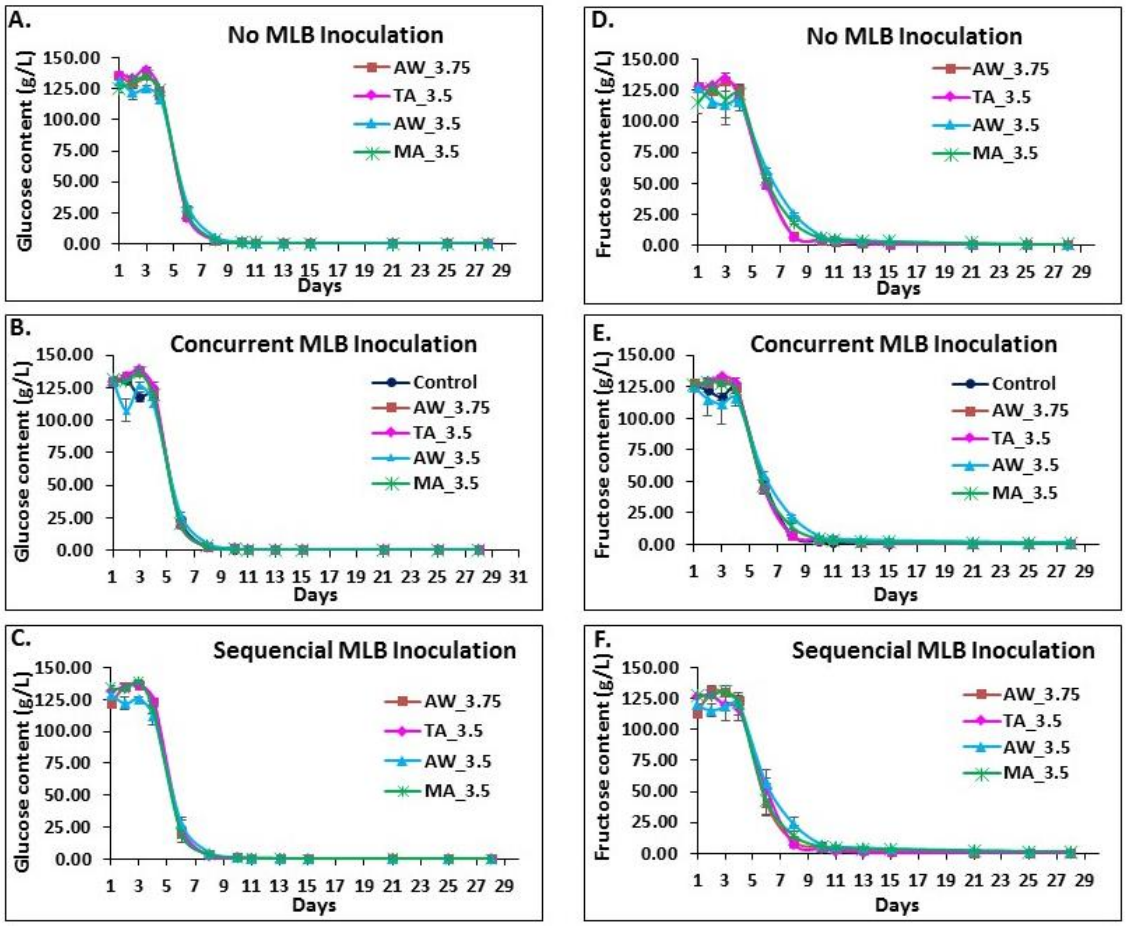


Figure 3. L-malic acid concentrations in 2017 wines.

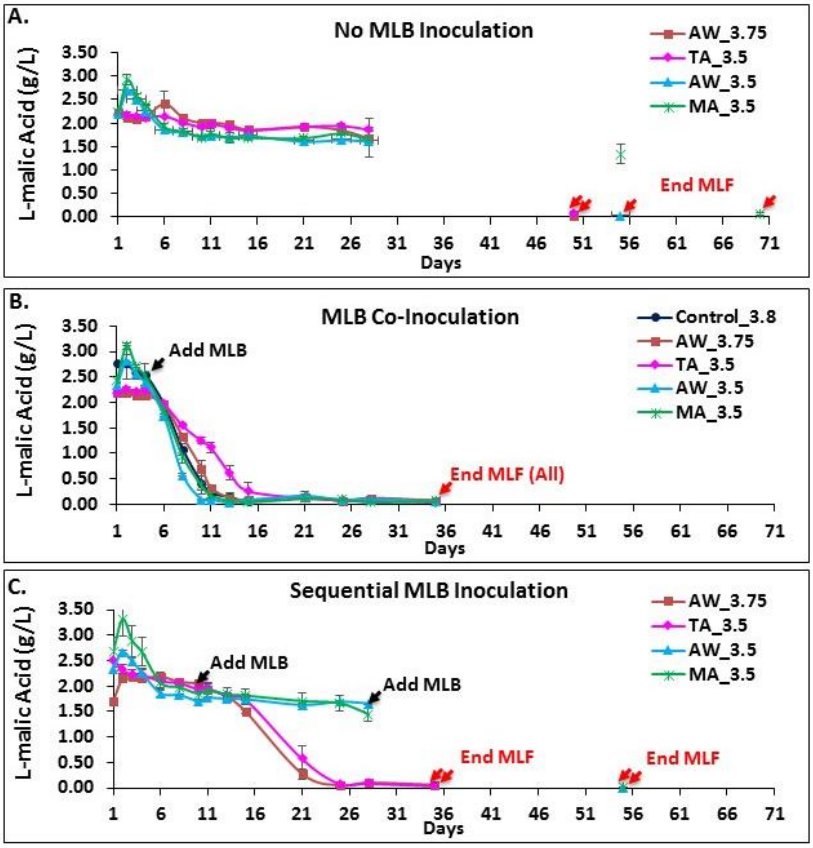


Figure 4. Acetic acid concentrations in 2017 wines.

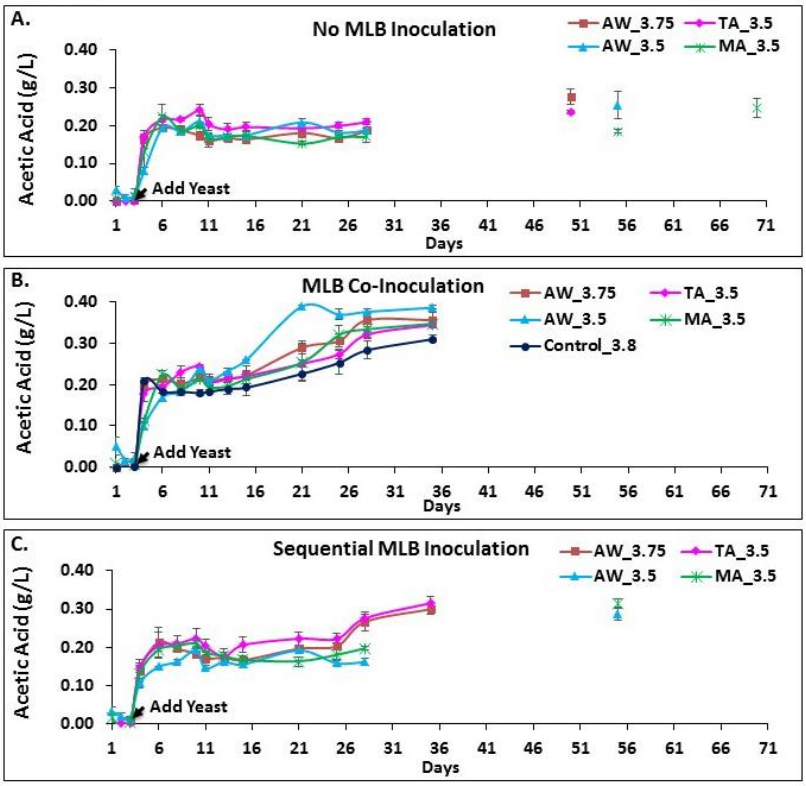
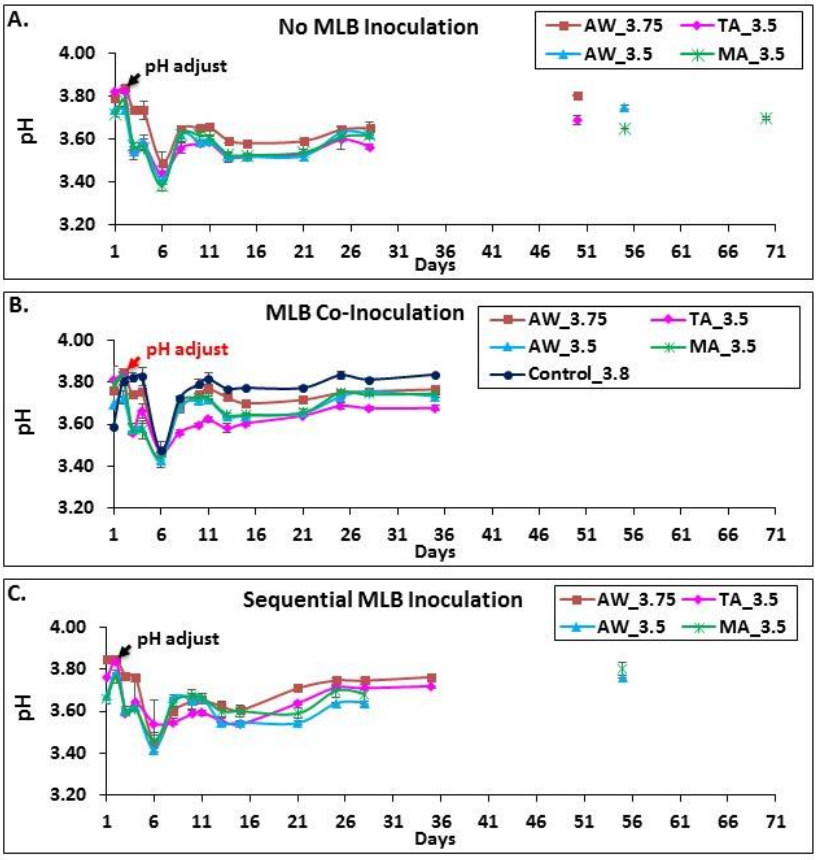


Figure 5. pH changes in 2017 wines.



es.

6.2.C. DNA extraction and microbial community sequencing analysis

Total genomic DNA was extracted from each wine sample, followed by paired-end sequencing of the hypervariable V4 region of 16S rRNA genes with the Illumina Miseq system. The quality-filtered Miseq reads were clustered into OTUs at a 97% of the sequence identity cutoff, which resulted in 22,985 – 98,366 quality filtered sequences from 72,785 – 114,630 raw reads in each analyzed wine samples (**Table S1**). We observed 1,517 - 2,193 OTUs in the grape juice samples (**Day 1, Table S1**), with SO2 addition, OTUs were decreased most in the tartaric acid adjusted pH 3.5 wine must (67.3%, **Day 2, Table S1**) and least reduction was observed in the acid wine adjusted pH 3.5 must (94.4%, **Day 2, Table S1**). With inoculation with ML bacteria starter culture, overall OTU numbers were decreased significantly (<14%, **Day 10, Table S1**) across all the fermentations. During MLF, OTUs continuously decreased, at the end of the MLF, the OTUs had decreased to less than 10% (**Table S1**). Those findings were supported by the Chao1 and Ace indices (**Table 3**). The invsimpson indices were notably decreased in the acid wine and tartaric acid adjusted (AW3.75, AW3.5, and TA3.5) must after SO2 addition, while the indices were not changed much in the high pH must (control) and DL-malic acid adjusted must (MA3.5) (**Day 2, Table 3**). This result indicates that the microbial diversity became less abundant in the acid wine and tartaric acid adjusted must with SO2 addition. Upon inoculation with malolactic bacteria (MLB) starter culture, invsimpson indices were further reduced in the all inoculated musts (**Day 10, Table 3**), demonstrating that MLB starter was able to strongly suppress the native microorganisms in the grape must.

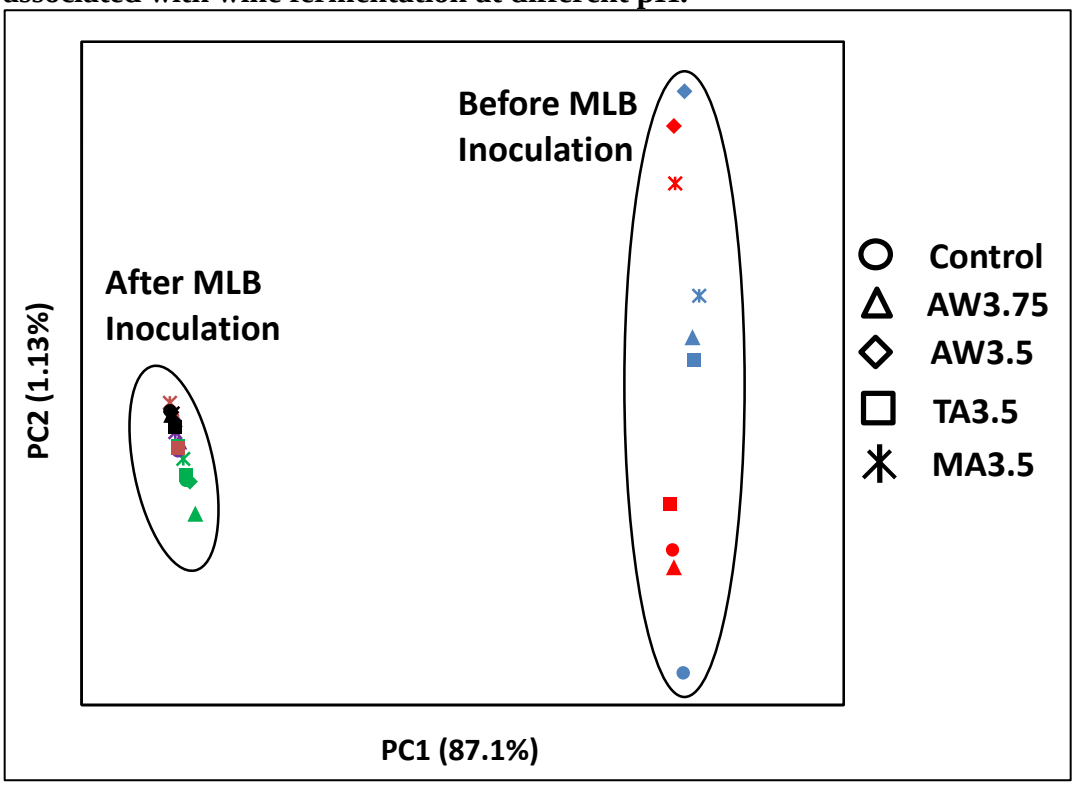
Table 3. Diversity metrics.

	Day1					Day 2					Day 10				
	Cont.	**AW3.75**	**AW3.5**	**TA3.5**	**MA3.5**	**Cont.**	**AW3.75**	**AW3.5**	**TA3.5**	**MA3.5**	**Cont.**	**AW3.75**	**AW3.5**	**TA3.5**	**MA3.5**
nSeqs	29624	42754	39396	35668	43956	22985	30517	53907	27335	30811	72906	70942	75705	61365	82940
Coverage	0.97	0.98	0.97	0.97	0.97	0.97	0.98	0.98	0.97	0.97	1.00	1.00	1.00	1.00	1.00
Chao	3103.98	3556.43	3868.04	3740.87	4102.01	2727.03	2801.37	3835.25	2846.07	3578.30	362.70	558.91	495.72	554.09	569.23
Ace	4742.66	4930.68	5439.84	5390.58	5480.64	3881.01	3931.07	5396.02	3982.24	5042.36	584.05	919.00	789.91	820.30	945.29
Invsimpson	2.62	4.07	6.16	4.18	4.32	2.17	2.14	3.18	2.37	4.15	1.08	1.12	1.09	1.10	1.06

	Day 15					Day 28					MLF_END				
	Cont.	**AW3.75**	**AW3.5**	**TA3.5**	**MA3.5**	**Cont.**	**AW3.75**	**AW3.5**	**TA3.5**	**MA3.5**	**Cont.**	**AW3.75**	**AW3.5**	**TA3.5**	**MA3.5**
nSeqs	80812	74556	107509	71216	98366	88040	68558	83694	69255	89104	83840	77506	91106	79647	87594
Coverage	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Chao	434.22	532.89	235.07	318.20	420.18	477.31	476.62	237.70	626.11	216.70	323.10	385.47	312.90	459.91	294.30
Ace	776.68	791.70	386.25	539.70	771.03	1053.66	1050.85	334.35	918.93	583.26	649.18	561.84	499.54	766.50	407.90
Invsimpson	1.05	1.05	1.02	1.05	1.03	1.03	1.02	1.02	1.07	1.01	1.02	1.02	1.02	1.04	1.02

Principal coordinates analysis comparing the temporal dynamics of wine microbiomes during fermentation showed that the native microbial community associated with grapes was distinct from the microbiomes after inoculation with a ML starter culture irrespective of must pH (**Figure 6**).

Figure 6. Principal Coordinates analysis (PCoA) of 16S rRNA data from microbiomes associated with wine fermentation at different pH.



The obtained operational taxonomic units (OTUs) were assigned to taxonomy, a total of 10 phyla (contributing ≥ 1 of reads) were observed in all the wine must samples at early stage of the fermentation (**Day 1 and 2, Figure 7**). In all the wine samples, Actinobacteria (9.5 – 15%) and Proteobacteria (11 – 25%) were the most abundant phyla; Bacteroidetes (4.4 – 7.9%), Firmicutes (2.9 – 5.5%), and Cyanobacteria (1.2 – 4.8%) were the moderately abundant phyla; Acidobacteria, Chloroflexi, Gemmatimonadetes, Planctomycetes, and Verrucomicrobia were the least abundant phyla (<1%) (**Table 5**). SO2 addition affected the abundance profile of phyla in the different acid adjusted musts. The abundance of Actinobacteria and Cyanobacteria was reduced in all the pH adjusted wine musts after SO2 addition (**Day2, Figure 7, Table 5**). The abundance of Bacteroidetes was reduced in the acid wine (AW3.75, AW3.5) and tartaric acid (TA3.5) adjusted musts, while the abundance of Firmicutes was reduced only in acid wine (AW3.75, AW3.5) adjusted musts (**Table 5**). SO2 also affected the presence of less abundant phyla (Acidobacteria, Chloroflexi, Gemmatimonadetes, Planctomycetes, and Verrucomicrobia to some degree. After inoculation with malolactic bacteria (MLB) starter culture, Firmicutes became the most abundant phylum (94 - 99%) during fermentation, while all other phyla were inhibited, most of them were unable to be detected during the fermentation. Only three phyla, Actinobacteria, Bacteroidetes, and Proteobacteria, were still observed through MLF (**Figure 7, Table 5**). ML bacteria in co-inoculated wines dominated the native bacteria populations at all wine pHs. They represented 94.4 to 9.6% of all bacteria present all through MLF. The addition of SO2 and MLB starter culture reduced the abundance of native bacteria in all must treatments in a similar way with only a small difference in the acid wine adjusted musts where the abundance of Fermicutes was reduced and not in the other treatments.

Figure 7. Relative abundance of bacterial phyla from co-inoculated fermentation wines in 2017

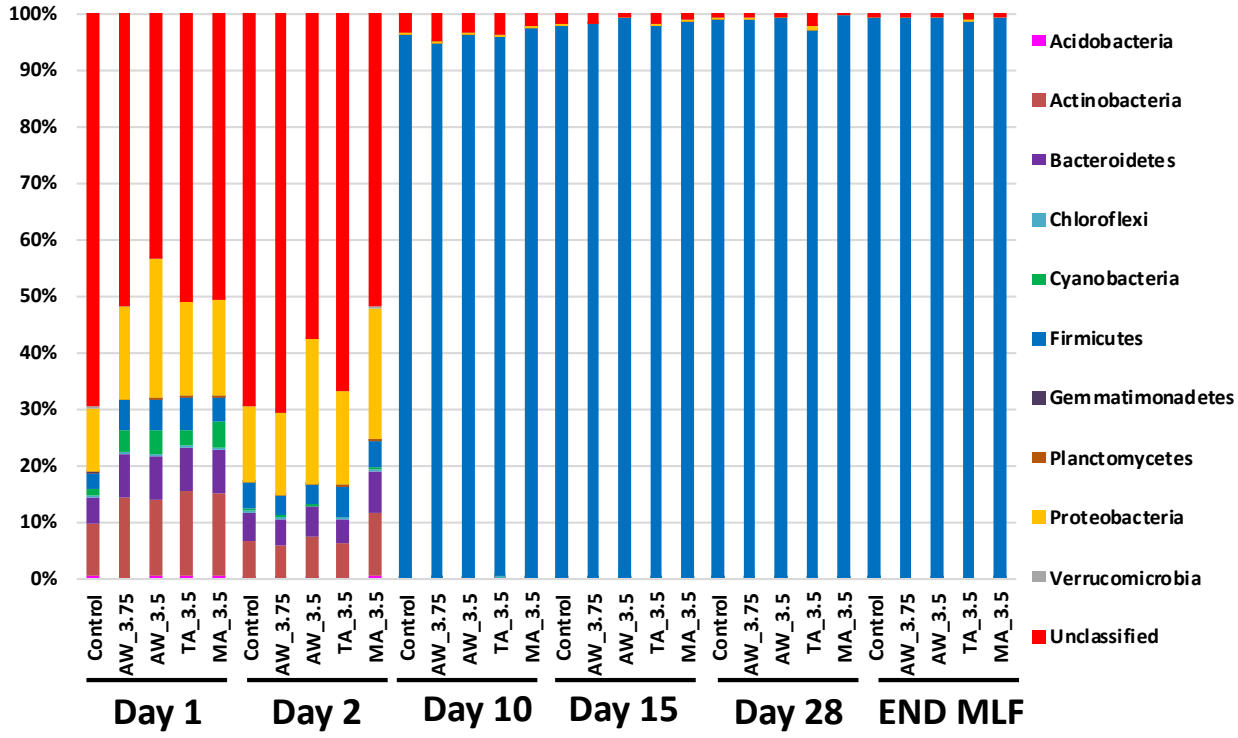


Table 5. Community composition of wine microbiome at the phylum level based on 16S rRNA MiSeq data

	Day1					Day 2					Day 10				
	Cont.	AW3.75	AW3.5	TA3.5	MA3.5	Cont.	AW3.75	AW3.5	TA3.5	MA3.5	Cont.	AW3.75	AW3.5	TA3.5	MA3.5
Acidobacteria	0.35	0.32	0.47	0.52	0.44	0.28	0.25	0.26	0.25	0.48	0.00	0.01	0.01	0.00	0.01
Actinobacteria	9.55	14.17	13.40	15.03	14.55	6.40	5.72	7.03	5.94	11.22	0.07	0.13	0.11	0.19	0.14
Bacteroidetes	4.43	7.50	7.90	7.45	7.80	4.97	4.69	5.34	4.31	7.36	0.05	0.09	0.07	0.13	0.06
Chloroflexi	0.29	0.31	0.35	0.43	0.28	0.22	0.25	0.28	0.24	0.39	0.00	0.01	0.00	0.01	0.00
Cyanobacteria	1.21	3.92	4.29	2.91	4.80	0.41	0.22	0.11	0.22	0.31	0.01	0.01	0.01	0.01	0.01
Firmicutes	2.90	5.29	5.18	5.54	3.97	4.63	3.42	3.50	5.42	4.48	96.27	94.45	96.07	95.50	97.32
Gemmatimonadetes	0.11	0.12	0.14	0.18	0.15	0.08	0.07	0.05	0.08	0.13	0.00	0.00	0.00	0.00	0.00
Planctomycetes	0.24	0.17	0.22	0.32	0.26	0.17	0.11	0.09	0.12	0.25	0.00	0.00	0.00	0.00	0.00
Proteobacteria	11.15	16.41	24.60	16.51	17.03	13.26	14.58	25.60	16.69	23.25	0.23	0.32	0.41	0.38	0.32
Verrucomicrobia	0.10	0.08	0.13	0.12	0.18	0.14	0.08	0.09	0.08	0.39	0.00	0.00	0.00	0.00	0.00
Unclassified	69.60	51.65	43.23	50.89	50.47	69.38	70.55	57.59	66.60	51.69	3.35	4.99	3.31	3.77	2.13

	Day 15					Day 28					MLF_END				
	Cont.	AW3.75	AW3.5	TA3.5	MA3.5	Cont.	AW3.75	AW3.5	TA3.5	MA3.5	Cont.	AW3.75	AW3.5	TA3.5	MA3.5
Acidobacteria	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Actinobacteria	0.03	0.14	0.02	0.03	0.06	0.03	0.04	0.02	0.09	0.02	0.01	0.04	0.03	0.06	0.03
Bacteroidetes	0.04	0.07	0.01	0.03	0.05	0.02	0.02	0.02	0.06	0.01	0.01	0.01	0.02	0.03	0.02
Chloroflexi	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00
Cyanobacteria	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Firmicutes	97.76	97.86	99.22	97.71	98.62	98.99	99.03	99.09	96.92	99.63	99.31	99.16	99.25	98.43	99.32
Gemmatimonadetes	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Planctomycetes	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Proteobacteria	0.26	0.22	0.16	0.25	0.24	0.25	0.12	0.29	0.57	0.10	0.12	0.14	0.19	0.27	0.10
Verrucomicrobia	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Unclassified	1.90	1.70	0.58	1.97	1.03	0.70	0.78	0.57	2.33	0.23	0.54	0.65	0.51	1.20	0.53

Actinobacteria were represented primarily by class of Actinobacteriia (9.0 -14%) and by the least abundant class of Rubrobacteria (0.39 – 0.69%) (**Table 6**). Although Alphaproteobacteria (8.3 - 21%) mostly resembled Proteobacteria, Betaproteobacteria (1.8 – 2.1%), Deltaproteobacteria (0.33 – 0.56%), and Gammaproteobacteria (0.66 -1.1%) were also represented in some portion of the Proteobacteria population. The majority classes of Firmicutes composed by Bacilli (2.2 - 4.7%) and Clostridia (0.51 – 0.92%) with small portion of Erysiprelotrichia. Bacteroidetes represented by five classes (Bacteroidia, Flavobacteriia, Chitinophagia, Cytophagia, and Sphingobacteriia). Among these classes, Cytophagia was the most abundant class (2.4 – 4.4%) and Chitinophagia was the next abundant class (1.0 – 2.3%) (**Table 6**). The relative abundance of seven classes (Actinobacteriia, Rubrobacteria, Chitinophagia, Cytophagia, Erysiprelotrichia, Gemmatimonadetes, and Planctomycecia) were decreased by SO2 addition in the all grape must. Interestingly, the relative abundance of Betaproteobacteria was increased in all the must samples. With SO2 addition, the relative abundance was reduced most of the classes (14 classes) in the acid wine adjusted high pH must (AW3.75), while it was increased most of the classes (14 classes) in the malic acid adjusted must (MA3.5).

Table 6. Community composition of wine microbiome at the class level based on 16S rRNA MiSeq data.

	Day1					**Day 2**					**Day 10**				
	Cont.	**AW3.75**	**AW3.5**	**TA3.5**	**MA3.5**	**Cont.**	**AW3.75**	**AW3.5**	**TA3.5**	**MA3.5**	**Cont.**	**AW3.75**	**AW3.5**	**TA3.5**	**MA3.5**
Acidobacteria; c_Acidobacteriia	0.35	0.30	0.44	0.49	0.42	0.27	0.23	0.25	0.24	0.43	0.00	0.00	0.01	0.00	0.01
Actinobacteria; c_Actinobacteriia	9.00	13.29	12.59	14.07	13.67	5.95	5.30	6.61	5.56	10.46	0.07	0.13	0.11	0.18	0.13
Actinobacteria; c_Coriobacteriia	0.06	0.11	0.04	0.08	0.06	0.09	0.04	0.06	0.03	0.03	0.00	0.00	0.00	0.00	0.00
Actinobacteria; c_Rubrobacteria	0.39	0.61	0.58	0.69	0.62	0.26	0.29	0.30	0.28	0.51	0.00	0.00	0.00	0.01	0.01
Bacteroidetes; c_Bacteroidia	0.24	0.64	0.58	0.49	0.52	0.57	0.53	0.56	0.74	0.54	0.00	0.01	0.01	0.01	0.00
Bacteroidetes; c_Flavobacteriia	0.05	0.09	0.06	0.09	0.09	0.06	0.04	0.06	0.05	0.39	0.00	0.00	0.00	0.00	0.01
Bacteroidetes; c_Chitinophagia	1.00	1.21	1.80	1.50	2.25	0.98	0.92	0.81	0.61	1.23	0.01	0.02	0.02	0.02	0.01
Bacteroidetes; c_Cytophagia	2.42	4.42	4.23	4.14	3.74	2.35	2.20	2.50	2.01	2.68	0.02	0.04	0.03	0.08	0.02
Bacteroidetes; c_Sphingobacteriia	0.63	1.01	1.08	1.09	1.08	0.93	0.94	1.39	0.85	2.46	0.01	0.01	0.01	0.02	0.01
Chloroflexi; c_Thermomicrobia	0.17	0.18	0.22	0.23	0.13	0.14	0.14	0.16	0.15	0.25	0.00	0.00	0.00	0.00	0.00
Firmicutes; c_Bacilli	2.23	4.29	3.84	4.67	3.11	3.31	2.69	2.88	4.40	3.21	96.26	94.41	96.05	95.46	97.31
Firmicutes; c_Clostridia	0.51	0.81	0.92	0.62	0.63	1.19	0.64	0.53	0.91	1.20	0.02	0.04	0.01	0.04	0.01
Firmicutes; c_Erysipelotrichia	0.04	0.07	0.18	0.11	0.09	0.04	0.02	0.02	0.03	0.02	0.00	0.00	0.00	0.01	0.00
Gemmatimonadetes; c_Gemmatimonadetes	0.11	0.12	0.14	0.18	0.15	0.08	0.07	0.05	0.08	0.13	0.00	0.00	0.00	0.00	0.00

Planctomycetes; c_Phycisphaerae	0.04	0.03	0.06	0.10	0.06	0.06	0.02	0.04	0.04	0.12	0.00	0.00	0.00	0.00	0.00
Planctomycetes; c_Planctomycecia	0.17	0.13	0.14	0.20	0.19	0.10	0.07	0.05	0.08	0.11	0.00	0.00	0.00	0.00	0.00
Proteobacteria; c_Alphaproteobacteria	8.32	13.21	20.64	12.88	13.52	9.83	10.73	14.00	13.04	16.17	0.15	0.21	0.20	0.29	0.21
Proteobacteria; c_Betaproteobacteria	1.77	1.82	2.13	2.00	1.92	2.06	2.70	3.58	2.17	4.12	0.05	0.07	0.06	0.05	0.06
Proteobacteria; c_Deltaproteobacteria	0.33	0.41	0.51	0.52	0.56	0.26	0.23	0.25	0.21	0.83	0.01	0.01	0.01	0.01	0.01
Proteobacteria; c_Gammaproteobacteria	0.66	0.87	1.14	0.91	0.89	1.03	0.85	7.70	1.17	2.02	0.03	0.03	0.15	0.03	0.04
Verrucomicrobia; c_Spartobacteria	0.06	0.05	0.08	0.06	0.11	0.09	0.04	0.07	0.06	0.19	0.00	0.00	0.00	0.00	0.00
Verrucomicrobia; c_Verrucomicrobiae	0.00	0.00	0.01	0.00	0.01	0.00	0.00	0.00	0.00	0.11	0.00	0.00	0.00	0.00	0.00
Unclassified	71.30	56.16	48.35	54.64	55.97	70.18	71.15	58.01	67.15	52.63	3.37	5.00	3.32	3.80	2.15

In the 2017 wines, we identified a total of 72 bacteria genera across all fermentation samples including lactic acid bacteria (LAB), such as *Enterococcus, Lactobacillus, Oenococcus, Streptococcus*. With addition of the malolactic starter culture, *Oenococcus* became the dominant genus and other genera were all suppressed within 6 days of ML starter culture inoculation (**Table 7_a**). Without ML starter culture, we identified 62 genera in the grape must samples and *Oenococcus* bacteria were the dominant at the end of the MLF (**Table 7_b**). We also identified 38 fungi species including *Pichia* sp., *Saccharomyces* sp., *Zygosaccharomyces* sp., *Hanseniaspora* spp. in 2017 wine must (**Table 7_c**). The 2017 wine samples yielded insufficient quantity and quality of DNA material, which did not allow us to sequence these samples and resulted in a lack of data during the progress of MLF for the non-ML inoculated wines. For 2018, we were able to extract higher quantity and quality of DNA material and completed community sequencing at more fermentation time points than 2017 (**Table S2_a, b, and c**). We expect that more detailed microbial profiles will be constructed by acid adjustment and starter culture inoculation method for 2018 samples.

Table 7_a. Bacterial populations identified in coinoculation fermentation in 2017

Coinoculation	**Day 1**					**Day 2**					**Day 10**				
Genus	**Cont.**	**AW3.75**	**AW3.5**	**TA3.5**	**MA3.5**	**Cont.**	**AW3.75**	**AW3.5**	**TA3.5**	**MA3.5**	**Cont.**	**AW3.75**	**AW3.5**	**TA3.5**	**MA3.5**
g_Rhodococcus	0.02	0.03	0.03	0.02	0.04	0.05	0.03	0.24	0.03	0.42	0.00	0.00	0.01	0.00	0.00
g_Blastococcus	0.20	0.31	0.37	0.39	0.36	0.14	0.12	0.08	0.10	0.16	0.00	0.01	0.00	0.00	0.00
g_Geodermatophilus	0.09	0.14	0.16	0.16	0.14	0.03	0.03	0.02	0.03	0.05	0.00	0.00	0.00	0.00	0.00
g_Modestobacter	1.38	1.98	1.64	1.80	2.16	0.52	0.45	0.38	0.55	0.54	0.01	0.02	0.00	0.02	0.01
g_Humicoccus	0.24	0.39	0.33	0.34	0.34	0.12	0.10	0.15	0.11	0.14	0.00	0.00	0.00	0.00	0.00
g_Kineococcus	0.32	0.52	0.49	0.64	0.58	0.18	0.17	0.20	0.23	0.22	0.00	0.00	0.00	0.01	0.00
g_Cellulomonas	0.19	0.28	0.22	0.37	0.28	0.12	0.11	0.08	0.10	0.22	0.00	0.00	0.00	0.01	0.00
g_Clavibacter	0.61	1.11	0.92	0.98	0.92	0.29	0.32	0.61	0.41	0.52	0.00	0.00	0.01	0.02	0.00
g_Curtobacterium	0.17	0.21	0.18	0.16	0.15	0.12	0.16	0.27	0.17	0.27	0.00	0.01	0.00	0.00	0.00
g_Frigoribacterium	0.09	0.14	0.09	0.09	0.08	0.09	0.07	0.18	0.07	0.22	0.00	0.00	0.00	0.00	0.00
g_Rathayibacter	0.39	0.57	0.47	0.47	0.48	0.26	0.22	0.34	0.25	0.53	0.00	0.01	0.01	0.00	0.01
g_Arthrobacter	0.23	0.32	0.45	0.49	0.43	0.25	0.25	0.22	0.24	0.45	0.00	0.01	0.00	0.00	0.00
g_Kocuria	0.13	0.27	0.42	0.33	0.35	0.12	0.09	0.08	0.09	0.13	0.00	0.00	0.00	0.00	0.00
g_Actinoplanes	0.07	0.02	0.07	0.02	0.02	0.01	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00
g_Aeromicrobium	0.04	0.03	0.04	0.04	0.02	0.02	0.02	0.03	0.03	0.23	0.00	0.00	0.00	0.00	0.00
g_Marmoricola	0.16	0.17	0.18	0.22	0.14	0.07	0.02	0.03	0.03	0.01	0.00	0.00	0.00	0.00	0.00

g_Nocardioides	0.28	0.36	0.51	0.48	0.48	0.33	0.29	0.34	0.25	0.91	0.00	0.00	0.00	0.01	0.01
g_Pseudonocardia	0.53	0.87	0.72	0.72	0.63	0.41	0.23	0.21	0.24	0.23	0.00	0.01	0.00	0.01	0.00
g_Streptomyces	0.07	0.12	0.13	0.16	0.10	0.04	0.11	0.10	0.07	0.17	0.00	0.00	0.00	0.00	0.00
g_Rubrobacter	0.03	0.08	0.09	0.06	0.04	0.03	0.04	0.03	0.03	0.06	0.00	0.00	0.00	0.00	0.00
g_Conexibacter	0.09	0.13	0.11	0.13	0.14	0.08	0.09	0.12	0.10	0.13	0.00	0.00	0.00	0.00	0.00
g_Solirubrobacter	0.12	0.19	0.16	0.24	0.19	0.07	0.08	0.04	0.08	0.16	0.00	0.00	0.00	0.00	0.00
g_Chryseobacterium	0.00	0.01	0.01	0.00	0.01	0.01	0.01	0.02	0.01	0.11	0.00	0.00	0.00	0.00	0.00
g_Flavobacterium	0.02	0.05	0.03	0.04	0.06	0.02	0.02	0.03	0.01	0.20	0.00	0.00	0.00	0.00	0.00
g_Sediminibacterium	0.11	0.14	0.09	0.16	0.27	0.09	0.09	0.12	0.07	0.11	0.00	0.00	0.00	0.00	0.00
g_Segetibacter	0.31	0.52	0.81	0.60	0.83	0.37	0.32	0.28	0.19	0.35	0.00	0.01	0.01	0.01	0.00
g_Dyadobacter	0.29	0.35	0.33	0.39	0.39	0.29	0.27	0.34	0.26	0.68	0.00	0.00	0.00	0.01	0.01
g_Hymenobacter	1.69	3.33	3.39	3.08	2.63	1.27	1.38	1.18	1.29	1.11	0.02	0.03	0.02	0.05	0.01
g_Spirosoma	0.40	0.64	0.40	0.55	0.58	0.73	0.50	0.92	0.39	0.79	0.00	0.00	0.01	0.01	0.00
g_Pedobacter	0.16	0.19	0.19	0.19	0.17	0.33	0.18	0.26	0.17	1.13	0.01	0.00	0.00	0.01	0.01
g_Bacillus	0.20	0.29	0.47	0.41	0.34	0.28	0.23	0.20	0.25	0.31	0.01	0.04	0.01	0.04	0.02
g_Geobacillus	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.10	0.00	0.00	0.00	0.00
g_Paenibacillus	0.04	0.08	0.15	0.11	0.10	0.08	0.14	0.11	0.13	0.27	0.01	0.02	0.01	0.01	0.01
g_Planococcus	0.09	0.07	0.15	0.14	0.14	0.05	0.07	0.07	0.06	0.09	0.00	0.00	0.00	0.00	0.00
g_Sporosarcina	0.00	0.01	0.01	0.01	0.00	0.06	0.07	0.04	0.06	0.07	0.00	0.01	0.00	0.01	0.00
g_Staphylococcus	0.03	0.04	0.12	0.06	0.17	0.05	0.04	0.18	0.04	0.10	0.00	0.00	0.00	0.00	0.00
g_Enterococcus	**0.25**	**0.36**	**0.16**	**0.65**	**0.15**	**0.42**	**0.38**	**0.23**	**0.42**	**0.16**	**0.00**	**0.01**	**0.00**	**0.01**	**0.00**
g_Lactobacillus	**0.60**	**1.16**	**0.84**	**1.23**	**0.81**	**1.08**	**0.70**	**0.91**	**0.95**	**1.01**	**0.02**	**0.01**	**0.01**	**0.03**	**0.01**
g_Oenococcus	**0.44**	**0.93**	**0.28**	**0.73**	**0.22**	**0.68**	**0.38**	**0.40**	**1.79**	**0.34**	**96.10**	**94.24**	**95.98**	**95.26**	**97.24**
g_Streptococcus	**0.00**	**0.01**	**0.00**	**0.01**	**0.01**	**0.02**	**0.01**	**0.01**	**0.02**	**0.01**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**
g_Clostridium	0.04	0.04	0.11	0.06	0.06	0.05	0.02	0.02	0.02	0.63	0.00	0.01	0.00	0.00	0.00
g_Turicibacter	0.04	0.06	0.18	0.10	0.09	0.02	0.02	0.02	0.03	0.02	0.00	0.00	0.00	0.01	0.00
g_Spiroplasma	0.03	0.04	0.08	0.07	0.07	0.03	0.04	0.04	0.03	0.03	0.00	0.00	0.00	0.00	0.00
g_Gemmatimonas	0.07	0.08	0.10	0.12	0.11	0.04	0.03	0.03	0.06	0.07	0.00	0.00	0.00	0.00	0.00
g_Singulisphaera	0.12	0.08	0.09	0.12	0.13	0.07	0.04	0.02	0.03	0.05	0.00	0.00	0.00	0.00	0.00
g_Brevundimonas	0.05	0.05	0.04	0.04	0.04	0.08	0.19	0.07	0.03	0.45	0.00	0.00	0.00	0.00	0.01
g_Caulobacter	0.01	0.01	0.00	0.01	0.01	0.06	0.01	0.01	0.00	0.07	0.00	0.00	0.00	0.00	0.00
g_Phenylobacterium	0.03	0.04	0.04	0.04	0.05	0.20	0.32	0.15	0.17	0.05	0.00	0.00	0.00	0.00	0.00
g_Aurantimonas	0.05	0.07	0.05	0.07	0.07	0.04	0.05	0.10	0.08	0.09	0.00	0.00	0.00	0.00	0.00
g_Rhodopseudomonas	0.03	0.04	0.04	0.04	0.06	0.02	0.04	0.05	0.03	0.07	0.00	0.00	0.00	0.00	0.00
g_Devosia	0.10	0.17	0.19	0.20	0.14	0.13	0.14	0.23	0.14	0.87	0.00	0.00	0.00	0.00	0.01
g_Methylobacterium	1.54	2.94	1.72	2.47	2.53	1.90	2.33	3.47	2.80	2.78	0.03	0.03	0.03	0.03	0.02
g_Paracoccus	0.04	0.10	0.09	0.09	0.09	0.02	0.03	0.03	0.02	0.04	0.00	0.00	0.00	0.00	0.00
g_Acetobacter	0.09	0.13	0.22	0.15	0.07	0.18	0.06	0.15	0.45	0.15	0.01	0.00	0.01	0.01	0.01
g_Acidisphaera	0.10	0.21	0.20	0.18	0.20	0.08	0.10	0.10	0.09	0.15	0.00	0.01	0.00	0.00	0.00
g_Gluconacetobacter	0.31	0.46	1.33	0.44	0.19	0.66	0.33	0.66	2.47	0.60	0.01	0.01	0.01	0.05	0.01
g_Gluconobacter	0.06	0.09	7.26	0.07	0.25	0.05	0.04	0.18	0.07	0.12	0.00	0.00	0.00	0.00	0.00
g_Kozakia	0.01	0.05	0.21	0.01	0.02	0.01	0.03	0.04	0.02	0.02	0.00	0.00	0.00	0.00	0.00
g_Roseomonas	0.17	0.18	0.19	0.20	0.25	0.15	0.17	0.22	0.14	0.23	0.01	0.01	0.00	0.00	0.00
g_Tanticharoenia	0.00	0.02	0.08	0.01	0.01	0.01	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00
g_Skermanella	0.35	0.45	0.73	0.55	0.63	0.20	0.14	0.10	0.17	0.26	0.00	0.00	0.00	0.00	0.01
g_Novosphingobium	0.04	0.07	0.06	0.08	0.09	0.13	0.15	0.06	0.06	0.06	0.00	0.00	0.00	0.00	0.00
g_Sphingomonas	2.12	3.12	3.11	3.14	3.48	2.52	2.55	4.02	2.46	4.79	0.03	0.07	0.07	0.07	0.06
g_Ralstonia	0.08	0.17	0.24	0.06	0.11	0.29	0.36	0.30	0.07	0.56	0.01	0.00	0.01	0.00	0.01
g_Massilia	0.60	0.53	0.64	0.55	0.60	0.62	0.75	0.77	0.64	1.16	0.01	0.02	0.02	0.01	0.02
g_Citrobacter	0.07	0.13	0.07	0.14	0.05	0.10	0.11	0.14	0.14	0.09	0.00	0.00	0.00	0.00	0.00
g_Pantoea	0.08	0.12	0.11	0.11	0.07	0.13	0.10	0.20	0.17	0.19	0.00	0.00	0.00	0.00	0.00
g_Acinetobacter	0.01	0.01	0.03	0.01	0.03	0.01	0.01	0.08	0.02	0.02	0.00	0.00	0.00	0.00	0.00
g_Cellvibrio	0.01	0.00	0.01	0.00	0.02	0.00	0.00	0.01	0.00	0.15	0.00	0.00	0.00	0.00	0.00
g_Pseudomonas	0.22	0.36	0.15	0.30	0.23	0.39	0.28	0.72	0.48	0.91	0.01	0.00	0.01	0.01	0.02
g_Arenimonas	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.07	0.00	0.00	0.00	0.00	0.00
g_Luteimonas	0.03	0.02	0.02	0.02	0.04	0.02	0.01	0.03	0.02	0.11	0.00	0.00	0.00	0.00	0.00

Unclassified	82.53	72.21	65.96	72.43	73.93	81.56	82.73	77.88	78.65	71.15	3.52	5.33	3.68	4.18	2.38

Table 7_b. Bacterial populations identified in without ML starter culture inoculation

No MLB Addition	**Day 1**			**Day 2**			**END_MLF**		
Genus	**AW3.75**	**AW3.5**	**TA3.5**	**AW3.75**	**AW3.5**	**TA3.5**	**AW3.75**	**AW3.5**	**TA3.5**
g_Blastococcus	0.31	0.37	0.39	0.15	0.09	0.15	0.00	0.00	0.00
g_Geodermatophilus	0.14	0.16	0.16	0.04	0.02	0.07	0.00	0.00	0.00
g_Modestobacter	1.98	1.64	1.80	0.52	0.42	0.62	0.00	0.00	0.00
g_Nakamurella (Humicoccus)	0.39	0.31	0.33	0.10	0.20	0.15	0.00	0.00	0.00
g_Kineococcus	0.52	0.49	0.64	0.24	0.16	0.17	0.00	0.00	0.00
g_Cellulomonas	0.28	0.22	0.37	0.13	0.11	0.12	0.00	0.00	0.00
g_Clavibacter	1.11	0.92	0.98	0.42	0.49	0.36	0.00	0.00	0.00
g_Curtobacterium	0.21	0.18	0.16	0.13	0.21	0.11	0.00	0.00	0.00
g_Frigoribacterium	0.14	0.09	0.09	0.07	0.14	0.04	0.00	0.00	0.00
g_Rathayibacter	0.57	0.47	0.47	0.28	0.26	0.24	0.00	0.00	0.00
g_Kocuria	0.27	0.42	0.33	0.16	0.03	0.15	0.00	0.00	0.00
g_Micrococcus	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
g_Actinoplanes	0.02	0.07	0.02	0.00	0.01	0.01	0.00	0.00	0.00
g_Marmoricola	0.17	0.18	0.22	0.06	0.12	0.06	0.00	0.00	0.00
g_Nocardioides	0.36	0.51	0.48	0.29	0.36	0.26	0.00	0.00	0.00
g_Pseudonocardia	0.87	0.72	0.72	0.25	0.26	0.26	0.00	0.00	0.00
g_Streptomyces	0.12	0.13	0.16	0.10	0.07	0.14	0.00	0.00	0.00
g_Rubrobacter	0.08	0.09	0.06	0.05	0.03	0.04	0.00	0.00	0.00
g_Conexibacter	0.13	0.11	0.13	0.07	0.09	0.07	0.00	0.00	0.00
g_Solirubrobacter	0.19	0.16	0.24	0.08	0.06	0.08	0.00	0.00	0.00
g_Sediminibacterium	0.14	0.09	0.16	0.11	0.12	0.04	0.00	0.00	0.00
g_Segetibacter	0.52	0.81	0.60	0.39	0.28	0.35	0.00	0.00	0.00
g_Dyadobacter	0.35	0.33	0.39	0.24	0.30	0.21	0.00	0.00	0.00
g_Hymenobacter	3.33	3.39	3.08	1.32	1.01	1.46	0.01	0.00	0.01
g_Spirosoma	0.64	0.40	0.55	0.43	0.92	0.54	0.00	0.00	0.00
g_Pedobacter	0.19	0.19	0.19	0.18	0.29	0.14	0.00	0.00	0.00
g_Bacillus	0.29	0.47	0.41	0.24	0.21	0.26	0.01	0.00	0.02
g_Brevibacillus	0.01	0.01	0.02	0.08	0.09	0.11	0.00	0.00	0.01
g_Paenibacillus	0.08	0.15	0.11	0.35	0.33	0.31	0.01	0.00	0.01
g_Planococcus	0.07	0.15	0.14	0.06	0.06	0.07	0.00	0.00	0.00
g_Staphylococcus	0.04	0.12	0.06	0.07	0.12	0.04	0.00	0.00	0.00
g_Thermoactinomyces	0.03	0.04	0.02	0.02	0.01	0.01	0.00	0.00	0.00
g_Enterococcus	**0.36**	**0.16**	**0.65**	**0.55**	**0.16**	**0.34**	**0.00**	**0.00**	**0.00**
g_Lactobacillus	**1.16**	**0.84**	**1.23**	**0.92**	**0.94**	**0.71**	**0.00**	**0.00**	**0.00**
g_Oenococcus	**0.93**	**0.28**	**0.73**	**0.64**	**0.29**	**0.39**	**99.58**	**99.77**	**99.06**
g_Weissella	**0.09**	**0.04**	**0.04**	**0.06**	**0.06**	**0.04**	**0.00**	**0.00**	**0.00**
g_Clostridium	0.04	0.11	0.06	0.01	0.04	0.03	0.00	0.00	0.00
g_Turicibacter	0.06	0.18	0.10	0.03	0.01	0.02	0.00	0.00	0.00

g_Spiroplasma	0.04	0.08	0.07	0.05	0.03	0.05	0.00	0.00	0.00
g_Gemmatimonas	0.08	0.10	0.12	0.06	0.03	0.05	0.00	0.00	0.00
g_Singulisphaera	0.08	0.09	0.12	0.05	0.02	0.07	0.00	0.00	0.00
g_Phenylobacterium	0.04	0.04	0.04	0.14	0.37	0.41	0.00	0.00	0.00
g_Bradyrhizobium	0.01	0.01	0.00	0.01	0.05	0.06	0.00	0.00	0.00
g_Devosia	0.17	0.19	0.20	0.10	0.19	0.09	0.00	0.00	0.00
g_Methylobacterium	2.94	1.72	2.47	1.68	3.66	2.20	0.00	0.00	0.02
g_Rhodobium	0.00	0.00	0.00	0.00	0.07	0.00	0.00	0.00	0.00
g_Paracoccus	0.10	0.09	0.09	0.05	0.03	0.04	0.00	0.00	0.00
g_Acetobacter	0.13	0.22	0.15	0.14	0.06	0.09	0.00	0.00	0.00
g_Acidisphaera	0.21	0.20	0.18	0.10	0.14	0.12	0.00	0.00	0.00
g_Gluconacetobacter	0.46	1.33	0.44	0.52	0.43	0.44	0.01	0.00	0.01
g_Gluconobacter	0.09	7.26	0.07	0.06	0.11	0.03	0.00	0.00	0.00
g_Kozakia	0.05	0.21	0.01	0.02	0.03	0.02	0.00	0.00	0.00
g_Roseomonas	0.18	0.19	0.20	0.14	0.25	0.18	0.00	0.00	0.00
g_Tanticharoenia	0.02	0.08	0.01	0.01	0.02	0.01	0.00	0.00	0.00
g_Skermanella	0.45	0.73	0.55	0.21	0.11	0.28	0.00	0.00	0.00
g_Novosphingobium	0.07	0.06	0.08	0.22	0.17	0.19	0.00	0.00	0.00
g_Sphingomonas	3.12	3.11	3.14	2.44	4.60	2.43	0.02	0.02	0.03
g_Ralstonia	0.17	0.24	0.06	0.30	0.99	0.21	0.00	0.00	0.00
g_Acidovorax	0.00	0.00	0.00	0.00	0.03	0.08	0.00	0.00	0.00
g_Massilia	0.53	0.64	0.55	0.55	0.82	0.58	0.00	0.00	0.00
g_Citrobacter	0.13	0.07	0.14	0.08	0.09	0.09	0.00	0.00	0.00
g_Pantoea	0.12	0.11	0.11	0.09	0.13	0.11	0.00	0.00	0.00
g_Pseudomonas	0.36	0.15	0.30	0.31	0.74	0.21	0.00	0.00	0.00
Unclassified	72.54	66.42	72.94	82.68	77.17	82.80	0.31	0.17	0.74

Table 7_c. Yeast species identified in in 2017 wines

	Day 1					**Day 2**				
Species	**Cont.**	**AW3.75**	**AW3.5**	**TA3.5**	**MA3.5**	**Cont.**	**AW3.75**	**AW3.5**	**TA3.5**	**MA3.5**
Cladosporium_delicatulum	0.69	1.16	0.04	1.26	0.37	0.86	0.47	0.06	0.77	0.32
Mycosphaerella_tassiana	2.16	2.04	0.14	5.00	1.18	1.69	0.99	0.08	1.94	0.99
Aureobasidium_pullulans	18.64	44.00	2.41	27.64	13.19	17.72	14.26	0.87	16.72	9.17
Dendrothyrium_variisporum	0.31	0.05	0.00	0.05	0.01	0.05	0.09	0.03	0.17	0.08
Neosetophoma_samararum	2.51	4.21	0.10	5.85	0.89	3.13	1.97	0.07	2.59	1.41
Aspergillus_piperis	15.30	0.38	0.01	0.26	0.05	0.54	0.38	0.04	0.24	0.04
Penicillium_bialowiezense	0.36	0.12	0.01	0.22	0.03	0.42	0.28	0.01	0.20	0.07
Penicillium_lapidosum	6.18	0.71	0.01	0.36	0.04	0.55	0.55	0.05	0.79	0.30
Erysiphe_necator	1.20	0.32	0.00	0.50	0.04	0.70	0.85	0.02	1.42	0.17
Botrytis_caroliniana	0.22	0.48	0.05	0.55	0.21	0.17	0.15	0.02	0.22	0.08
Schwanniomyces_occidentalis	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.61	0.00	0.00
Kregervanrija_fluxuum	0.07	0.05	0.02	0.06	0.01	0.11	0.03	0.00	0.17	0.06
Pichia_terricola	**0.01**	**0.00**	**0.00**	**0.01**	**0.00**	**0.02**	**0.00**	**0.18**	**0.01**	**0.00**

Kazachstania_aerobia	0.06	0.06	0.00	0.26	0.02	0.25	0.17	0.02	0.36	0.15
Saccharomyces_cerevisiae	**0.04**	**0.02**	**0.02**	**0.04**	**0.01**	**0.08**	**0.03**	**3.63**	**0.07**	**0.02**
Torulaspora_delbrueckii	0.43	0.47	1.89	1.13	0.92	0.78	1.47	0.14	1.86	1.87
Zygosaccharomyces_parabailii	**0.01**	**0.02**	**0.00**	**0.04**	**0.01**	**0.04**	**0.02**	**0.00**	**0.07**	**0.03**
Hanseniaspora_osmophila	**3.53**	**5.06**	**24.11**	**7.56**	**22.63**	**8.11**	**37.25**	**0.75**	**14.46**	**12.36**
Hanseniaspora_uvarum	**0.04**	**0.01**	**0.14**	**0.04**	**0.14**	**0.10**	**0.08**	**0.26**	**0.07**	**0.12**
Hanseniaspora_valbyensis	**0.03**	**0.03**	**0.01**	**0.02**	**0.02**	**0.09**	**0.04**	**0.01**	**0.13**	**0.63**
Hanseniaspora_vineae	**0.14**	**0.05**	**0.24**	**0.14**	**0.16**	**0.40**	**0.18**	**0.02**	**0.29**	**0.23**
Saccharomycodes_ludwigii	0.23	0.05	0.00	0.19	0.01	0.23	0.09	0.01	0.37	0.06
Zygoascus_hellenicus	0.00	0.00	0.00	0.02	0.00	0.00	0.02	0.00	0.30	0.00
Trigonopsis_cantarellii	0.03	0.07	0.00	0.10	0.02	0.10	0.07	0.03	0.23	0.08
Phaeoacremonium_hungaricum	0.00	0.00	0.00	0.00	0.00	0.00	0.24	0.01	0.07	0.00
Cystobasidium_pinicola	0.10	0.14	0.01	0.36	0.06	0.22	0.16	0.01	0.25	0.14
Rhodosporidiobolus_colostri	0.29	1.73	0.07	0.69	0.63	0.36	0.15	0.03	0.33	0.27
Rhodotorula_nothofagi	0.32	2.01	0.36	1.08	1.30	1.27	1.03	0.19	1.28	0.88
Sporobolomyces_roseus	0.10	0.27	0.01	0.31	0.07	0.26	0.19	0.00	0.30	0.08
Cystofilobasidium_capitatum	0.05	1.79	0.01	0.59	0.10	0.16	0.03	0.00	0.06	0.04
Cystofilobasidium_ infirmominiatum	0.06	0.58	0.00	0.15	0.11	0.07	0.03	0.01	0.03	0.04
Guehomyces_pullulans	0.09	0.47	0.01	0.16	0.06	0.11	0.02	0.00	0.07	0.03
Krasilnikovozyma_huempii	0.03	0.04	0.00	0.02	0.02	0.09	0.02	0.00	0.03	0.03
Udeniomyces_puniceus	0.58	3.00	0.02	2.12	0.78	0.80	0.51	0.02	0.83	0.38
Naganishia_albida	0.68	2.61	0.03	1.58	0.36	1.23	0.68	0.06	1.00	0.54
Naganishia_friedmannii	1.31	3.11	0.10	4.50	0.74	1.94	1.53	0.12	2.24	1.53
Vishniacozyma_victoriae	0.70	0.70	0.01	0.66	0.09	0.69	0.29	0.04	0.57	0.56
Ustilago_hordei	0.05	0.02	0.00	0.10	0.01	0.03	0.01	0.00	0.02	0.01

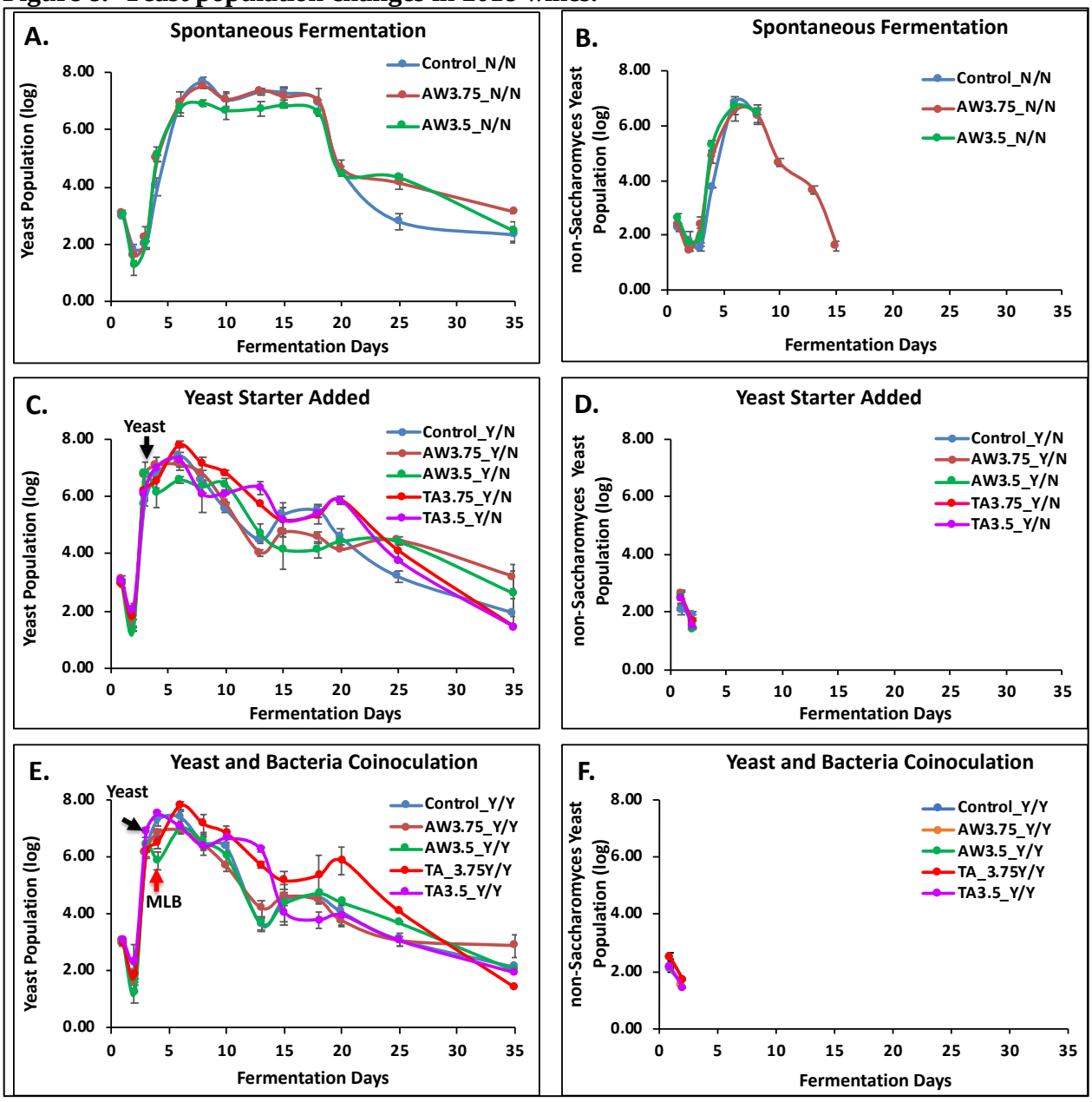
6.3 Results of 2018 wines

6.3.A. Yeast and bacteria population changes by plating

6.3.A.1. Yeast populations changes in response to pH adjustments of the must and use of starter cultures

In 2018, we applied three different inoculation methods, i.e. spontaneous fermentation (no yeast and no ML bacteria inoculation), yeast only fermentation (only yeast inoculated, no ML bacteria inoculated), and co-inoculation (inoculated with yeast and ML bacteria). In the 2018 grape musts, total native yeast populations were about 10^3 CFU/mL of must (**Figure 8, A, C, and E, Day 1**). With SO_2 addition (30 mg/L), the native yeast population decreased more than 10 fold (**Figure 8, A, C, and E, Day 2)**. Yeast starter culture was added on day 3 in yeast only inoculated and co-inoculated fermentation. Similar as in 2017 fermentations, the yeast population reached its peak on day 6, then total yeast population declined progressively throughout fermentation (**Figure 8, C and E)**. In spontaneous fermentations (no yeast inoculation), the total yeast population reached the same maximum population density around 10^8 CFU/mL. Yeast populations in the musts with acid additions (AW3.75 and AW3.5) increased earlier than in the high pH, non-adjusted (Control) must (**Figure 8, A, Day 4**). The population of non-saccharomyces yeasts only increased in the no yeast inoculated control must (no acid addition) and in the must with acid wine addition (AW3.75 and AW3.5). In all musts which had been inoculated with a yeast starter, the non-saccharomcyes were quickly suppressed by the added starter culture yeast (**Figure 8, B, D, and F**). Unlike inoculated starter culture yeast, native yeast in the non-inoculated fermentations maintained high populations longer (between day 6-18) compared to the starter culture yeast (between day 3-8).

Figure 8. Yeast population changes in 2018 wines.

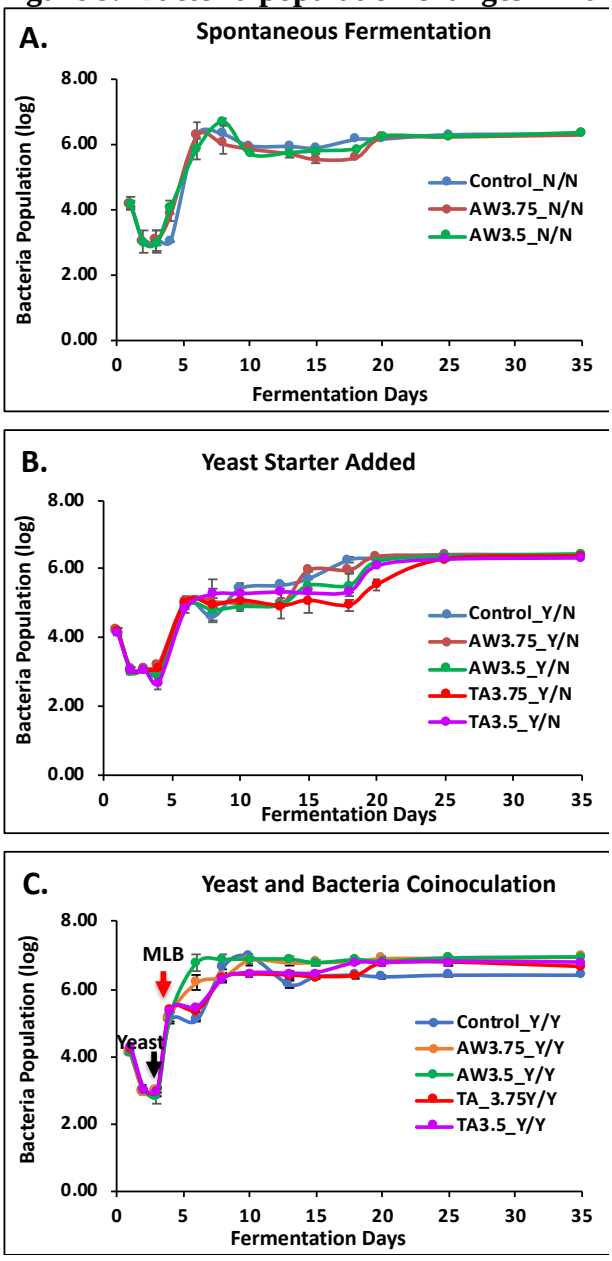


6.3.A.2. Impact of pH adjustments and starter culture on bacteria populations and rate of MLF

In spontaneous fermentation, the total bacteria populations at each of the three pHs developed at the same rate and same total numbers (cell density). The bacteria population reached over 10^6 cells/ml between day 6 and day 8, then overall population decrease slightly over the next 10 days of fermentation (**Figure 9, A**). In yeast only fermentation, native ML bacteria took more than 15 days and slowly reached over 10^6 CFU/mL. Yeast starter culture addition delayed the growth of LAB bacteria in the musts without ML starter cultures (**Figure 9, B**). Growth of native (non-inoculated) LAB was fastest in the high pH must, followed by target pH 3.75 and 3.5 (**Figure 9,**

B, Day 18 – Day 20). In co-inoculated fermentations, ML bacteria population quickly (in 2 days) reached over 10_6 CFU/mL in the acid wine adjusted must, while it was taking about 4 days to reach over 10_6 CFU/mL in the no acid addition and tartaric acid added wine must (**Figure 9, C)**. The acid wine contained 4.9 g/L of L-malic acid, by addition with acid wine also raised the L-malic acid concentration, which might be stimulated quick ML growth.

Figure 9. Bacteria population changes in 2018 wines.

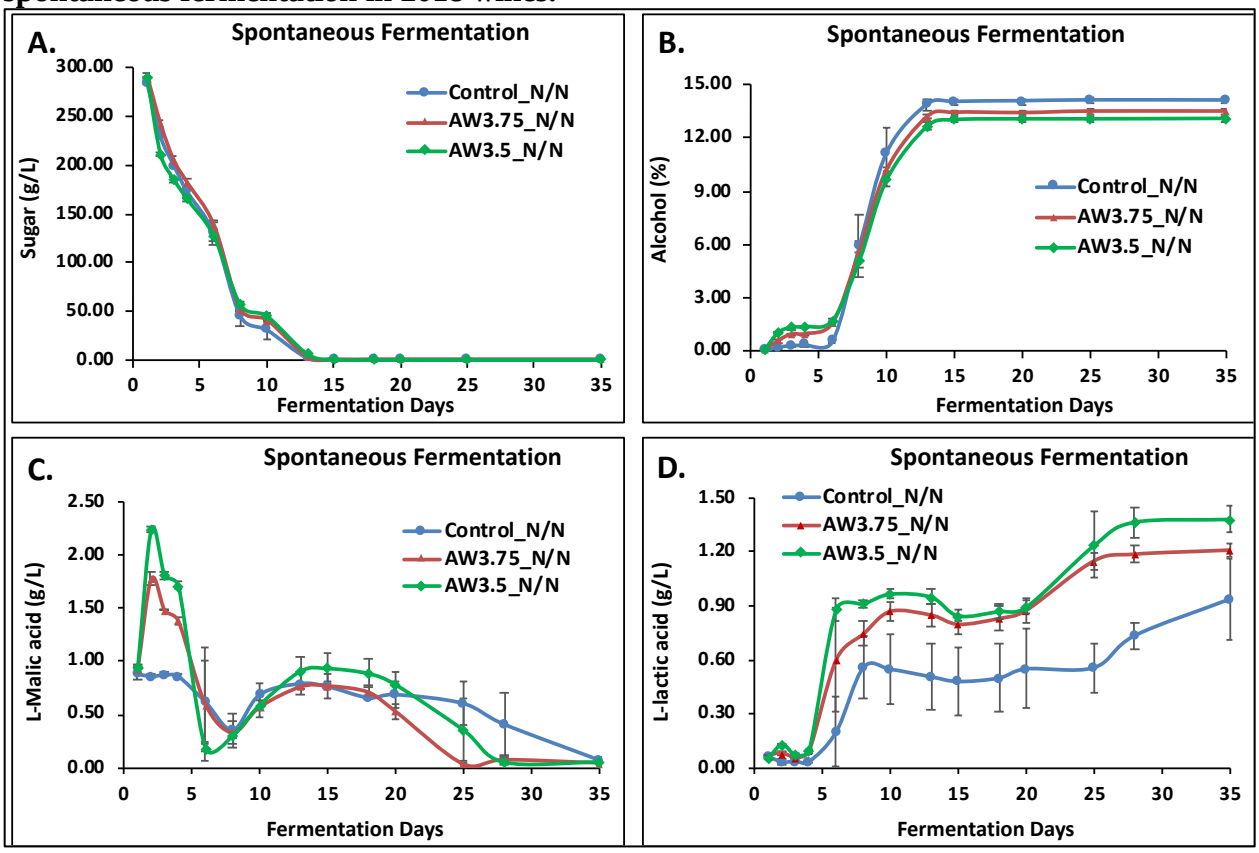


6.3.B. The impact of pH adjustments on the alcoholic and malolactic fermentation in the fermentations with different starter culture additions

6.3.B.1. Spontaneous fermentations, no yeast and no bacteria added

In 2018, one of our fermentation trials was spontaneous fermentation (no yeast and no ML bacteria added). During spontaneous fermentation, pH adjustments to the must had no impact on the alcoholic (yeast) fermentation rate. Lag phase, exponential phase, decline phase, and total length of fermentation were the same. Also, alcohol production was the same in all three wine musts, no acid addition (control), adjusted to pH 3.75 (AW3.75), and pH 3.5 (AW3.5) with acid wine (**Figure 10, A and B**). Spontaneous MLF was fastest in wine adjusted initially to pH 3.75, followed by pH 3.5, slowest in non-adjusted pH 4.3 (control) (**Figure 10, C)**. The malic acid utilization curves indicate at each of the different pH wines two different populations were active in succession (**Figure 10, C**). L-lactic acid production curves confirm the MLF rates (**Figure 10, D**).

Figure 10. Impact of pH adjustment on primary and secondary fermentation in the spontaneous fermentation in 2018 wines.

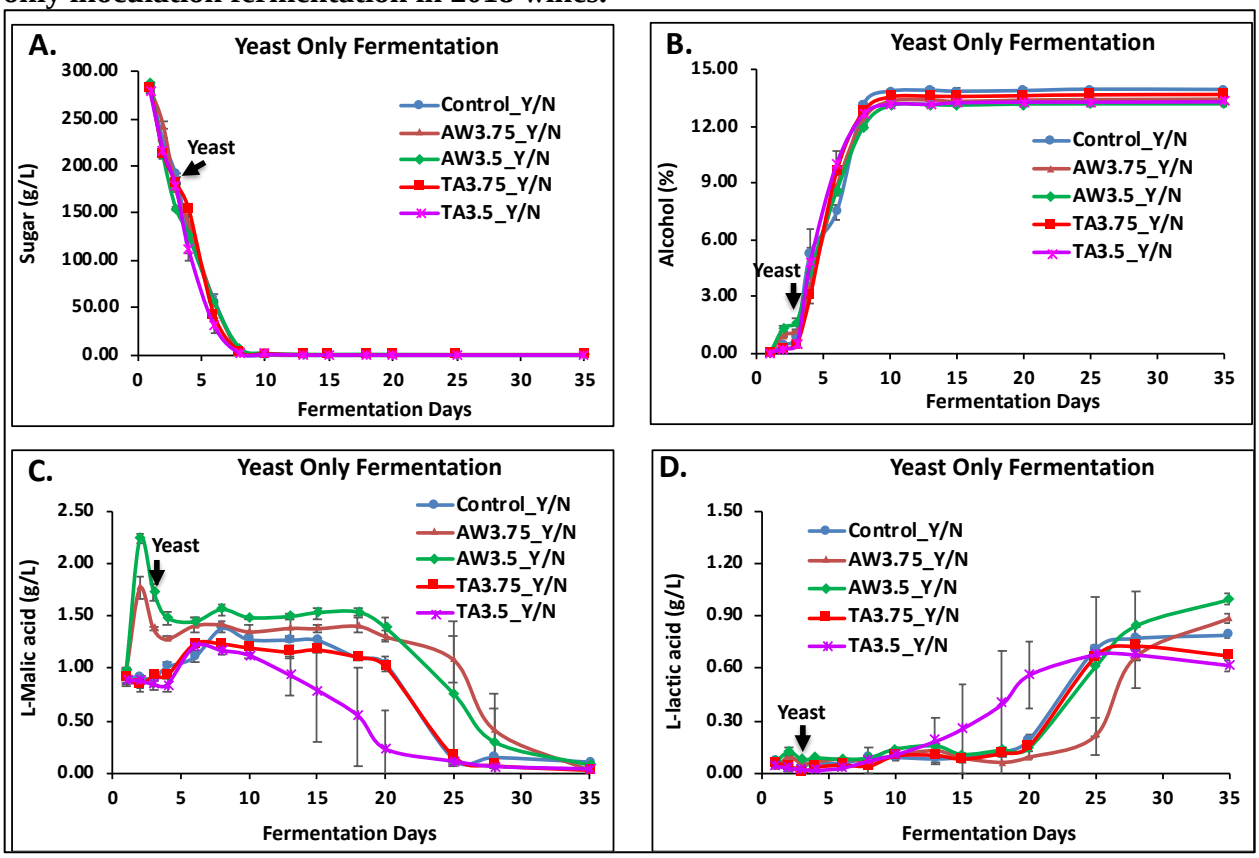


6.3.B.2. Fermentations with yeast starter culture, no bacteria added

In yeast only fermentations, a yeast starter culture was added at day 3, but no ML starter culture was added. The initial pH and the additions of either acid wine or tartaric acid had no impact on the AF rates (**Figure 11, A and B**). Spontaneous MLF completed sooner in the wines with tartaric acid additions (TA3.75 and TA3.5) and the non-adjusted wines (Control). Spontaneous MLF took about 10 days longer in the wines to which acid wine had been added (**Figure 11, C**). This might be due to the higher amount of malic acid present with the malic acid that was brought in by the acid wine. Initial malic acid concentration in the wines with acid wine addition was about 1.75 and 2.25 g/L compared to only 1 g/L in the non-adjusted must and in the musts to

which tartaric acid was added. The acid wine used for the initial pH adjustments also carried some SO2 (5.5 mg/L to pH 3.75 and 9.3 mg/L to pH 3.5) into the must. This SO2 addition likely had some inhibitory effect on the bacteria. MLF performance curves were confirmed by L-lactic acid production (**Figure 11, D**).

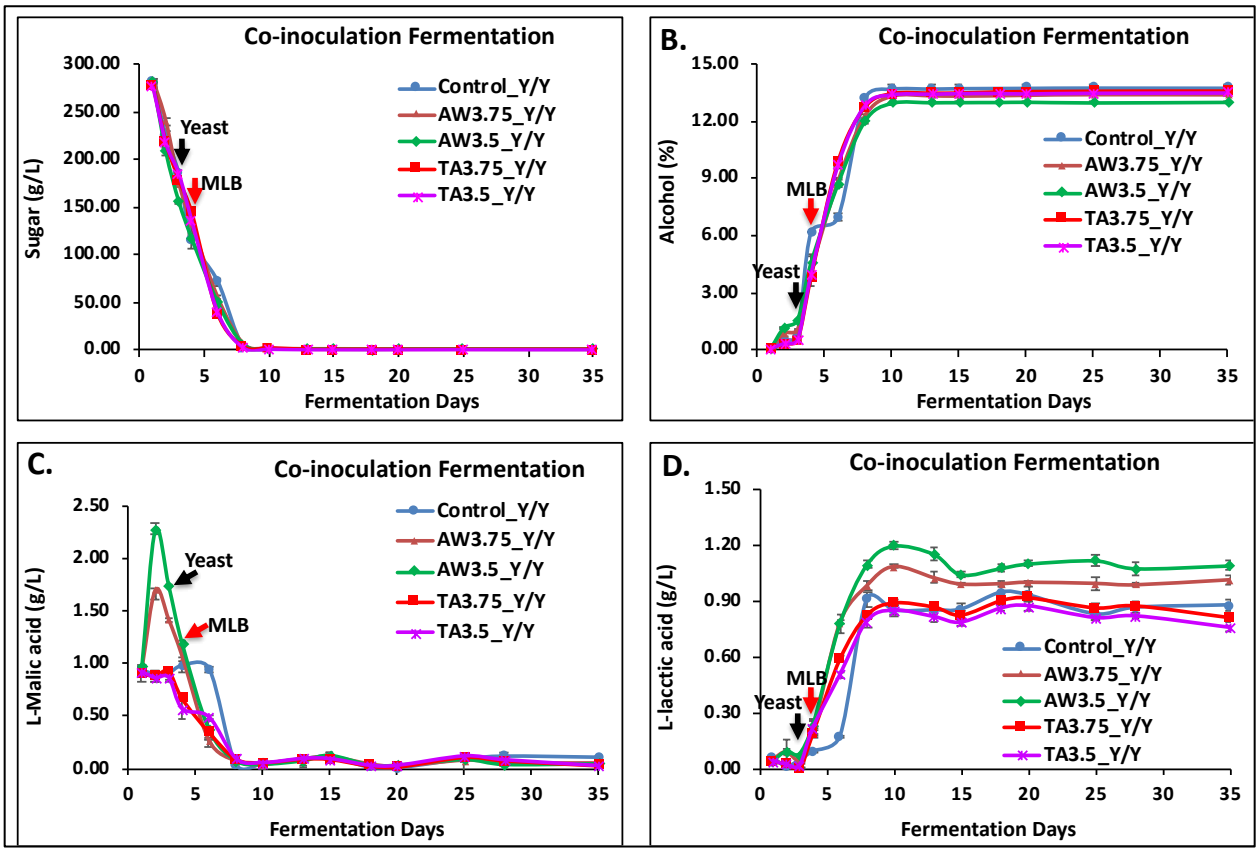
Figure 11. Impact of pH adjustment on primary and secondary fermentation in the yeast only inoculation fermentation in 2018 wines.



6.3.B.3. Co-inoculation fermentation, yeast and bacteria starter cultures added

In the co-inoculation fermentation, we added yeast and ML bacteria starter culture at day 3 and day 4, respectively. The additions of tartaric acid (TA3.75 and TA3.5) and acid wine (AW3.75 and AW3.5) to the must had no impact on the AFs (**Figure 12, A and B**). The addition of the bacteria starter culture also shows no impact on onset and rate of AFs. Although in high pH must (control, initial pH 4.3), MLF started 2 days later compare to acid added wines, all co-inoculated wines finished MLF within 10 days (**Figure 12, C**). The rate of MLF in the co-inoculated wines, tartaric acid adjustments to the must showed slower than control or acid wine adjusted must, which consistent to the 2017 result (**Figure 3**). L-lactic acid production confirmed MLF rates (**Figure 12, D**).

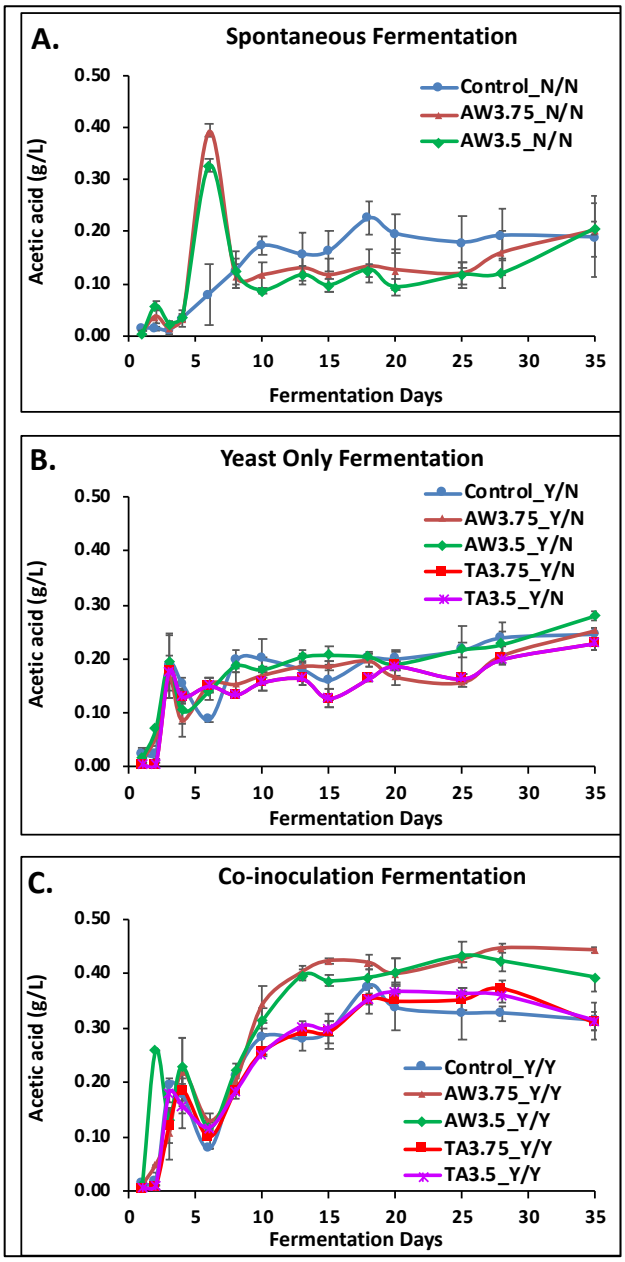
Figure 12. Impact of pH adjustment on the primary and secondary fermentation in the co-inoculation fermentation in 2018 wines.



6.3.C. Acetic acid content in the wines

The addition of ML starter culture led to increased acetic acid production in all musts, irrespective of starting pH resulting in a somewhat higher final acetic acid content, between 0.3 and 0.4 g/L in the ML inoculated musts (**Figure 13, C**) compare to 0.2 g/L in the musts without ML starter culture (**Figure 13, A and B**). Final acetic acid concentrations were well below sensory threshold in all wines.

Figure 13. Impact of pH adjustments and starter culture on acetic acid content in the 2018 wines

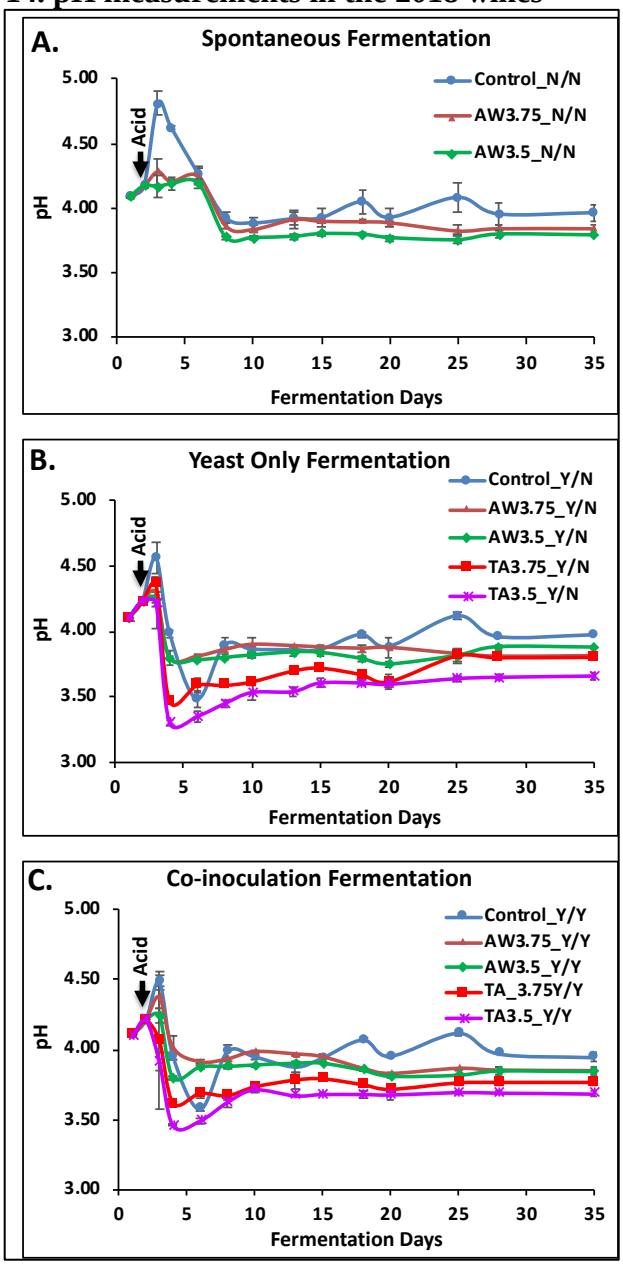


6.3.D. Effectiveness of acid additions

The acid additions with tartaric acid and with acid wine made in this experiment were only successful in lowering the must pH in the first few days. Must pH in all acidulated musts increased from the initial target pH values of 3.75 and 3.5 back up to about 3.8 in the acids wines and held a little lower in the musts with tartaric acid additions. In general, the acid additions held different must pH for only about a week. There were some small differences in the final wine pHs based on the acid additions. In this very high pH must, the addition of tartaric acid was more effective in lowering the pH than addition of acid wine whether starter cultures were

added or not (**Figure 14**). In order to lower the pH in such high pH must to the target pH and hold it, we estimate that we would have to double the amount of tartaric acid added.

Figure 14. pH measurements in the 2018 wines



6.3.E. Yeast and bacteria populations assessed by DNA sequence analysis

In this experiment with prepared DNA extractions from 13 treatments with 2 sample reps for 14 time points = 364 sample points. These samples were submitted for 16S rRNA sequencing (bacteria population) and ITS sequencing (yeast population). From the sequence results, we will able to evaluate the impact of pH adjustments and starter culture inoculation on the yeast and bacteria populations changes.

The DNA extracts have been submitted for sequencing. Extraction and concentration of DNA from the many samples took much longer than we had anticipated. We expect to complete the analysis of the sequencing results in August 2020.

6.3.F. Chemical Analysis of the 2018 Wines

At the time of sensory evaluation, approximately one year following production, the wines were analyzed for pH, titratable acidity (expressed in g/L tartaric acid), ethanol, and acetic acid. Enological Testing Services analyzed the wines for the Sulfides Panel (carbon disulfide, methyl thioacetate, ethyl thioacetate, diethyl disulfide, diethyl sulfide, dimethyl disulfide, dimethyl sulfide, ethyl mercaptan, hydrogen sulfide, and methyl mercaptan) and Biogenic Amines Panel (cadaverine, histamine, putrescine, and tyramine). The only compounds that produced values above the detection threshold for these analyses were dimethyl sulfide and putrescine. We thank ETS for the generous donation of these analyses free of charge.

All chemical data were analyzed by two-way ANOVA for the treatment effects of Inoculation Method and Target Initial pH. These analyses are summarized in Table 8 for the Tartaric Acid-treated Merlot and in Table 9 for the Acid Wine-Treated Merlot. Tartaric Acid and Acid Wine treatments were evaluated separately in all statistical analysis of the 2018 wines due to inherent differences in treatment application. Statistical analysis and data visualization for the 2018 wines were conducted in SAS and in R, with packages FactoMineR and ggplot2 (R Core Team 2019, Sebastian et al. 2008, Whickam 2016). In all tables, means represented with different letters are significantly different for the 0.05 confidence level, with letter “a” representing the greatest value.

It is important to note that the Target Initial pH does not reflect the pH in the finished wines. We decided to keep the acid additions within ranges used in the wine industry. The aim was to start the fermentations at the decreased pH targets. No attempt was made to maintain the target pH with additional acid additions during the course of the fermentations. For both the Acid Wine and Tartaric Acid treatments, the finished pH numbers are as much as 0.45 units higher than targeted. Acid addition decisions were based on bench trials at the beginning of fermentation, and the amount of acid that produces the desired pH reading on the benchtop is often an underapproximation of the amount required to maintain that pH when the must reaches equilibrium at the end of fermentation. The pH of wine is not directly correlated with acid concentration, as it is also influenced by the positive cation concentration (Schneider and Troxell 2018). Instead, the degree to which an acid addition effectively changes the pH of a wine is related to the buffering capacity, or the amount of acid required to change the pH by one unit (Schneider and Troxell 2018). In wines with high harvest pH numbers (> pH 4), such as the 2018 experiment wines, the buffering capacity is increased by proximity to the second pKA for tartaric acid, and therefore the amount of acid required to change the pH to a lasting degree is similarly increased.

Table 8. Tartaric Acid-Treated Merlot: Treatment Averages for Basic Chemical Analysis after 1 Year Aging

Treatment		Finished pH	Titratable Acidity g/L	Ethanol %v/v	Acetic Acid g/L	Dimethyl Sulfide µg/L	Putrescine µg/L
Inoculation Method	Yeast Only	3.92	4.73	13.93	0.24 b	47.01 b	2.14
	Coinoculation	3.91	4.78	13.75	0.33 a	52.96 a	2.52
	error	*0.03*	*0.05*	*0.14*	*0.02*	*1.45*	*0.22*
Target Initial pH	4.30 (Unadjusted)	4.09 a	4.19 b	14.09	0.29	43.17 b	2.72
	3.75	3.87 b	4.92 a	13.98	0.28	53.08 a	2.13
	3.50	3.79 b	5.16 a	13.45	0.29	53.70 a	2.15
	error	*0.03*	*0.06*	*0.18*	*0.02*	*1.77*	*0.27*

Differing letters indicate significantly different means at the 95% confidence level

Table 9. Acid Wine-Treated Merlot: Treatment Averages for Basic Chemical Analysis after 1 Year Aging

Treatment		Finished pH	Titratable Acidity g/L	Ethanol %v/v	Acetic Acid g/L	Dimethyl Sulfide µg/L	Putrescine µg/L
Inoculation Method	Yeast Only	4.03	4.29	13.69	0.24 b	34.88 b	2.13
	Coinoculation	3.99	4.32	13.59	0.38 a	39.24 a	2.47
	error	*0.03*	*0.05*	*0.11*	*0.01*	*0.97*	*0.26*
Target Initial pH	4.30 (Unadjusted)	4.09 a	4.19 b	14.10 a	0.29	43.17 a	2.72
	3.75	4.00 ab	4.39 ab	13.70 a	0.34	36.20 b	2.17
	3.50	3.94 b	4.43 a	13.12 b	0.30	21.82 b	2.02
	error	*0.03*	*0.06*	*0.14*	*0.02*	*1.18*	*0.31*

Differing letters indicate significantly different means at the 95% confidence level

In both Tartaric and Acid Wine treated Merlot, the pH 3.5 and pH 3.75 treatments were not statistically different at the 0.05 confidence level for the pH and titratable acidity in the finished wine. The pH 3.5 treatments were statistically different from the unadjusted wines, although these differences may be of limited enological consequence, particularly in the Acid Wine treatments which all remain near pH 4.0. The significantly lower percent ethanol in the pH 3.5 Acid Wine treatment is the result of the dilution by Acid Wine, which comprised 6.8% and 11.3% of the must volume for pH adjustments to 3.75 and 3.5, respectively (**Table 1**). Acetic Acid content was statistically higher in coinoculated treatments than in treatments inoculated only with yeast (which completed a sequential spontaneous malolactic fermentation), however final concentrations for both treatments were still well below the reported sensory threshold. Dimethyl sulfide (DMS) was highest in coinoculated treatments; Target Initial pH treatments also varied significantly, but trends were opposite in each acid treatment. In the Acid Wine Treatment, the unadjusted pH 4.3 treatment had the highest average DMS concentration, in the Tartaric Acid treatment, the Target pH 3.5 and pH 3.75 treatments had the highest quantities of DMS. Putrescine did not vary significantly by target initial pH and Inoculation method.

Analysis for anthocyanin (malvidin 3-glucoside equivalents), small polymeric pigments (SPP), large polymeric pigments (LPP), tannins (catechin equivalents), and phenols (catechin equivalents) was conducted in duplicate within six months of the sensory analysis by previously published methods (Harbertson et al. 2003, 2015). These data are summarized in Tables 10 and 11.

Table 10. Tartaric Acid- Treated Merlot: Treatment Averages for Phenolics Assays

Treatment		Anthocyanin mg/L	SPP mg/L	LPP mg/L	Total Tannin mg/L	Total Phenolics mg/L	Non-Tannin Phenolics mg/L
Inoculation Method	Yeast Only	462	1.08	**0.77 b**	375	1367	991
	Coinoculation	492	1.09	**0.88 a**	376	1356	980
	error	*42.3*	*0.047*	*0.034*	*8.67*	*42.9*	*42.6*
Target Initial pH	4.30 (Unadjusted)	503	1.18	0.82	**391 a**	1386	995
	3.75	495	1.11	0.90	**386 ab**	1389	1003
	3.50	434	0.96	0.75	**350 b**	1310	959
	error	*57.9*	*0.057*	*0.041*	*10.6*	*52.6*	*52.1*

Differing letters indicate significantly different means at the 95% confidence level

Table 11. Acid Wine- Treated Merlot: Treatment Averages for Phenolics Assays

Treatment		Anthocyanin mg/L	SPP mg/L	LPP mg/L	Total Tannin mg/L	Total Phenolics mg/L	Non-Tannin Phenolics mg/L
Inoculation Method	Yeast Only	411	1.10	0.79	365	1261	896
	Coinoculation	495	1.01	0.76	330	1315	986
	error	*42.6*	*0.034*	*0.042*	*6.40*	*48.0*	*44.1*
Target Initial pH	4.30 (Unadjusted)	503	**1.18 a**	0.82	391	1386	995
	3.75	415	**1.01 b**	0.72	333	1241	908
	3.50	442	**0.98 b**	0.78	317	1237	920
	error	*52.2*	*0.043*	*0.051*	*7.84*	*58.8*	*54.0*
Target Initial pH × Inoculation Method	4.30 × Yeast Only	501	1.24	0.79	**401 a**	1356	954
	4.30 × Coinoculation	504	1.11	0.86	**381 ab**	1417	1035
	3.75 × Yeast Only	361	1.03	0.73	**340 bc**	1242	902
	3.75 × Coinoculation	468	1.00	0.71	**327 cd**	1240	914
	3.50 × Yeast Only	372	1.04	0.85	**353 abc**	1186	833
	3.50 × Coinoculation	512	0.92	0.71	**281 d**	1288	1008
	error	*73.9*	*0.059*	*0.072*	*11.1*	*83.1*	*76.4*

Differing letters indicate significantly different means at the 95% confidence level

6.3.G. Sensory Analysis

In November 2019, a trained panel performed a descriptive sensory analysis on thirty of the 2018 experiment wines listed in Table 12. Fifteen panelists representing students, employees, and former associates of WSU Tri-Cities were recruited. This research was approved under WSU IRB #12546-011. Panelists completed six hours of training to develop the list of aroma attributes

for the descriptive analysis (Table S3). Taste attributes were sweet, sour, hot, bitter, and astringent. Formal evaluations were held over nine hour-long sessions. Ten wines were evaluated in each session: two sets of 5 with 75 second breaks between samples, and a mandatory 5-minute break between sets. Twelve panelists completed the evaluation.

Table 12. 2018 Treatments Assessed by Descriptive Analysis

pH 4.30 – No Acid – Coinoculated
pH 4 .30 – No Acid – Yeast Only
pH 3.75 – Acid Wine – Coinoculated
pH 3.75 – Acid Wine – Yeast Only
pH 3.50 – Acid Wine – Coinoculated
pH 3.50 – Acid Wine – Yeast Only
pH 3.75 – Tartaric Acid – Coinoculated
pH 3.75 – Tartaric Acid – Yeast Only
pH 3.50 – Tartaric Acid – Coinoculated
pH 3.50 – Tartaric Acid – Yeast Only

Three fermentation replicates were produced for each treatment

Sensory data were analyzed by mixed model ANOVA to assess the main effects of target initial pH, inoculation method, and the random panelist effect with SAS proc mixed. Tartaric Acid and Acid Wine data were considered separately. Significance was evaluated for the 0.05 confidence level. Post-hoc analysis for significant F-tests were conducted with Tukey's comparison at the 0.05 confidence level. In all bar plots, bars labeled with differing letters are significantly different.

In the Tartaric Acid-treated wines, "Artificial Fruit" and "Floral" aromas differed significantly by inoculation method; "Artificial Fruit" was scored higher for coinoculated treatments and "Floral" was scored higher for treatments that were only inoculated with yeast (Figure 15, Note that fermentations inoculated only for primary fermentation completed malolactic fermentation spontaneously). "Strawberry" aroma was significant by target initial pH and was highest in the pH 3.5 wines and lowest in the pH 4.0 wines (Figure 16). The attribute "Sour" was significant for target initial pH at the 0.10 confidence level with a p-value of 0.058 and was scored highest in the pH 3.5 wines and lowest in the pH 4.0 wines. This trend in the sensory data aligns with the titratable acidity numbers, which are highest in the pH 3.5 treatment.

Figure 15. Significant Sensory Attributes by Inoculation Method in Tartaric Acid-Treated Wines

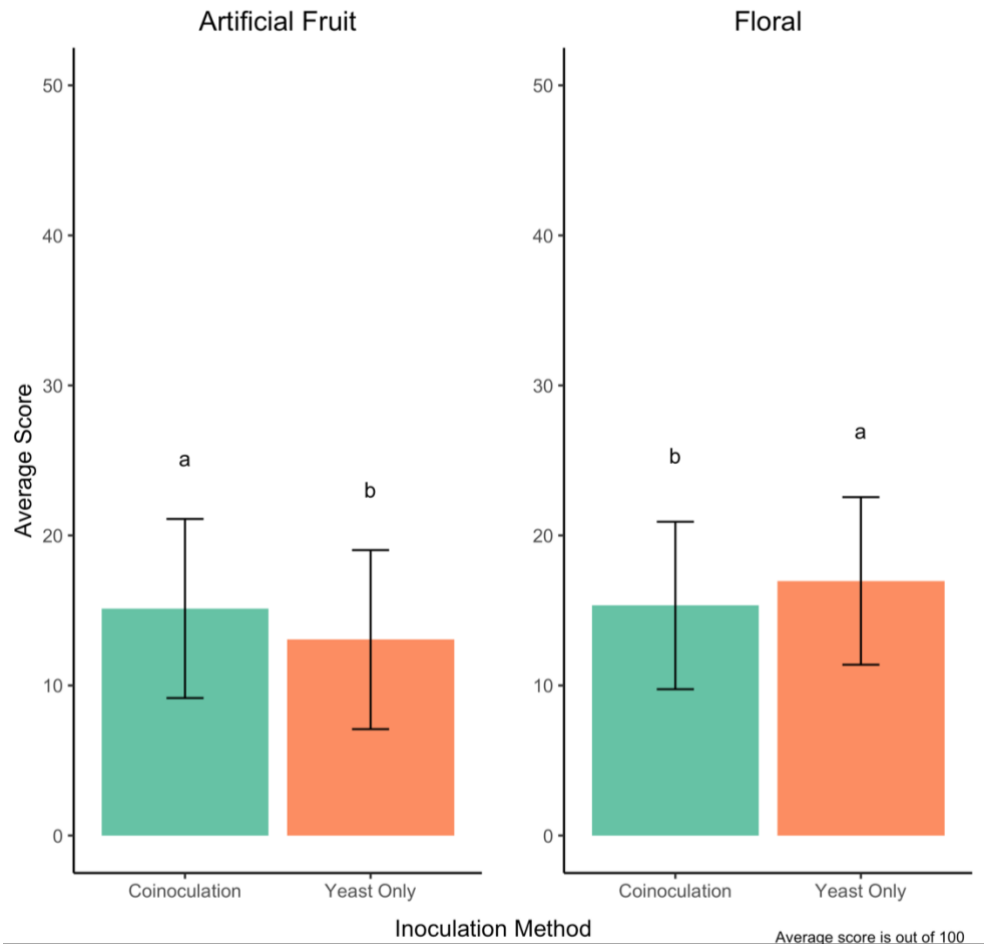
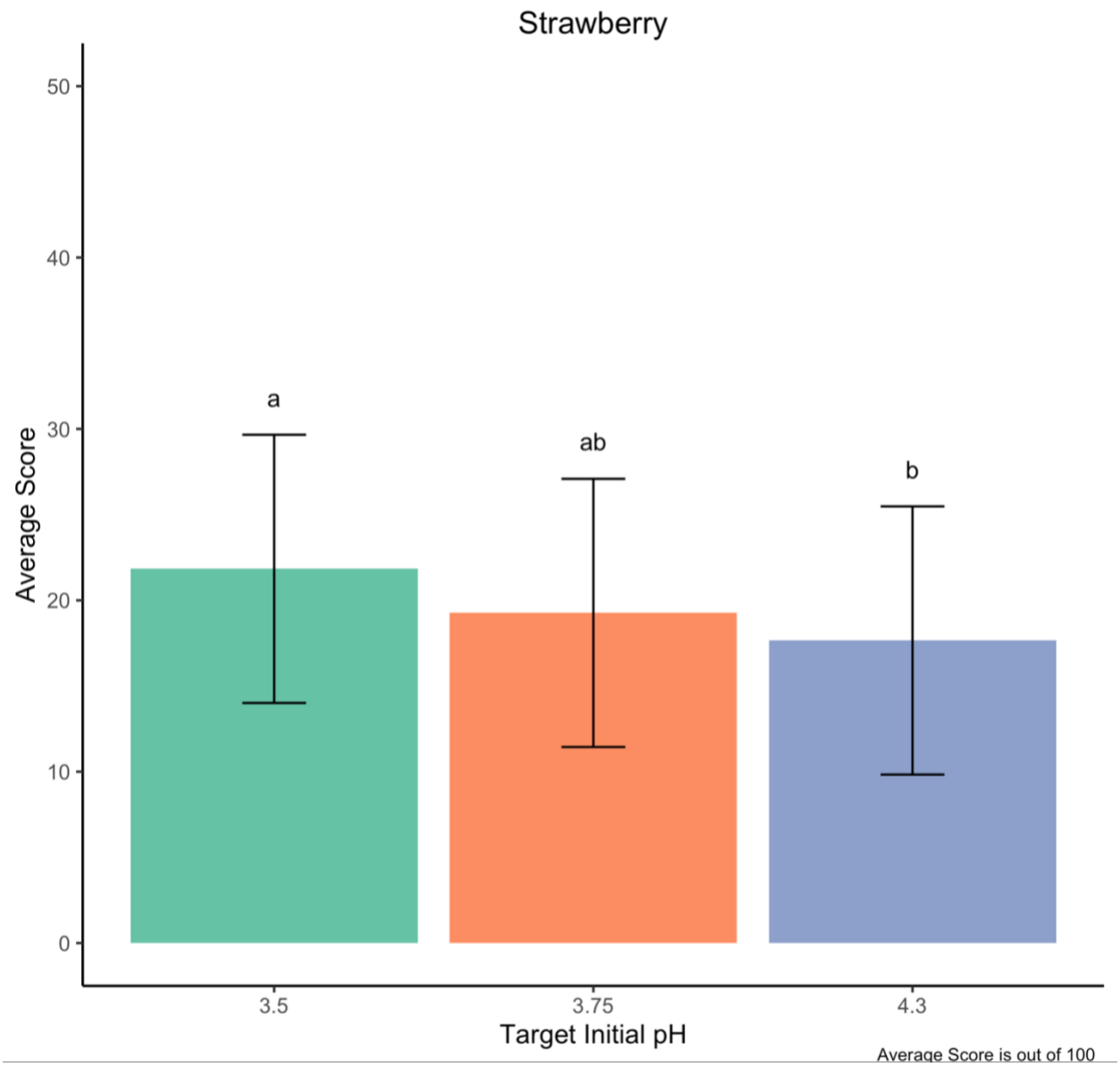
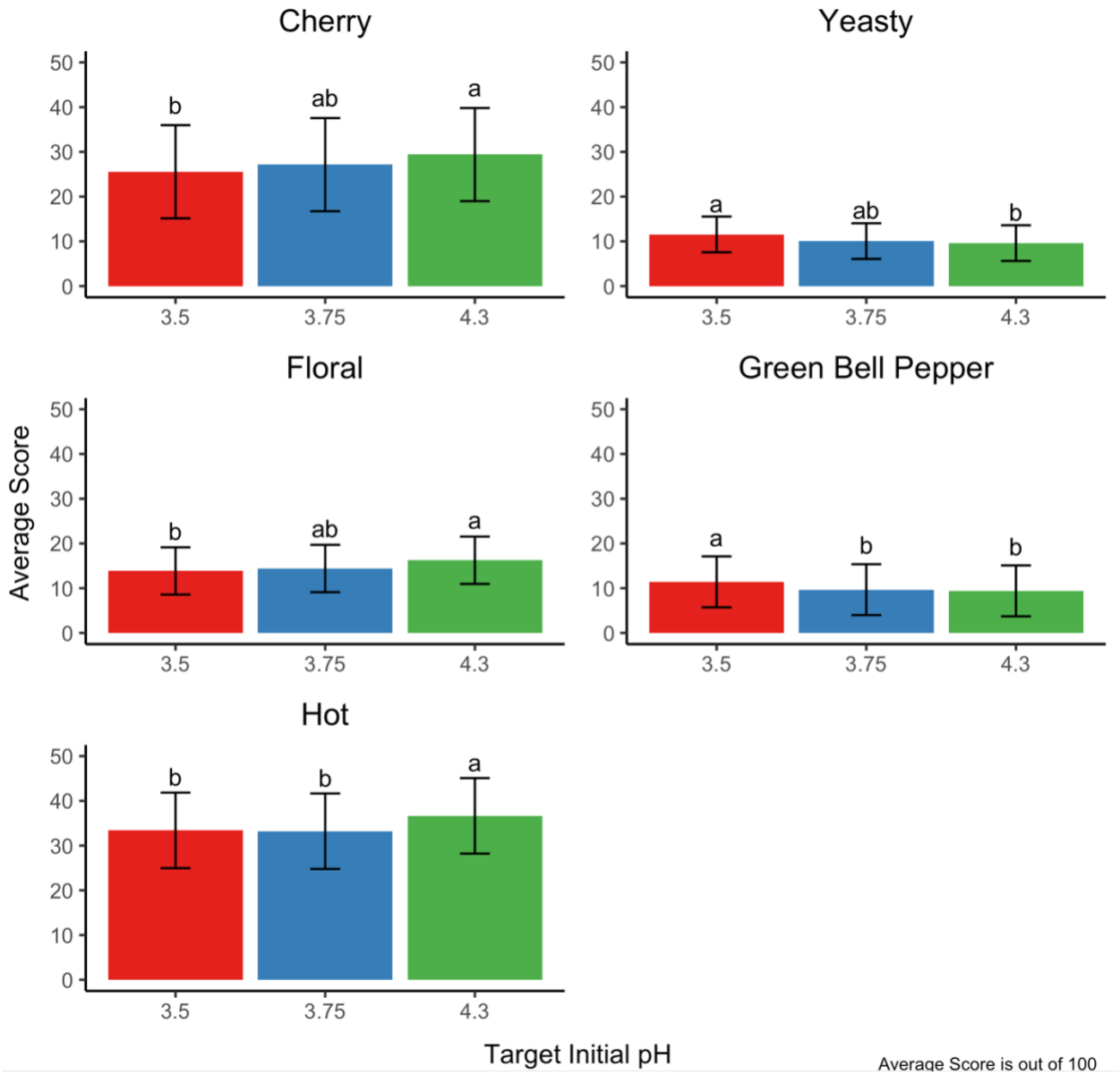


Figure 16. Significant Sensory Attributes by Target Initial pH in Tartaric Acid-Treated Wines



The effect of the Target Initial pH treatment in the Acid Wine-treated wines produced differences in the aroma attributes "Cherry," "Floral," "Hot," "Yeasty," and "Green Bell Pepper." (Figure 17.) "Cherry" and "Floral" Aromas and "Hot" taste were rated highest in the pH 4.0 wines and lowest in the pH 3.5 wines. "Yeasty" and "Green Bell Pepper" aromas were highest in the pH 3.5 wines and lowest in the pH 4.0 wines. It is possible that the character of the pH 3.5 wines, which are perceived as less fruity and more vegetal, is due to some sensory effect of the Acid Wine. There were no significant sensory differences resulting from inoculation method in the Acid Wine-treated wines.

Figure 17. Significant Sensory Attributes by Target Initial pH in Acid Wine-Treated Wines



A multifactor analysis (MFA) for all significant compounds by GC-MS, chemical measures, and sensory attributes was used to visualize the relationships between these datasets and are represented in Figure 18and Figure 19. Metabolites are numbered by retention time (RT), and are detailed in Tables 13 through 18.

Figure 18. 2018 Tartaric Acid-Treated Merlot: Chemistry, Metabolite, and Sensory MFA

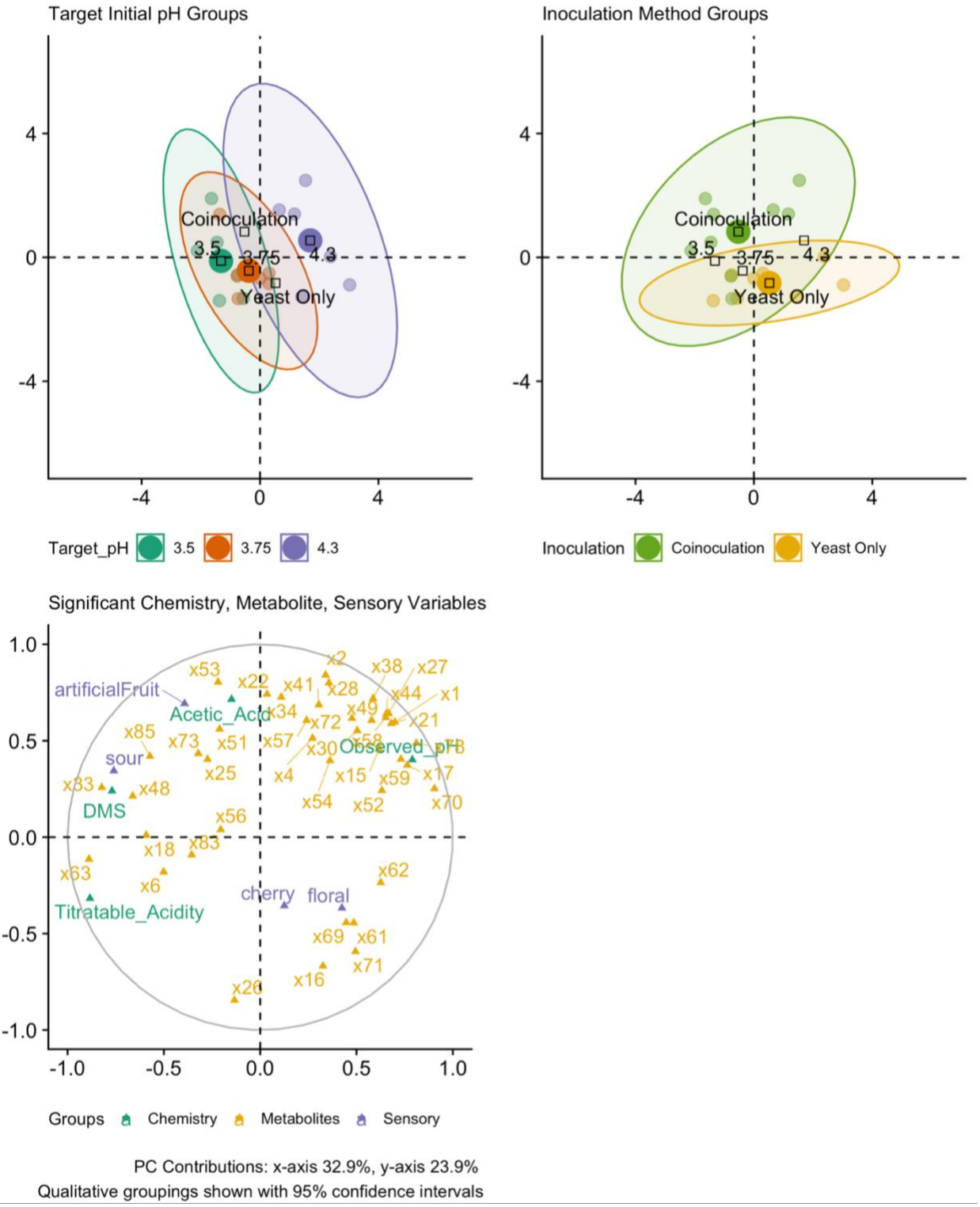
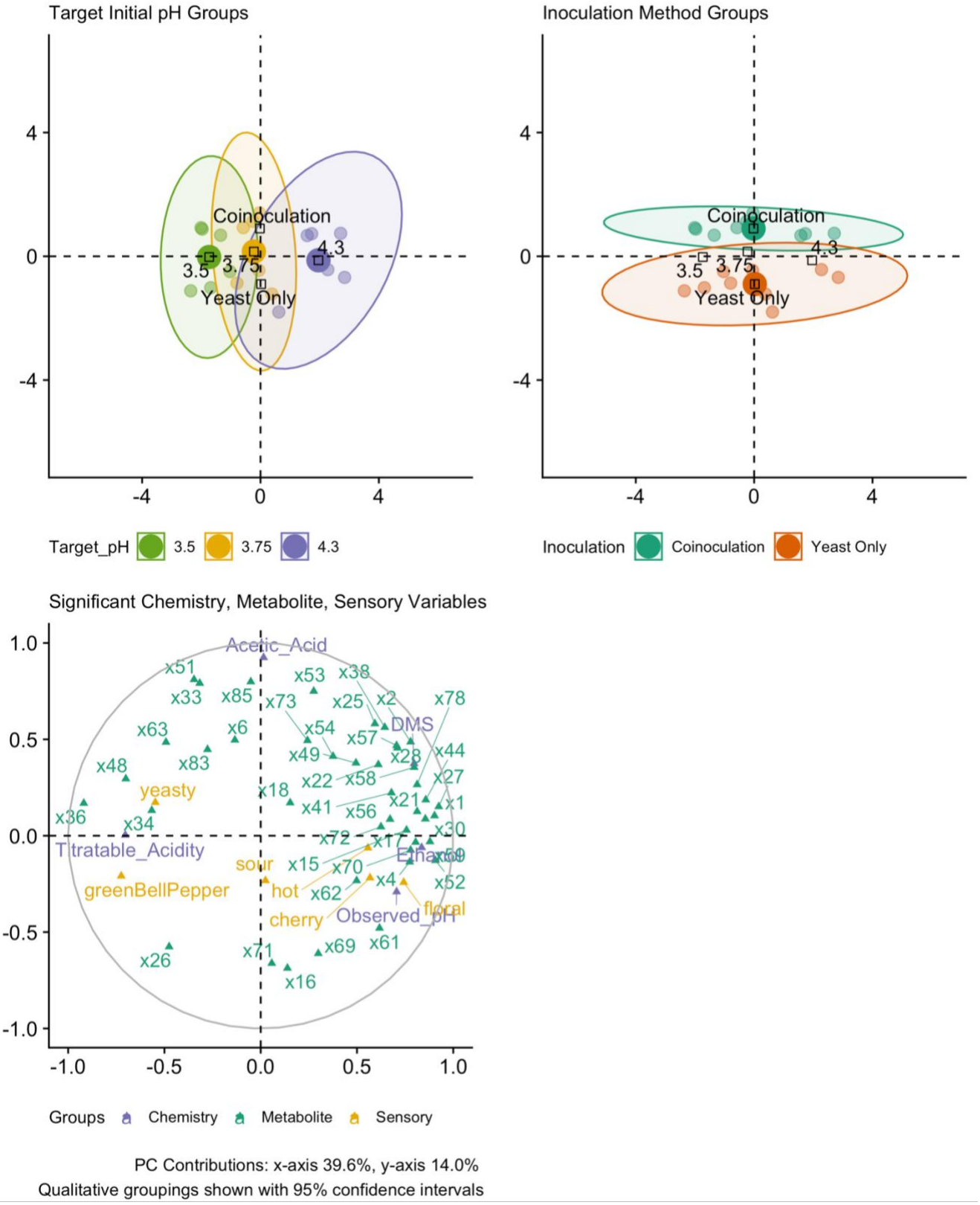

Target Initial pH Groups
Coinoculation
3.5
3.75
4.3
Yeast Only
Target_pH 3.5 3.75 4.3
Inoculation Method Groups
Coinoculation
3.5
3.75
4.3
Yeast Only
Inoculation Coinoculation Yeast Only
Significant Chemistry, Metabolite, Sensory Variables
artificialFruit
Acetic_Acid
sour
DMS
Titratable_Acidity
cherry
floral
Observed_pH
Groups Chemistry Metabolites Sensory
PC Contributions: x-axis 32.9%, y-axis 23.9%
Qualitative groupings shown with 95% confidence intervals

Figure 19. 2018 Acid Wine-Treated Merlot: Chemistry, Metabolite, and Sensory MFA



6.3.H. Metabolite Analysis by GC-MS

Metabolite Analysis by Gas Chromatography–Mass Spectrometry (GC-MS) for the 2018 experiment wines was conducted in March 2020. All wines were randomized and tested in triplicate with SPME headspace sampling. A 2-Undecanone internal standard was spiked at 50ug/L in each vial. The data were analyzed by PARADISe software (), which uses a multi-way model to deconvolute user-selected intervals on the collective total ion chromatogram. 90 compounds in total were identified in the initial report.

Statistical analysis was conducted in R Studio (Cite). Tartaric acid and Acid Wine treatments were analyzed separately with the same control treatment data. A 2-way ANOVA model for the effects of inoculation method and target initial pH was used to identify compounds that varied significantly among treatments at the 0.05 confidence level. Post-hoc analysis were conducted for significant F tests with Tukey's analysis also at the 0.05 confidence level. Statistically significant compounds are listed in Table X-X.

Compound identification was completed by NIST database comparisons in PARADISe and comparison to values in the literature. The Kovats mix was run on the instrument protocol and used to calculate the Kovats retention index. In Table X, named compounds were identified if the NIST probability score was 60% or greater, or if other published Kovats retention indices on DB-WAX columns were found within ± 10 KI by the Pherobase Search tool.

Conclusion

This analysis shows the wines made from the acid-adjusted musts differ from the non-adjusted, pH 4.3 must. Both sets if wines, acidified with tartaric acid and with acid wine, show a consistent change in metabolites following the pH from 4.3, to adjusted target 3.75, and target 3.5. This clearly demonstrates that the pH of the must has a strong influence on the metabolites produced during wine fermentation – even in musts with such small changes in pH! The acid-adjusted wines also show a clear separation between coinoculated and yeast only inoculated wines, indicating a difference in the metabolites formed by the starter culture ML bacteria versus the native ML bacteria (yeast only). This agrees with other studies that show the sensory impact of various ML starter cultures and starter culture versus native ML bacteria. It is to note that the type of fermentation management with yeast and ML bacteria have an impact beyond the starting must pH. For both acid adjustments, the coinoculated and the yeast only fermentation separated!

Table 13. Tartaric Acid-Treated Merlot: Significant GC-MS Metabolites by Target Initial pH

Treatment		Compound Number				
		2	15	16	17	18
		Compound ID				
		Ethyl Acetate	Isoamyl Acetate	Ethyl Hexanoate	Unknown	Hexanol
Target Initial pH	4.00	1.48 b	0.360 b	1.57 b	0.014 b	0.009 a
	3.75	1.61 ab	0.380 ab	1.80 a	0.015 b	0.008 a
	3.50	1.77 ab	0.430 a	1.62 ab	0.018 a	0.007 b
	p-value	*0.003*	*0.008*	*0.023*	*<0.001*	*<0.001*

Treatment		Compound Number				
		21	33	41	44	48
		Compound ID				
		Unknown	Unknown	Isopentyl Hexanoate	Unknown	Unknown
Target Initial pH	4.00	9.98 b	0.233 a	0.075 a	0.015 b	0.012 a
	3.75	10.59 b	0.206 b	0.069 ab	0.016 b	0.011 a
	3.50	12.27 a	0.168 c	0.064 b	0.020 a	0.010 b
	p-value	*<0.001*	<0.001	*0.011*	*<0.001*	*<0.001*

Treatment		Compound Number				
		49	58	59	63	70
		Compound ID				
		Unknown	Ethyl Hexadecanoate	Unknown	Methionol	Unknown
Target Initial pH	4.00	0.017 b	0.072 b	0.12 b	0.006 a	0.020 b
	3.75	0.018 b	0.073 b	0.32 b	0.005 b	0.022 b
	3.50	0.021 a	0.083 a	0.15 a	0.003 c	0.300 a
	p-value	*0.001*	*0.003*	*<0.001*	*<0.001*	*<0.001*

Treatment		Compound Number			
		72	78	83	85
		Compound ID			
		Beta Damascenone	Phenylethyl Alcohol	Octanol	Ethyl Hexadecanoate
Target Initial pH	4.00	0.019 b	4.06 a	0.98 b	0.015 b
	3.75	0.020 b	3.22 b	1.00 b	0.016 b
	3.50	0.022 a	2.99 b	1.09 a	0.02 a
	p-value	*<0.001*	*<0.001*	*0.007*	*<0.001*

Table 14. Acid Wine-Treated Merlot: Significant GC-MS Metabolites by Target Initial pH

Treatment		Compound Number				
		1	2	6	15	17
		Compound ID				
		Unknown	Ethyl Acetate	Unknown	Isobutanol	Butanol
Target Initial pH	4.00	0.126 a	1.77 a	0.026 b	0.427 a	0.018 a
	3.75	0.104 b	1.4 b	0.031 ab	0.346 b	0.015 b
	3.50	0.094 c	1.29 b	0.041 a	0.336 b	0.014 b
	p-value	*<0.001*	*<0.001*	*0.030*	*<0.001*	*<0.001*

Treatment		Compound Number				
		21	22	28	41	44
		Compound ID				
		Unknown	Ethyl Hexanoate	Unknown	Isopentyl Hexanoate	Unknown
Target Initial pH	4.00	12.27 a	3.80 a	0.005 a	0.075 a	0.020 a
	3.75	10.26 b	3.24 b	0.003 b	0.061 b	0.015 b
	3.50	9.82 b	3.19 b	0.003 b	0.059 b	0.014 b
	p-value	*<0.001*	0.002	*<0.001*	*<0.001*	*<0.001*

Treatment		Compound Number				
		49	56	57	58	63
		Compound ID				
		Unknown	Butanoic Acid	Ethyl Decanoate	Unknown	Methionol
Target Initial pH	4.00	0.021 a	0.01141 a	11.1 a	0.083 a	0.003 b
	3.75	0.020 ab	0.00948 b	9.4 b	0.074 ab	0.004 ab
	3.50	0.016 b	0.00905 b	8.9 b	0.066 b	0.004 a
	p-value	*0.036*	*<0.001*	*<0.001*	*<0.001*	*0.006*

Treatment		Compound Number			
		71	72	78	85
		Compound ID			
		2-Phenylethyl Acetate	Beta Damascenone	Phenylethyl Alcohol	Ethyl Hexadecanoate
Target Initial pH	4.00	0.066 b	0.0222 a	4.06 a	0.006 b
	3.75	0.077 a	0.0188 b	3.73 b	0.007 a
	3.50	0.067 b	0.0175 b	3.27 c	0.007 ab
	p-value	*0.007*	*<0.001*	*<0.001*	*0.038*

Table 15. Tartaric Acid-Treated Merlot: Significant GC-MS Metabolites by Inoculation Method

Treatment		Compound Number				
		2	16	22	33	34
		Compound ID				
		Ethyl Acetate	Isopentyl Acetate	Ethyl Hexanoate	Unknown	Hexanol
Inoculation Method	Yeast Only	1.52 b	1.86 a	3.54 b	0.18 b	0.15 b
	Coinoculation	1.72 a	1.47 b	3.86 a	0.23 a	0.17 a
	p-value	*0.003*	*<0.001*	*0.045*	*<0.001*	*0.005*

Treatment		Compound Number				
		48	51	53	63	71
		Compound ID				
		Unknown	Octanol	Unknown	Methionol	2-Phenylethyl Acetate
Inoculation Method	Yeast Only	0.01 b	0.23 b	0.04 b	0.004 b	0.055 b
	Coinoculation	0.012 a	0.032 a	0.05 a	0.005 a	0.071 a
	p-value	*<0.001*	*<0.001*	*<0.001*	*0.005*	*<0.001*

Treatment		Compound Number	
		72	51
		Compound ID	
		Beta Damascenone	Ethyl Hexadecanoate
Inoculation Method	Yeast Only	0.02 b	0.006 b
	Coinoculation	0.021 a	0.008 a
	p-value	*0.008*	*<0.001*

Table 16. Acid Wine-Treated Merlot: Significant GC-MS Metabolites by Inoculation Method

Treatment		Compound Number				
		2	6	16	22	28
		Compound ID				
		Ethyl Acetate	Unknown	Isopentyl Acetate	Ethyl Heaxanoate	Unknown
Inoculation Method	Yeast Only	1.35 b	0.02 b	1.94 a	3.23 b	0.003 b
	Coinoculation	1.62 a	0.04 a	1.28 b	3.59 a	0.004 a
	p-value	*<0.001*	*<0.001*	*<0.001*	*0.019*	*<0.001*

Treatment		Compound Number		
		57	63	83
		Compound ID		
		Unknown	Methionol	Octonol
Inoculation Method	Yeast Only	9.11 b	0.003 b	0.999 b
	Coinoculation	10.49 a	0.004 a	1.073 a
	p-value	*<0.001*	*<0.001*	*0.023*

Table 17. Tartaric Acid Treated Merlot: GC-MS Metabolites with Significant Inoculation x Target Initial pH Interaction

Treatment		Compound Number				
		1	6	25	26	27
		Compound ID				
		Unknown	Unknown	Hexyl Acetate	Unknown	Unknown
Inoculation × Target Initial pH	Yeast Only × pH 4.30	0.121 ab	0.011 b	0.022 b	0.016 a	0.036 ab
	Coinoculation × pH 4.30	0.131 a	0.041 a	0.03 a	0.008 b	0.04 a
	Yeast Only × pH 3.75	0.116 abc	0.059 a	0.029 a	0.018 a	0.034 abc
	Coinoculation × pH 3.75	0.103 bc	0.044 a	0.028 a	0.017 a	0.03 c
	Yeast Only × pH 3.50	0.099 c	0.065 a	0.027 a	0.018 a	0.03 c
	Coinoculation × pH 3.50	0.107 bc	0.058 a	0.028 a	0.014 a	0.032 bc
	p-value	***0.031***	**0.001**	***<0.001***	***0.042***	***0.027***

Treatment		Compound Number				
		28	30	38	52	61
		Compound ID				
		Unknown	3-Methyl Pentanol	Unknown	2-Methyl Propanoic Acid	Diethyl Succinate
Inoculation × Target Initial pH	Yeast Only × pH 4.30	0.004 b	0.018 b	0.029 b	0.045 ab	2.39 a
	Coinoculation × pH 4.30	0.006 a	0.021 a	0.037 a	0.048 a	1.32 d
	Yeast Only × pH 3.75	0.003 b	0.019 ab	0.023 b	0.045 ab	1.6 bc
	Coinoculation × pH 3.75	0.004 b	0.017 b	0.026 bc	0.039 b	1.45 cd
	Yeast Only × pH 3.50	0.003 b	0.017 b	0.22 c	0.042 ab	1.77 b
	Coinoculation × pH 3.50	0.004 b	0.017 b	0.26 b	0.038 b	1.56 bcd
	p-value	***0.037***	***0.003***	***<0.001***	***0.048***	***<0.001***

Treatment		Compound Number	
		62	69
		Compound ID	
		Unknown	Unknown
Inoculation × Target Initial pH	Yeast Only × pH 4.30	0.205 a	0.008 a
	Coinoculation × pH 4.30	0.103 b	0.002 c
	Yeast Only × pH 3.75	0.101 b	0.003 bc
	Coinoculation × pH 3.75	0.087 b	0.003 bc
	Yeast Only × pH 3.50	0.137 b	0.004 b
	Coinoculation × pH 3.50	0.077 b	0.003 bc
	p-value	***0.024***	***<0.001***

Table 18. Acid Wine Treated Merlot: GC-MS Metabolites with Significant Inoculation x Target Initial pH Interaction

Treatment		Compound Number				
		4	25	26	30	33
		Compound ID				
		Ethyl Propanoate	Hexyl Acetate	Unknown	2-Methyl Pentanol	Unknown
Inoculation × Target Initial pH	Yeast Only × pH 4.30	0.0157 ab	0.0215 bc	0.01594 b	0.0181 b	0.148 cd
	Coinoculation × pH 4.30	0.017 a	0.0295 a	0.00766 c	0.0212 a	0.188 ab
	Yeast Only × pH 3.75	0.0144 abc	0.0193 c	0.03881 a	0.0141 c	0.136 d
	Coinoculation × pH 3.75	0.0125 c	0.0247 ab	0.01674 b	0.014 c	0.22 a
	Yeast Only × pH 3.50	0.0132 bc	0.0199 bc	0.03751 a	0.0143 c	0.181 bc
	Coinoculation × pH 3.50	0.0118 c	0.0209 bc	0.01793 b	0.0136 c	0.216 a
	p-value	*0.029*	0.027	*<0.001*	*0.015*	*0.006*

Treatment		Compound Number				
		34	36	38	48	51
		Compound ID				
		Hexanol	Unknown	Unknown	Unknown	Octanol
Inoculation × Target Initial pH	Yeast Only × pH 4.30	0.158 c	4.3E-05 e	0.0293 b	0.00884 b	0.0243 c
	Coinoculation × pH 4.30	0.187 bc	3.6E-05 e	0.0368 a	0.01059 a	0.0332 b
	Yeast Only × pH 3.75	0.22 a	1.7E-03 d	0.0275 b	0.01084 a	0.0241 c
	Coinoculation × pH 3.75	0.207 ab	2.4E-03 c	0.0311 b	0.01143 a	0.04 a
	Yeast Only × pH 3.50	0.224 a	3.6E-03 b	0.0269 b	0.01206 a	0.0278 c
	Coinoculation × pH 3.50	0.207 ab	4.1E-03 a	0.0286 b	0.01159 a	0.0419 a
	p-value	*0.004*	*<0.001*	*0.040*	*0.013*	*0.024*

Treatment		Compound Number				
		52	53	54	59	61
		Compound ID				
		2-Methyl Propanoic Acid	Unknown	2,3-Butanediol	Unknown	Diethyl Succinate
Inoculation × Target Initial pH	Yeast Only × pH 4.30	0.0448 a	0.0436 c	0.0476 bc	0.143 a	2.393 a
	Coinoculation × pH 4.30	0.0476 a	0.0558 b	0.0502 bc	0.157 a	1.321 b
	Yeast Only × pH 3.75	0.0304 b	0.0417 c	0.0404 c	0.122 b	0.997 c
	Coinoculation × pH 3.75	0.0264 bc	0.0543 b	0.0564 b	0.117 bc	0.929 c
	Yeast Only × pH 3.50	0.0279 b	0.0444 c	0.043 c	0.111 bc	1.044 bc
	Coinoculation × pH 3.50	0.022 c	0.0482 bc	0.0419 c	0.1 c	0.82 c
	p-value	*0.004*	*0.043*	*0.011*	*0.038*	*<0.001*

Treatment		Compound Number			
		62	69	70	85
		Compound ID			
		Unknown	Unknown	Unknown	Ethyl Hexadecanoate
Inoculation × Target Initial pH	Yeast Only × pH 4.00	0.205 a	0.00751 a	0.0303 a	0.00539 c
	Coinoculation × pH 4.00	0.103 b	0.00216 b	0.0299 ab	0.0075 b
	Yeast Only × pH 3.75	0.106 b	0.00229 b	0.0251 bcd	0.00519 c
	Coinoculation × pH 3.75	0.1 b	0.00164 b	0.0271 abc	0.0095 a
	Yeast Only × pH 3.50	0.116 b	0.00329 b	0.0245 cd	0.00581 c
	Coinoculation × pH 3.50	0.105 b	0.00154 b	0.0204 d	0.00745 b
	p-value	*0.003*	*<0.001*	*0.033*	*<0.001*

6.3.I. Metabolite Analysis by LC-MS (2018)

Metabolite Analysis by Liquid Chromatography-Mass Spectrometry (LC-MS) was conducted in January 2020 for the 2018 wines listed in Table 12. Single instrument replicates were tested

with a simple 15-minute method in negative mode with a mobile phase ramp from 0.1% acetic acid in water to 0.05% acetic acid in methanol.

Only observations that appeared in all three fermentation replicates for at least one Target Initial pH and Inoculation Method treatment combination were included in the analysis (298 compounds in the Acid Wine treatment, 305 compounds in the Tartaric Acid Treatment). In figures 20 and 21, LC-MS metabolite data were analyzed by Principle Component Analysis (PCA). The relative importance of the contributions of each observation to the depicted components are indicated by color gradient. In both the Tartaric Acid and Acid Wine treatments, the variation in the first component (x-axis) appears to be well explained by the difference in Target Initial pH. In the Acid Wine treatment, the second component shows good separation between "Coinoculation" and "Yeast Only" treatments. The variation described by the second component in the Tartaric Acid data appears to be driven by differences between Target pH 3.5 and 3.75 within the "Yeast Only" inoculation treatment.

The compounds of greatest importance to the first two principle components are listed in Table 19. This evaluation may reasonably be extended to approximate identification by comparison to published mass databases such as Metlin, however external verification is required to reliably identify compounds and should be pursued in a separate analysis.

In both acid treatments, the majority of these defining compounds are characterized by relatively high mass, greater than 300 m/z, the maximum threshold considered by GC-MS. All compounds in Table 19 elute in the first half of the 15-minute run, when the liquid phase is primarily water and acetic acid, suggesting these compounds are also comparatively polar. The following masses were found only in Acid Wine treated samples: 144.0605 and 676.2366. The following masses were found only in Tartaric Acid-treated samples: 880.2133, 847.2932, 536.1857, 698.2211, 586.179, 564.2174, 648.2013, 726.2536, 622.2245.

Table 19. Top 15 Contributing LC-MS Observations Principle Components 1&2

Tartaric Acid Treatment				Acid Wine Treatment			
Principle Component 1 (42.2%)		Principle Component 2 (22.7%)		Principle Component 1 (36.6%)		Principle Component 2 (22.3%)	
Mass	RT	Mass	RT	Mass	RT	Mass	RT
367.1543	3.159	390.2555	3.81	280.1406	1.423	899.2885	3.163
510.1693	3.882	450.2778	3.809	326.1266	2.602	816.2306	5.221
566.1971	3.644	370.0646	1.791	734.2916	3.159	348.1107	2.6
566.1985	3.73	866.2462	2.407	580.2145	4.818	136.0705	1.472
552.1815	2.87	882.2427	2.889	510.1693	3.882	510.2094	2.691
550.202	4.144	312.087	2.165	400.1449	4.265	374.148	1.92
354.1589	4.719	348.1117	2.6	877.3051	3.16	626.1834	3.99
552.1807	2.769	316.0849	2.794	508.1548	3.532	664.1799	2.867

488.1844	3.413	334.0959	2.239	818.2229	3.719	512.1842	3.182
194.0794	1.844	318.1002	2.788	1028.3232	4.39	448.1311	4.743
330.1215	2.877	320.0793	2.447	164.0665	1.093	928.2329	4.309
580.2131	4.268	304.0847	2.931	891.3207	3.16	634.1569	1.607
704.2298	4.472	348.1126	3.986	442.2141	3.22	383.1362	1.691
360.1333	2.075	350.2355	6.841	782.2463	4.189	182.0787	1.47
348.1523	3.03	642.2644	2.332	367.1543	3.159	164.0666	2.6

Data are presented in descending order of importance

Figure 20. 2018 Tartaric Acid-Treated Merlot PCA: LC-MS Metabolite Plot

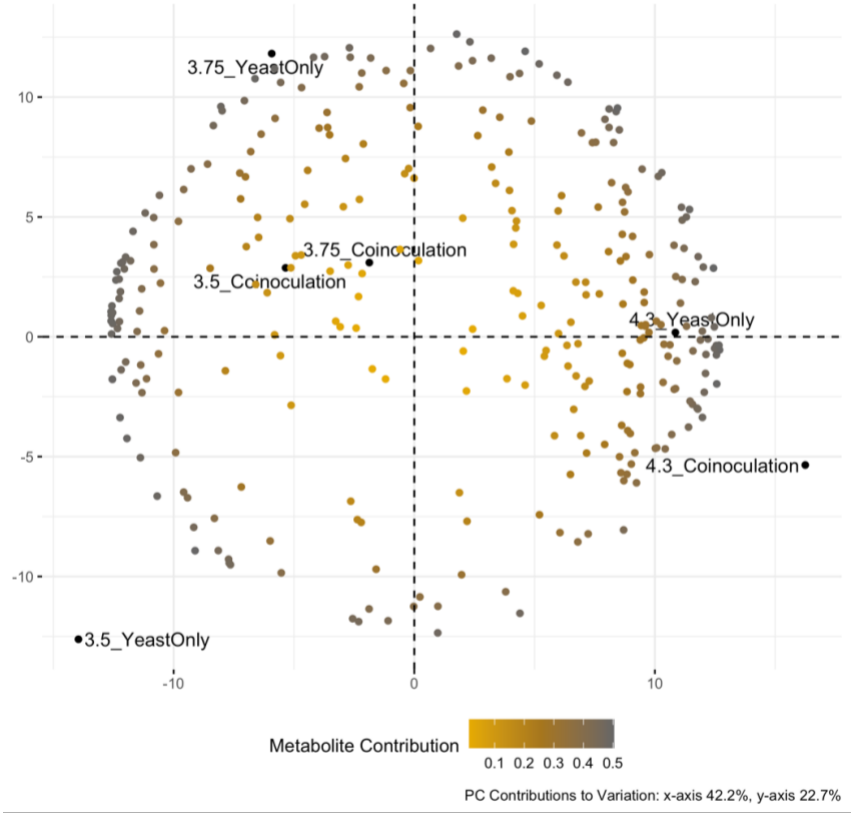
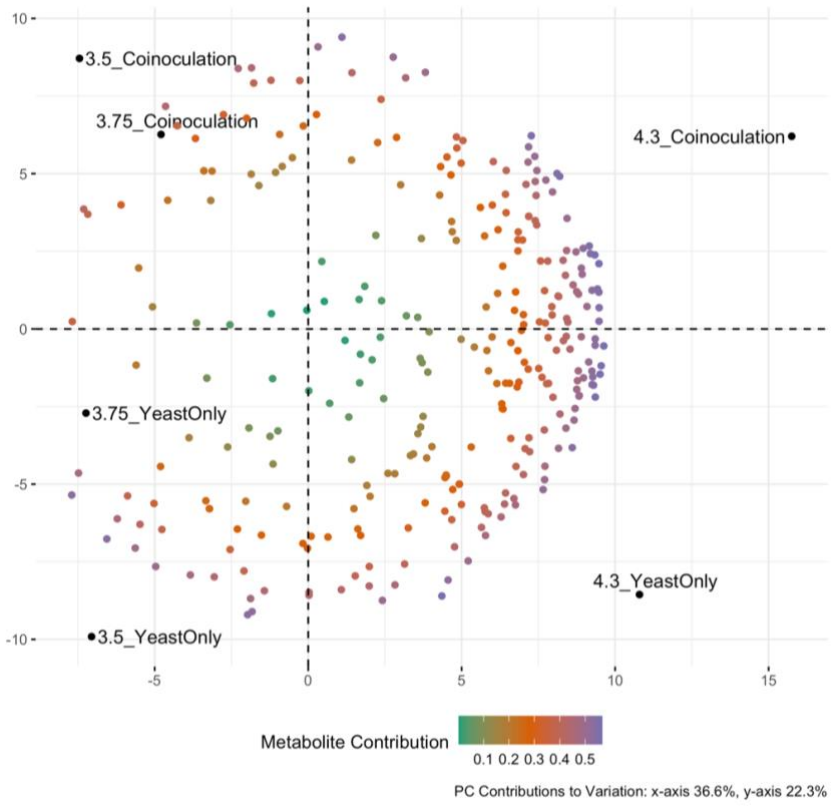


Figure 21. 2018 Acid Wine-Treated Merlot PCA: LC-MS Metabolite Plot



Conclusion

The metabolite analysis by LC-MS found fewer individual compounds separating the various must treatments. Nevertheless, the metabolites distribution found in this analysis also separates clearly the wines based on must acidification. The separation between the 3 different must pHs is most clearly seen in the tartaric acid treatments, less so in the acid wine added musts. We suspect that the relatively large addition of acid wine had more impact on the final wine composition than the small pH adjustments achieved. In both acid treatments, tartaric acid and acid wine, the impact of yeast only inoculation vs coinoculation show a very noticeable impact on the wine. Whether the wines were made with a starter culture ML (coinoculated) or with spontaneous MLF (yeast only) made a clear difference in all wines, especially in the high pH musts.

6.3.J. Metabolite Analysis by LC-MS (2017)

Metabolite analysis by LC-MS was conducted in January-March 2019 for all fermentation samples of the 2017 wines. The same instrumentation used as in the 2018 LC-MS analysis. A PCA of the treatments described in Table 20 was used to assess the 208 discrete mass observations that occurred in all fermentation replicates for at least one treatment. Fifty-nine samples were excluded from the analysis due to poor instrument sensitivity. The first two components of this analysis describe relatively little variation among the samples, 19.4% and 13.2%, respectively. In Figure 22, where 95% confidence intervals describe fermentation day, differences in fermentation progression appear to reasonably describe the first component. In Figure 23, confidence ellipses visualize the differences in target initial pH, and the overlap of the

3.8 unadjusted treatment, 3.75, and 3.5 treatment confidence intervals suggests there were not strong differences produced as a result of these treatments during fermentation. Inoculation methods are compared in Figure 24 and do not appear to describe any of the variation in the first two components.

Table 20. 2017 Treatments Analyzed by LC-MS

Control – pH 3.8 – No Acid – Coinoculated
pH 3.75 – Acid Wine – Coinoculated
pH 3.75 – Acid Wine – Sequential Inoculation
pH 3.75 – Acid Wine – Yeast Only
pH 3.50 – Acid Wine – Coinoculated
pH 3.50 – Tartaric Acid – Coinoculated
pH 3.50 – Malic Acid – Coinoculated

Three fermentation replicates were produced for each treatment

Conclusions

The treatments for the 2017 wines produced few differences in the metabolite compositions as measurable by the selected LC-MS method. It is possible this is due to the fact that the starting must pH was 3.8 in 2017, and that as a result, relatively small acid adjustments were required, with low consequence for must composition. These findings in the 2017 wines do not agree with those of the 2018s, in which differences were attributed to both the Target Initial pH and the Inoculation Method. This disparity may lie inherent to the differing chemistries of a must harvested at pH 3.8 (2017) and one harvested at pH 4.3 (2018). The similarities between the 2017 outcomes for the selected inoculation methods, acid additions, and initial target pH values may indicate that the degree to which a fermentation is robust to variation in some enological protocols may have some relationship to the starting pH. The data here are insufficient to suggest large differences exist between treatments in the 2017 wines. The instrument method was designed around the detection of phenolic compounds, and not small-polar compounds such as those frequently associated with microbial metabolism, therefore further investigations are required to characterize the 2017 wines, including profiling by GC-MS.

Figure 22. 2017 Wines Fermentation Day PCA: LC-MS Metabolite Biplot

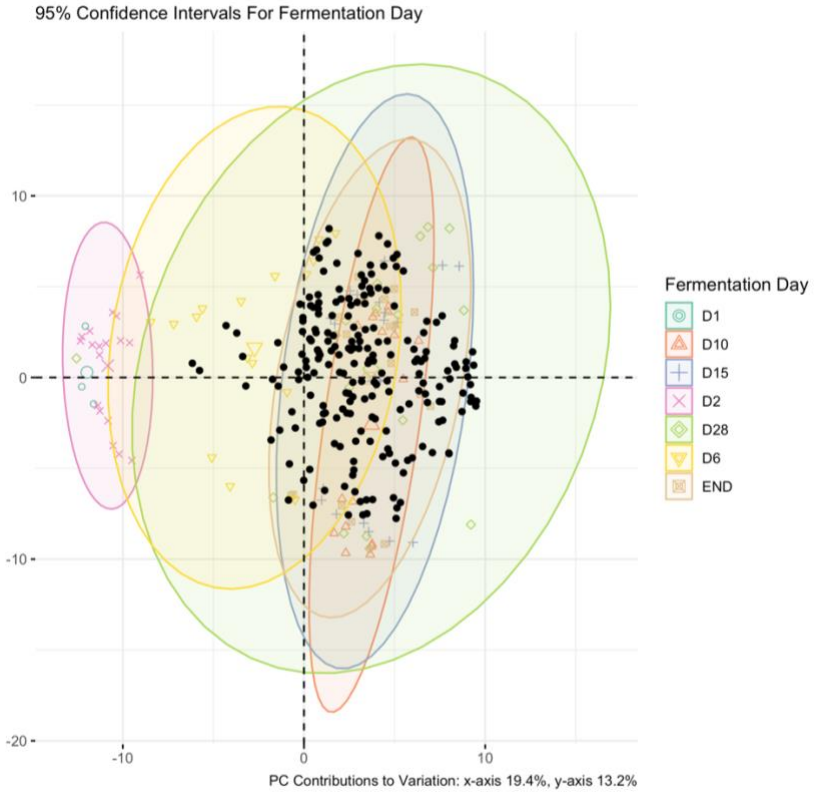


Figure 23. 2017 Wine Target Initial pH PCA: LC-MS Metabolite Biplot

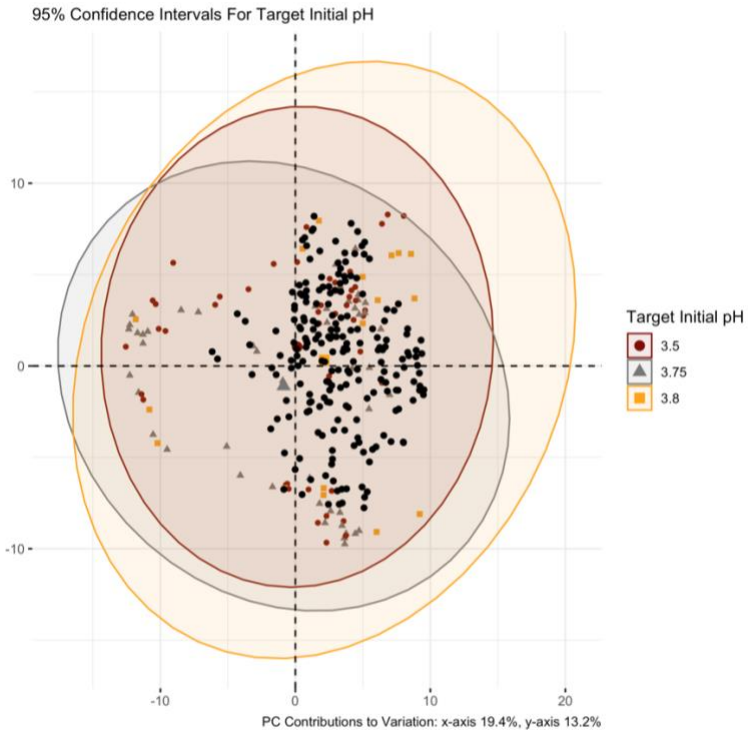
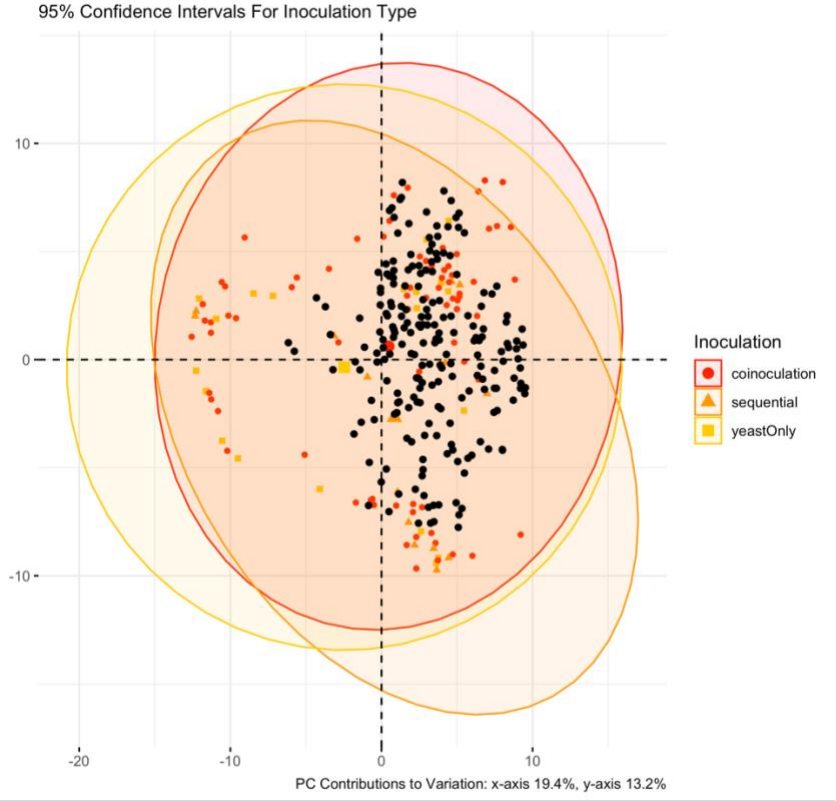


Figure 24. 2017 Wine Inoculation Method PCA: LC-MS Metabolite Biplot



7. **Outreach and Education Efforts - Presentations of Research**: List of journals, publications, reports, speeches, posters and other presentations developed for this research. Describe your strategy for communicating research results to end-users and stakeholders.

Preliminary results of this project were presented to the Washington wine industry at a WAVE seminar on 10 July 2019 in Woodinville, WA. Now that the results of the 2017 wines are complete and with the complete results from the 2018 wines we are planning more seminar presentation in WAVE, in WSU VE Extension seminars, and in the Annual meeting of the Washington Winegrowers in 2021. We will prepare manuscripts for scientific papers.

8. **Research Success Statements**: In a few sentences, describe in detail how your research program benefits the wine industry.

These research findings show practical ways to manage high pH musts. The relatively small pH adjustments compared to the target pHs nevertheless resulted in significant sensory differences in the wines and equally significant differences in the metabolites (aroma compounds). This shows that pH adjustment is a powerful tool to affect the wine sensory profile. This project demonstrated that the addition of yeast and bacteria starter is a very effective tool to manage native yeast and bacteria populations and to avoid spoilage problems in high pH musts. The added starter cultures strongly dominated the native yeast and bacteria all through the fermentation. These diverse yeast and bacteria populations, inoculated vs spontaneous, and different populations based on pH produced clear significant differences in the wine composition

as analyzed by LC (non-volatile compounds) and by GC (volatile compounds). The sensory analysis showed a similar separation of the wines based on starting pH and use of starter culture or fermentation with native microorganisms.
For high pH musts (pH 3.6-4.3) acid adjustments and use of yeast and/or bacteria starter cultures have significant impact on the metabolites formed during fermentation and on the perceived flavors. This was seen even in the wines of this study where the pH adjustments were rather small. The results indicate that it would be beneficial to explore these winemaking tools more to help differentiate our wines. Yeast and bacteria starter cultures are a great tool to work with high pH musts if a winemaker does not or cannot make large acid adjustments using tartaric and/or malic acid or acid wine.
The detailed microbial genomic data and metabolic data are providing deep insight into the fermentations pathways as affected by different must pH and starter culture additions. We were able to see that specific microbial populations form specific metabolite profiles. Additional research is needed to identify and quantify specific flavor impact compounds and the metabolic links of specific microorganisms and to major flavor impact compounds.

9. **Funds Status**: Include a general summary of how funds were spent. (Copy of budget tables is acceptable if accurate.)

As of 29 June 2020, there remained $2,042.41 in the project account. We would like permission to use this remainder to pay wages for graduate student Lindsey Kornowske during the summer of 2020 while she continues to work on the wine metabolite data and the sequencing data from the 2018 wines. She will also be helping to prepare manuscripts publication and slide shows for industry presentations.

Please submit continuing project report with your proposal to the WSU-ARC Grants Coordinator at: arcgrants@wsu.edu. Final reports should be sent to: arcgrants@wsu.edu.

Citations

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Supplemental Data

Table S1. Summary of Illumine MiSeq reads generated and OTUs observed from wine must samples with initial pH adjusted with acid wine or organic acids

	Day1					Day 2					Day 10				
	Cont.	**AW3.75**	**AW3.5**	**TA3.5**	**MA3.5**	**Cont.**	**AW3.75**	**AW3.5**	**TA3.5**	**MA3.5**	**Cont.**	**AW3.75**	**AW3.5**	**TA3.5**	**MA3.5**
Raw reads	91842	106647	83922	87989	88417	106324	105598	153825	99392	96497	86353	86363	88731	77408	92307
Quality filtered reads	29625	42754	39396	35668	43956	22985	30517	53907	27335	30811	72906	70942	75705	61365	82940
OTUs	1517	2035	2103	2105	2193	1386	1446	1988	1416	1942	188	261	229	279	273
OTUs Changes (%)	100%	100%	100%	100%	100%	91.4%	71.1%	94.4%	67.3%	88.6%	12.4%	12.8%	10.9%	13.3%	12.4%

	Day 15					Day 28					MLF_END				
	Cont.	**AW3.75**	**AW3.5**	**TA3.5**	**MA3.5**	**Cont.**	**AW3.75**	**AW3.5**	**TA3.5**	**MA3.5**	**Cont.**	**AW3.75**	**AW3.5**	**TA3.5**	**MA3.5**
Raw reads	90909	82348	114630	79307	106065	95282	72785	89620	77040	94607	88974	82919	97002	85757	92158

Quality filtered reads	80812	74556	107509	71216	98366	88040	68558	83694	69255	89104	83840	77506	91106	79647	87594
OTUs	147	171	103	148	187	170	150	109	242	92	123	147	127	208	130
OTUs Changes (%)	9.7%	8.4%	4.9%	7.0%	8.5%	11.2%	7.4%	5.2%	11.5%	4.2%	8.1%	7.2%	6.0%	9.9%	5.9%

Table S2_a. 16S and ITS sequencing samples lists: no yeast, no MLB

	Rep1			**Rep2**			**R1**	**R2**
Sample	**DNA Conc. (ng/uL)**	**Volume (µL)**	**DNA quantity (ng)**	**DNA Conc. (ng/uL)**	**Volume (µL)**	**DNA quantity (ng)**	**PCR (ul)**	**PCR (ul)**
Control_NN_D1	2.28	30.00	68.40	3.09	28.00	86.52	yes	yes
Control_NN_D2	3.34	36.00	120.24	2.77	36.00	99.72	yes	yes
Control_NN_D3	1.71	36.00	61.56	1.37	36.00	49.32	yes	yes
Control_NN_D6	25.20	80.00	2016.00	47.50	80.00	3800.00	yes	yes
Control_NN_D10	20.50	200.00	4100.00	14.40	140.00	2016.00	yes	yes
Control_NN_D15	20.40	170.00	3468.00	10.20	90.00	918.00	yes	yes
Control_NN_D20	4.03	40.00	161.20	6.34	40.00	253.60	yes	yes
Control_NN_D25	17.50	45.00	787.50	13.20	40.00	528.00	yes	yes
Control_NN_D35	21.20	40.00	848.00	24.20	35.00	847.00	yes	yes
AW3.75_NN_D1	10.40	23.00	239.20	15.50	20.00	310.00	yes	yes
AW3.75_NN_D2	2.58	30.00	77.40	2.62	30.00	78.60	yes	yes
AW3.75_NN_D3	0.84	30.00	25.32	0.90	30.00	26.94	yes	yes
AW3.75_NN_D6	44.40	75.00	3330.00	5.31	120.00	637.20	yes	yes
AW3.75_NN_D10	14.00	160.00	2240.00	29.70	150.00	4455.00	yes	yes
AW3.75_NN_D15	6.58	90.00	592.20	17.20	85.00	1462.00	yes	yes
AW3.75_NN_D20	11.00	35.00	385.00	12.80	35.00	448.00	yes	yes
AW3.75_NN_D25	20.00	60.00	1200.00	14.90	60.00	894.00	yes	yes
AW3.75_NN_D35	19.90	35.00	696.50	45.30	35.00	1585.50	yes	yes
AW3.5_NN_D1	10.50	23.00	241.50	6.76	25.00	169.00	yes	yes
AW3.5_NN_D2	1.31	56.00	73.36	1.42	56.00	79.52	yes	yes
AW3.5_NN_D3	1.33	36.00	47.88	1.37	36.00	49.32	yes	yes
AW3.5_NN_D6	8.78	120.00	1053.60	12.40	120.00	1488.00	yes	yes
AW3.5_NN_D10	19.20	150.00	2880.00	22.00	70.00	1540.00	yes	yes
AW3.5_NN_D15	25.30	28.00	708.40	24.50	28.00	686.00	yes	yes
AW3.5_NN_D20	4.94	33.00	163.02	4.94	38.00	187.72	yes	yes
AW3.5_NN_D25	29.50	30.00	885.00	15.70	40.00	628.00	yes	yes
AW3.5_NN_D35	22.30	35.00	780.50	12.70	35.00	444.50	yes	yes

Table S2_b. 16S and ITS sequencing samples lists: with yeast, no MLB

	Rep1			**Rep2**			**R1**	**R2**
Sample	**DNA Conc. (ng/uL)**	**Volume (µL)**	**DNA quantity (ng)**	**DNA Conc. (ng/uL)**	**Volume (µL)**	**DNA quantity (ng)**	**PCR (ul)**	**PCR (ul)**

Control_+N_D1	4.52	38.00	171.76	4.45	40.00	178.00	yes	yes
Control_+N_D2	1.94	35.00	67.90	1.89	33.00	62.37	yes	yes
Control_+N_D3	22.20	125.00	2775.00	19.70	125.00	2462.50	yes	yes
Control_+N_D6	13.00	80.00	1040.00	18.70	75.00	1402.50	yes	yes
Control_+N_D20	4.37	20.00	87.40	3.45	42.00	144.90	yes	yes
Control_+N_D25	28.90	40.00	1156.00	25.30	40.00	1012.00	yes	yes
Control_+N_D35	16.70	35.00	584.50	8.68	40.00	347.20	yes	yes
AW3.75_+N_D1	10.90	23.00	250.70	13.40	19.00	254.60	yes	yes
AW3.75_+N_D2	2.97	27.00	80.19	2.60	27.00	70.20	yes	yes
AW3.75_+N_D3	10.80	80.00	864.00	12.40	80.00	992.00	yes	yes
AW3.75_+N_D6	13.70	110.00	1507.00	15.00	160.00	2400.00	yes	yes
AW3.75_+N_D20	1.04	52.00	54.08	0.53	52.00	27.35	yes	yes
AW3.75_+N_D25	4.66	60.00	279.60	1.49	60.00	89.40	yes	yes
AW3.75_+N_D35	24.50	35.00	857.50	19.30	40.00	772.00	yes	yes
AW3.5_+N_D1	2.50	50.00	125.00	3.20	50.00	160.00	yes	yes
AW3.5_+N_D2	1.65	47.00	77.55	1.88	48.00	90.24	yes	yes
AW3.5_+N_D3	7.04	170.00	1196.80	6.28	170.00	1067.60	yes	yes
AW3.5_+N_D6	17.30	170.00	2941.00	13.50	170.00	2295.00	yes	yes
AW3.5_+N_D20	1.24	49.00	60.76	3.10	48.00	148.80	yes	yes
AW3.5_+N_D25	3.45	58.00	200.10	1.23	58.00	71.34	yes	yes
AW3.5_+N_D35	7.24	35.00	253.40	24.20	35.00	847.00	yes	yes
TA3.75_+N_D1	2.17	190.00	412.30	1.64	180.00	295.20	yes	yes
TA3.75_+N_D2	0.49	62.00	30.07	0.71	62.00	44.14	yes	yes
TA3.75_+N_D3	2.46	45.00	110.70	4.48	50.00	224.00	yes	yes
TA3.75_+N_D6	18.90	120.00	2268.00	13.70	170.00	2329.00	yes	yes
TA3.75_+N_D20	5.53	45.00	248.85	11.60	45.00	522.00	yes	yes
TA3.75_+N_D25	10.20	90.00	918.00	19.40	45.00	873.00	yes	yes
TA3.75_+N_D35	17.90	45.00	805.50	16.90	40.00	676.00	yes	yes
TA3.5_+N_D1	0.84	370.00	311.54	0.67	350.00	235.55	yes	yes
TA3.5_+N_D2	0.98	60.00	58.74	1.31	57.00	74.67	yes	yes
TA3.5_+N_D3	3.68	190.00	699.20	3.35	170.00	569.50	yes	yes
TA3.5_+N_D6	16.10	300.00	4830.00	16.40	250.00	4100.00	yes	yes
TA3.5_+N_D20	15.10	80.00	1208.00	10.90	40.00	436.00	yes	yes
TA3.5_+N_D25	17.00	40.00	680.00	17.80	40.00	712.00	yes	yes
TA3.5_+N_D35	14.30	57.00	815.10	14.80	57.00	843.60	yes	yes

Table S2_c. 16S and ITS sequencing samples lists: with yeast, with MLB

	Rep1			**Rep2**			**R1**	**R2**
Sample	**DNA Conc. (ng/uL)**	**Volume (µL)**	**DNA quantity (ng)**	**DNA Conc. (ng/uL)**	**Volume (µL)**	**DNA quantity (ng)**	**PCR (ul)**	**PCR (ul)**
Control_++_D1	3.53	18.00	63.54	4.34	18.00	78.12	yes	yes
Control_++_D2	0.97	37.00	35.89	0.98	30.00	29.40	yes	yes

Control_++_D3	23.90	125.00	2987.50	17.20	125.00	2150.00	yes	yes
Control_++_D4	11.30	125.00	1412.50	13.20	125.00	1650.00	yes	yes
Control_++_D6	17.60	78.00	1372.80	11.00	72.00	792.00	yes	yes
Control_++_D10	28.30	90.00	2547.00	33.50	120.00	4020.00	yes	yes
Control_++_D35	6.60	35.00	231.00	21.60	40.00	864.00	yes	yes
AW3.75_++_D1	8.97	20.00	179.40	10.10	20.00	202.00	yes	yes
AW3.75_++_D2	1.60	28.00	44.80	1.55	24.00	37.20	yes	yes
AW3.75_++_D3	20.00	40.00	800.00	13.50	85.00	1147.50	yes	yes
AW3.75_++_D4	6.08	195.00	1185.60	5.41	180.00	973.80	yes	yes
AW3.75_++_D6	7.89	180.00	1420.20	10.10	160.00	1616.00	yes	yes
AW3.75_++_D10	59.80	80.00	4784.00	66.00	80.00	5280.00	yes	yes
AW3.75_++_D35	3.21	75.00	240.75	7.61	75.00	570.75	yes	yes
AW3.5_++_D1	2.70	58.00	156.60	5.25	58.00	304.50	yes	yes
AW3.5_++_D2	1.61	56.00	90.16	2.03	54.00	109.62	yes	yes
AW3.5_++_D3	7.05	90.00	634.50	5.75	85.00	488.75	yes	yes
AW3.5_++_D4	3.15	180.00	567.00	3.70	180.00	666.00	yes	yes
AW3.5_++_D6	6.95	170.00	1181.50	9.87	150.00	1480.50	yes	yes
AW3.5_++_D10	59.40	80.00	4752.00	80.80	35.00	2828.00	yes	yes
AW3.5_++_D35	8.78	35.00	307.30	5.64	35.00	197.40	yes	yes
TA3.75_++_D1	1.38	190.00	262.20	1.14	180.00	205.20	yes	yes
TA3.75_++_D2	0.93	62.00	57.66	1.18	64.00	75.52	yes	yes
TA3.75_++_D3	9.07	45.00	408.15	9.11	50.00	455.50	yes	yes
TA3.75_++_D4	5.89	170.00	1001.30	7.24	95.00	687.80	yes	yes
TA3.75_++_D6	3.59	130.00	466.70	11.60	145.00	1682.00	yes	yes
TA3.75_++_D10	25.60	130.00	3328.00	24.10	100.00	2410.00	yes	yes
TA3.75_++_D35	12.30	42.00	516.60	16.40	40.00	656.00	yes	yes
TA3.5_++_D1	0.56	450.00	252.00	0.92	200.00	184.00	yes	yes
TA3.5_++_D2	0.76	56.00	42.56	0.70	56.00	39.20	yes	yes
TA3.5_++_D3	2.83	140.00	396.20	3.92	140.00	548.80	yes	yes
TA3.5_++_D4	3.52	200.00	704.00	4.10	200.00	820.00	yes	yes
TA3.5_++_D6	15.00	260.00	3900.00	28.50	250.00	7125.00	yes	yes
TA3.5_++_D10	10.20	90.00	918.00	12.70	80.00	1016.00	yes	yes
TA3.5_++_D35	9.12	40.00	364.80	6.06	42.00	254.52	yes	yes

Table S3. Aroma Standards, Preparation, and Description for the 2018 Wine Sensory Trial

Aroma Standard[1]	Preparation	Description
Dried Fruit	2 SunSweet© prunes, 8-10 SunMaid© raisins	Aromas of raisins and prunes.
Blackberry	1 tsp blackberry jam	The aroma of blackberry jam.
Cassis	1 oz Kirkland brand Crème de Cassis	The aroma of cassis liquor.
Cherry	1 tsp cherry jam	The aroma of cherry jam.
Strawberry	1 tsp strawberry jam	The aroma of strawberry jam
Artificial Fruit	8 red jujubes candies	A confectionary aroma associated with candy or perfume
Stone Fruit	1 tbsp apricot jam	Aromas of apricot or other pit-containing tree-grown fruits
Floral	8 Violet and Lilac "Jujubes" candies	Aromas of violet, rose, or other blossoms.
Baking Spice	½ tsp anise, ½ tsp whole cloves	Aromas of clove, anise, and vanilla.
Black Tea	Steep 1 black Lipton Brand tea bag for 2 min	The aroma of Lipton black tea.
Black Pepper	1 tsp fresh ground black pepper	The aroma of black pepper.
White Pepper	1 tsp white pepper	An earthy, animal-like aroma associated with white pepper spice
Yeasty	Pinch of baker's yeast	A cereal, lactic aroma associated with rehydrated baker's yeast.
Smokey/Oak	French oak large chips, Medium Toast, steeped for > 1 week	Aromas of smoke, toasted oak, and pipe tobacco.
Chemical	1 oz Vodka	A pungency associated with the aroma of ethanol, ethyl acetate, or solvent.
Sulfur/Burnt Match	2 matches, struck and extinguished into 2 oz Franzia© Chillable Red	A sulfurous aroma associated with the smell of burnt matches.
Asparagus	1 oz of canned asparagus water	A vegetal aroma associated with canned asparagus.
Green Bell Pepper	1" piece fresh cut green bell pepper	A fresh vegetal aroma associated with green bell peppers
Green Olive	1 oz brine and 1 olive (Lindsay brand manzanita olives)	A vegetal and briny aroma associated with green olives.