Washington State Grape and Wine Research Program
FINAL REPORT
2022-23 Funding Cycle

Project Title: Impact of Yeast and Malolactic Bacteria on Wine Flavor Precursors

Principal Investigator: Hailan Piao**,** Washington State University, Richland

Co-PI: Thomas Henick-Kling, Thomas Collins, James Harbertson, WSU-Richland

Summary: This research project aimed to deepen our understanding of how wine yeast and malolactic bacteria influence the expression of smoke taint in wine. Smoke-taint compounds, released during fermentation and aging, are largely linked to sugars, forming glycosides. Wine yeast and bacteria possess glycosidase enzymes capable of breaking these glycosides, liberating active aroma compounds. Genetic analysis suggests that *Saccharomyces cerevisiae* yeast potentially have numerous glycosidases (48 at the time of this report). However, it is unclear which enzymes are active during fermentation, which aroma precursors are hydrolyzed, and their impact on wine aroma. This study seeks to identify yeast and malolactic bacteria (MLB) strains that could reduce undesirable aromas in smoke-exposed wine. Selected yeast and bacteria could be used to hydrolyze specific smoke-taint glycosides and not release desirable aroma glycosides. This would allow us to remove the free, undesirable compounds via reverse osmosis and retain desirable glycosides. Some yeast and bacteria also could enhance desirable aromas in wines. The project involved various scale fermentations and extensive chemical analyses in elucidating the glycosidic activity of different wine yeast and bacteria strains and their specific activity on wine glycosides.

We wanted to see how diverse the *Saccharomyces cerevisiae* yeast glycosidic activity is in their action against wine aroma glycosides. In the first year, 27 *S. cerevisiae* yeast and 18 *Oenococcus oeni* (MLB) bacteria strains were obtained from the University of California, Davis, culture collection and screened. These strains originated in various countries and diverse wines. A flavor extract containing glycosides was prepared from a smoke-tainted wine, and pre-screening tests were conducted to assess the fermentation ability of the various strains in the model wine with the wine glycoside extract added. Ten yeast and ten bacteria strains were selected based on their fermentation performance. Most yeast strains showed good fermentation activity in the synthetic medium, while only a few bacteria strains could conduct malolactic fermentation in the synthetic wine with flavor extracts. Approximately 1,550 compounds were identified in yeast fermentation, including 110-120 glycoside compounds. Isomer analysis revealed 95 compound isomers across all the samples, and 27 out of 95 compounds were found exclusively by yeast fermentation. A similar analysis of malolactic fermentation detected around 2,650 compounds, but only a small number of glycoside compounds were found. Further insight observation revealed that 16 and 7 smoke taint glycosides were able to be hydrolyzed by yeast and bacteria glycosidase, respectively.

In the second year, we evaluated the glycosidic activity of five commercial yeast and five commercial bacteria strains for their activity in wine fermentations. The comparison of fermentations with smoke-exposed fruit and non-smoke-exposed fruit allowed us to see whether the glycosidic activity of the selected yeast and bacteria varied in the presence and absence of smoke-taint compounds. Five commercial yeast strains (Lalvin EC1118, QA23, GRE, D254, 58W3) and five bacteria strains (Lalvin 31, O-Mega, Beta, Alpha, K54H) were used. The

sequential malolactic fermentation allowed us to see which aroma precursors were cleaved by yeast and which were cleaved by bacteria. The alcoholic fermentation progressed at the same rate for both types of grapes, and all yeast strains completed fermentation within 7 days. Malolactic fermentation was similar for both, smoke-exposed and non-smoke-exposed grapes, with all wines completing the process within 21 to 26 days. Metabolite analysis was conducted to analyze aroma compounds released by yeast and bacteria strains. Subsequent observations of yeast glycosidase activity revealed that GRE and QA23 were the most effective strains for hydrolyzing glycosides (94 and 120, respectively), while EC1118 and D254 demonstrated intermediate capability in cleaving glycosides (47 and 33, respectively), and 58W3 showed to be the least effective (14 glycosides). Individual yeasts exhibited similar degrees of glycosidase activities on smoke-tainted compounds, with 56, 41, 21, 17, and 8 glycosides cleaved by QA23, GRE, EC1118, D254, and 58W3, respectively. Following an analysis of the ability of malolactic bacteria (MLB) to cleave the glycosides in combination with individual yeast strains compared to wines with no MLB, the most effective combination was found to be GRE yeast and Beta MLB, capable of cleaving 131 total glycosides and 62 smoke-taint compounds. Each MLB's glycosidase activity varied with different yeast strain combinations. The least effective combination, QA23 and Alpha, only hydrolyzed a total of 8 glycosides, with 6 of them being smoke-tainted. Considering the glycosidase activity on both total and smoke-taint compounds of individual yeast strains and their corresponding MLB combinations, we selected GRE (high), EC1118 (intermediate), and 58W3 (low) - three yeast strains exhibiting different levels of glycosidase activity - and chose Beta, Alpha, and Lalvin 31 for the MLF. Analysis of glycoside compounds revealed the presence of some smoke-tainted compounds in non-smoke-exposed wine, likely due to natural wildfire smoke exposure. Different yeast strains showed varying effectiveness on smoke-taint and non-smoke-taint glycosides. The bacteria tested exhibited comparable glycosidic activity.

In the third year, full-scale wine fermentations were conducted using three selected commercial yeast strains (EC1118, 58W3, GRE) and three MLB strains (Lalvin31, Beta, and Alpha). Natural wildfire exposed Cabernet Sauvignon grapes were fermented in 200 L fermenters. In full-scale fermentation, 58W3 showed a similar level of glycosidase activity as EC1118 and GRE, cleaved with 12, 11, and 9 smoke-taint glycosides by 58W3, GRE, and EC1118, respectively. Among these smoke-tainted glycosides, 7 compounds were able to be hydrolyzed by all three yeast strains. In subsequent MLF, the three MLB strains showed variable activity in different yeast-fermented wines; however, the degree of variation is less significant compared to bucket fermentation. Overall, the three MLB strains demonstrated higher efficiency in 58W3 and GRE fermented wines, while they were less effective in EC1118 fermented wine. Unlike bucket fermentation, glycosidase activity in MLB surpasses yeast.

Over three years, the study assessed the glycosidic activity of various yeast and MLB strains in wine fermentation, focusing on smoke-tainted compounds. The initial preliminary study demonstrated strain-specific glycosidase activity for unique compounds, such as syringol/vanillyl alcohol sinapoyl, guaiacol fertaroyl, and cresol caftaroylhexoside, by UCD514 (yeast), UCD525 (yeast), and UCD199 (MLB), respectively. Subsequent evaluations of five commercial strains using small-scale fermentation further revealed varied glycosidic activity among different yeast and MLB strains, both in smoke-tainted and non-smoke-tainted glycosides. In full-scale fermentation, selected yeast and bacteria strains displayed variable glycosidic activity, with MLB strains demonstrating higher efficiency in certain yeast-fermented wines, particularly with GRE and 58W3 strains. For the full-scale fermentation, we used natural wildfire-exposed grapes, which, compared to controlled in-house smoke-exposed grapes, exhibited less extensive exposure, resulting in fewer detected smoke-tainted compounds in full-scale fermentation. Overall, the study emphasized the complexity of glycosidic activity in wine fermentation, influenced by both yeast and bacteria strains.

Impact of Yeast and Malolactic Bacteria on Wine Flavor Precursors	
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	

5.1. Project Year 1 Objectives and experiments

In the first year (2020-2021), we proposed to screen a wide range of wine yeast and malolactic bacteria (MLB) strains for their glycosidic activity to estimate how wide ranging this activity is and to help find effective strains that could reduce undesired flavors and possibly enhance desired wine flavors. We acquired 27 wine yeast (*Saccharomyces cerevisiae*) and 18 MLB strains (*Oenococcus oeni*) from the University of California, Davis culture collection to initiate this project. We used synthetic grape juice and synthetic wine containing 20% flavor extract for the yeast and MLB strains, respectively. The glycoside flavor extract was prepared from a smoke-tainted wine with reverse osmosis filtration, which filtered out all compounds larger than the targeted glycoside flavor precursors.

Each 125 mL of synthetic grape juice ferment was inoculated with a precultured yeast strain (10^7 cells/mL). All samples were incubated at 20 °C (68°F), and the sugar content of each sample was measured periodically until the completion of alcoholic fermentation.

Each 50 mL of synthetic wine was inoculated with a precultured MLB strain (10^8 cells/mL). All wine samples were incubated at 25 °C (77 °F), and the L-malic acid content of each sample was measured periodically until completion of malolactic fermentation.

At the end of alcoholic and malolactic fermentation, all the wine samples were frozen until analysis for free and bound forms of flavor compounds. Synthetic grape juice and synthetic wine were frozen as a control for yeast and MLB screening, respectively.

Samples were analyzed for free and bound forms of flavor compounds using LC-MS (King et al., 2014). All the data were analyzed using Agilent's MassHunter and Mass Profiler Professional software packages.

5.2. Project Year 2 objectives and experiments

In the second year (2021-2022), we evaluated glycosidic activities in wine fermentations with smoke-exposed and non-smoke-exposed Merlot grapes (control). Due to the limited amount of fruit available, these fermentations were carried out in buckets (20 L) with 5 commercial yeast (Lalvin EC1118, QA23, GRE, D254, 58W3) strains. The yeast and MLB strains were selected based on the commercial experience available of their glycosidic activity in wine. We added 50 mg/L of SO_2 to the grapes at crush. The grape must pH was 3.6, and the tartaric acid content was about 3.2 $\pm$ 0.1. The pH adjustments are made to help avoid spontaneous MLF in these musts. Sugar content was adjusted to 22 Brix, and yeast available nutrients were adjusted to 235-240 mg/L. Fermentation with each selected yeast strain was carried out in triplicate. At the completion of alcoholic fermentation, each bucket of wine was split into five 250 mL glass bottles for malolactic fermentation (MLF) with the selected 5 commercial malolactic bacteria (MLB) strains. We also collected 1L of each wine sample at the completion of alcoholic fermentation for evaluating the impact of yeast strains only.

The remaining wine from each yeast fermentation was split into 250 mL glass jars, inoculated with 5 selected commercial MLB (Lalvin 31, O-Mega, Beta, Alpha, K54H) stains, and incubated at 20°C. The L-malic acid content of each sample was measured periodically until the completion of malolactic fermentation. At the end of malolactic fermentation, samples were taken and frozen for analysis of aroma compounds.
Samples were analyzed for free and bound forms of flavor compounds using the LC-MS method (King et al., 2014), Agilent 1290 Infinity II UHPLC coupled with an Agilent 6545 QTOF-MS system. Data were analyzed using Agilent's MassHunter Qualitative Analysis software (v10.0) and Mass Profiler Professional software.

5.3. Project Year 3 Objectives and experiments

In the third year (2022-2023), we evaluated 3 commercial yeast (EC1118, 58W3, GRE) strains for their glycosidic activity under full-scale wine fermentations, using Cabernet Sauvignon grapes that had been exposed to natural wildfire smoke and fermented wines in 150 L batches in triplicates. We chose individual yeast strains with strong (GRE), moderate (EC1118), and low (58W) glycosidase activity based on the screening tests carried out in the fall of 2021 in the bucket fermentation of smoke-tainted and non-smoke-tainted (control) grapes. Yeast starter cultures were added to the manufacturer's instructions. Yeast Available Nitrogen (YAN) was adjusted to 250 mg/L with a combination of yeast organic nitrogen and diammonium hydrogen phosphate (DAP).

To separate the action of the yeast and bacteria, we used sequential alcoholic and malolactic fermentation. Three Cabernet Sauvignon wines fermented with 3 yeasts in triplicate, each followed by 3 malolactic bacteria. We took samples after alcoholic fermentation to evaluate the impact of yeast strains. The remaining wines from each ferment were divided into 20 L kegs for malolactic fermentation (MLF) with the selected 3 MLB (Lalvin31, Beta, and Alpha) strains. The malolactic fermentations were carried out at 20°C. At the end of malolactic fermentation, 100 mL of each wine was frozen for the following analysis of flavor compounds by LCMS. Again, the MLF with each selected strain was also carried out in triplicate.

Samples were analyzed for free and bound forms of aroma compounds using the LC-MS method (King et al., 2014), Agilent 1290 Infinity II UHPLC coupled with an Agilent 6545 QTOF-MS system. Data were analyzed using Agilent's MassHunter Qualitative Analysis software (v10.0) and Mass Profiler Professional software.

6.1. Summary of mini-scale fermentation

6.1.1. Prescreening yeast and bacteria strains

To select various characteristic yeast and bacteria strains containing glycosidase acidity, we first conducted prescreening fermentations using synthetic grape juice and synthetic wine, respectively. We measured each yeast strain's alcoholic fermentation activity periodically by measuring glucose and fructose content. The alcoholic fermentation results showed that most of the yeast strains tested in the small-scale fermentation were

actively fermenting except one strain, UCD158. Since most of the yeast strains were effectively fermented, we selected 10 yeast strains based on the diverse strain descriptions and origins (Table S1).

To examine bacteria strains' glycosidase enzyme activity, we also selected candidate strains through prescreening malolactic fermentation activity in synthetic wine. Through the prescreening, 10 bacteria strains showed good malolactic fermentation activity. We selected these 10 bacteria strains (Table S2) and next applied them to mini-scale malolactic fermentations for the glycosidase activity assay with the glycoside extracts added.

6.1.2. Mini-scale fermentations

To evaluate yeast strains' glycosidase activity on the smoke-tainted glycoside precursor compounds, we conducted mini-scale alcoholic fermentation in the synthetic grape juice containing glycoside extract. We included a control without yeast added. Most of the yeast strains were able to complete fermentation within 19 days. UCD522 and UCD557 needed an additional 10 days to complete, and in the UCD2784 fermentation, 18% of fructose remained by day 31 (**Figure S1**).

To evaluate bacteria strains glycosidase activity on the smoke-tainted glycoside precursor compounds, we performed mini-scale malolactic fermentation in the synthetic wine containing flavor extract. Synthetic wine without bacteria addition was used as a control. Unfortunately, only a few bacteria strains were able to conduct malolactic fermentation in the mini-scale ferments in synthetic wine medium containing flavor extracts. We conducted experiments twice and collected malolactic fermentation samples from 5 different bacterial strains. In the first test, 3 strains (UCD167, UCD176, and UCD224) were able to conduct malolactic fermentation, and during the second test, UCD139 was able to conduct malolactic fermentation, but not the UCD167.

6.1.3. Glycoside analysis

After the completion of mini-scale alcoholic fermentation in the synthetic juice and malolactic fermentation in the synthetic wine, the wines were analyzed by LC-MS for the flavor compounds modified by yeast or bacteria glycosidase enzymes.

Through qualitative and quantitative analysis, we identified about 1,550 compounds across the yeast fermentations which were modified by some of the yeast and bacteria. To further determine glycoside compounds, we applied an in-house glycoside database and detected 147 across all the samples, and about 110-120 glycoside compounds in individual yeast fermentation, among them 16 smoke-taint glycosides and 15 non-smoke-taint glycosides were hydrolyzed by 10 different yeast strains (**Table 1**). Some smoke-tainted glycosides such as guaiacol galloylpentoside, guaiacol glutathionylpentosylhexoside, were cleaved by all 10 tested yeast strains. While certain smoke-tainted glycosides were only cleaved by some strains, i.e. 4-methyl syringol hexuronide and syringol/vanillyl alcohol sinapoyl only by UCD514. For the mini-scale fermentation, we added filtered flavor extract, which reduced the number of compounds and resulted in a limited number of compounds being detected. The results showed that different yeast strains have diverse glycosidase activity.

Qualitative and quantitative analysis with the LC-MS raw data identified about 2,650 compounds across all the malolactic fermentation samples, however after further screening for the glycoside compounds using in-house glycosides database, we observed that 7 smoke-taint and 17 non-smoke-taint glycosides were hydrolyzed by 6 MLB strains with variable activity (**Table 2**). We observed non-strain specific smoke-taint compound, guaiacol tartaroylpentoside, was cleaved by all the MLB strains, while cresol caftaroylhexoside was only targeted by UCD199.

Principal component analysis (PCA) was performed to compare the yeast strains glycosidase activities against control (No yeast) samples. The PCA plot shows that all the tested yeast strains have similar glycolytic activity in the synthetic grape juice, differing significantly from the control (**Figure S2**). Similarly, we used PCA analysis for the malolactic fermentation samples. This showed two groups of bacteria, bacteria strains (UCD167 and UCD176) had a different glycoside activity from the other 3 strains (UCD139, UCD199, UCD22). (**Figure**

S3). These results indicate that yeast and bacterial strains could effectively cleave smoke-tainted glycoside precursor compounds and potential tools to help mitigate smoke-tainted compounds during wine fermentation.

Table 1. Smoke-taint and non-smoke-taint glycosides identified during mini-scale fermentation with 10 yeast strains

	UCD506	UCD509	UCD512	UCD514	UCD522	UCD525	UCD557	UCD932	UCD2784	UCD2790	No yeast
Compounds	**Smoke-taint glycoconjugates**										
Number of compounds	**7**	**8**	**9**	**11**	**3**	**10**	**7**	**7**	**7**	**7**	**0**
4-ethylguaiacol hexonate	+	+	-	-	+	-	+	-	-	-	+
4-ethylguaiacol hexosylhexoside	+	-	+	+	+	+	+	+	-	+	+
4-methyl guaiacol caftaroyl	-	-	-	-	-	-	-	-	+	-	+
4-methyl guaiacol coumaroylpentoside	+	+	+	-	+	-	+	+	-	-	+
4-methyl guaiacol coutaroyl	-	+	-	+	+	+	-	+	+	+	+
4-methyl guaiacol tartaroyl	-	+	-	-	+	-	+	+	+	+	+
4-methyl guaiacol tartaroyl	-	-	-	+	+	+	+	-	-	-	+
4-methyl syringol fertaroylhexoside	+	+	-	-	+	-	+	-	+	+	+
4-methyl syringol hexuronide	+	+	+	-	+	+	+	+	+	+	+
4-methyl syringol hexuronide	+	-	+	-	+	-	-	+	+	+	+
guaiacol caftaroylpentoside	-	-	+	-	+	+	-	+	-	+	+
guaiacol fertaroyl	+	+	+	+	+	-	+	+	+	+	+
guaiacol galloylpentoside	-	-	-	-	-	-	-	-	-	-	+
guaiacol glutathionylpentosylhexoside	-	-	-	-	-	-	-	-	-	-	+
guaiacol malonyl	+	-	-	+	+	-	-	-	+	-	+
syringol/vanillyl alcohol sinapoyl	+	+	+	-	+	+	+	+	+	+	+
Compounds	**Non-smoke-taint glycoconjugates**										
Number of compounds	**10**	**8**	**10**	**10**	**10**	**11**	**11**	**7**	**10**	**7**	**0**
4-ethyl syringol hexuronide	+	+	+	+	+	-	+	+	+	+	+
4-vinyl phenol malyl	+	+	+	-	+	-	-	+	-	+	+
4-vinylguaiacol feruloylpentosylhexoside	-	-	-	-	-	-	-	-	-	-	+
4-vinylguaiacol galloylpentoside	+	+	+	+	+	+	+	+	-	+	+
4-vinylguaiacol malyl	-	-	-	-	-	-	-	-	-	-	+
ethyl vanillin galloylhexoside	+	+	+	+	-	+	-	+	-	+	+
ethyl vanillin hexuronide	-	+	+	-	+	-	+	+	+	+	+
sinapoyl alcohol caftaroyldihexoside	-	-	-	-	-	-	-	-	-	-	+
sinapoyl alcohol hexosyl	-	-	-	-	-	-	+	+	+	+	+
syringyl alcohol caffeoyl	+	+	-	+	-	-	-	+	+	+	+

syringyl alcohol caffeoylhexoside	-	-	-	-	-	-	-	-	-	-	+
syringyl alcohol coumaroyl	-	+	-	+	-	+	-	-	-	+	+
syringyl alcohol coumaroylhexoside	-	-	-	-	-	-	-	-	-	-	+
syringyl alcohol coumaroylhexoside	-	-	-	-	-	-	-	-	-	-	+
vanillin galloylhexoside	-	-	-	-	+	+	-	+	+	-	+

"+" indicates present in the fermentation and not hydrolyzed by yeast, while "-" indicates that hydrolyzed by yeast glycosidase.

Table 2. Smoke-taint and non-smoke-taint glycosides identified during mini-scale MLF with MLB strains

	UCD224	UCD167	UCD176	UCD139	UCD199	No MLB
Compounds	**Smoke-taint glycoconjugates**					
Number of compounds	**3**	**4**	**4**	**3**	**6**	**0**
4-ethylguaiacol sinapoylhexoside	+	-	-	+	+	+
4-methyl guaiacol galloylhexoside	+	+	+	-	-	+
4-methyl syringol sinapoylhexoside	+	+	+	-	-	+
cresol caftaroylhexoside	+	+	+	+	-	+
cresol coumaroyldipentoside	-	-	-	+	-	+
guaiacol tartaroylpentoside	-	-	-	+	-	+
guaiacol tartaroylpentoside	-	-	-	-	-	+
Compounds	**Non-smoke-taint glycoconjugates**					
Number of compounds	**3**	**9**	**10**	**11**	**10**	**0**
4-ethylphenol glutathionyl	+	+	+	-	-	+
4-vinyl catechol coutaroyl	+	-	-	+	+	+
4-vinyl catechol galloylhexoside	+	-	-	-	-	+
4-vinyl phenol coumaroylhexoside	-	+	+	-	-	+
coniferaldehyde malylpentoside	+	-	-	-	+	+
coniferol/4-vinyl syringol feruloyl	-	+	+	-	-	+
sinapaldehyde tartaroylpentoside	+	-	-	+	+	+
sinapoyl alcohol caffeoylpentoside	+	+	+	-	-	+
sinapoyl alcohol maloylpentosylhexoside	+	+	+	-	-	+
sinapoyl alcohol sinapoylhexoside	+	-	-	-	-	+
sinapoyl alcohol tartaroylpentoside	+	+	+	+	-	+
sinapoyl alcohol tartaroylpentoside	+	+	-	+	+	+
syringol/vanillyl alcohol galloyldipentoside	-	+	+	-	-	+
syringyl alcohol tartaroyldihexoside	+	-	-	-	-	+
syringyl alcohol caftaroylpentoside	+	-	-	+	+	+
syringyl alcohol caftaroylpentoside	+	-	-	-	+	+
syringyl alcohol coutaroylhexoside	+	-	-	+	+	+

"+" indicates present in the fermentation and not hydrolyzed by MLB, while "-" indicates that hydrolyzed by MLB glycosidase.

6.2. Summary of bucket fermentations

6.2.1. Bucket fermentation using smoke-exposed and non-smoke-exposed grapes

To evaluate glycosidase activity on flavor compounds, including the smoke-trained glycosides in the real grape must, we conducted 25 L quantity bucket fermentations with five selected commercial yeast (Lalvin EC1118, QA23, GRE, D254, 58W3) strains. Both smoked-exposed and non-smoke-exposed Merlot grapes had the same fermentation rates, and all the yeast strains were able to complete alcoholic fermentation within 7 days.

After the completion of alcoholic fermentation, each wine fermented with the various yeast strains was divided into 5 glass bottles (250 mL) and inoculated with the different bacteria strains. Both, smoke-exposed and non-smoke-exposed wines completed malolactic fermentation in the same manner. To evaluate the glycosidase activities of different bacteria, we used four *O. oeni* strains (Lalvin31, Omega, Beta, and Alpha) and one *Lactiplantibacillus plantarum* (K45H) strain as malolactic bacteria starter culture. Malolactic fermentation was completed in all the wines within 21 to 26 days.

After the completion of bucket fermentation with the smoke-exposed and non-smoke-exposed (control) grapes, samples were collected and analyzed on the LC-MS for glycoside compounds hydrolyzed by the yeast glycosidase enzymes. In total, we analyzed 90 samples for yeast fermentation and 450 samples for malolactic fermentation, which allowed interactions between yeast and bacteria strains.

6.2.2. Glycoside analysis

We identified about 15,000 compounds in all yeast fermentation through qualitative and quantitative analysis, including smoke-exposed and non-smoke-exposed wines. We utilized an in-house glycoside database to determine which glycoside compounds were hydrolyzed by yeast strains and identified about 300-400 glycoside compounds in each yeast fermentation. In-sight observation of yeast glycosidase activity revealed that GRE and QA23 were the most effective strains for hydrolyzing glycosides (94 and 120, respectively), while EC1118 and D254 demonstrated intermediate capability in cleaving glycosides (47 and 33, respectively), and 58W3 showed to be the least effective (14 glycosides). Individual yeasts exhibited similar degrees of glycosidase activities on smoke-taint associated compounds, with 56, 41, 21, 17, and 8 glycosides cleaved by QA23, GRE, EC1118, D254, and 58W3, respectively (**Table 3**). This indicates that we might be able to select yeasts that express smoke-taint less and enhance desired aroma compounds. Conversely, we could ferment with a yeast that liberates most or all smoke-tainted compounds and then remove the free aroma compounds from the wine with filtration and selective adsorbents. All the smoke-tainted compounds listed hydrolyzed by different yeast strains are available upon request.

Each wine underwent primary fermentation with yeast and secondary malolactic fermentation with 5 different bacteria (Lalvin 31, O-Mega, Beta, Alpha, K54H) strains. After completion of malolactic fermentation, we analyzed the metabolites in these wines. Through both qualitative and quantitative assessments, we successfully identified approximately 18,444, 15,326, 17,264, 18,326, and 16,758 compounds in EC1118, 58W3, GRE, D254, QA23 fermented wines, respectively, thus being able to characterize each yeast. To further determine which glycoside

compounds were liberated by bacteria strains, we utilized an in-house glycoside database and identified about 300-450 glycoside compounds in each malolactic fermentation wine. We found some bacteria-specific glycoside compounds that were only presented in the malolactic fermentation wines but not in the control (no MLB) wine. Subsequent evaluation of the ability of malolactic bacteria (MLB) to cleave the glycosides in combination with individual yeast strains compared to wines with no MLB, the most effective combination was found to be GRE yeast and Beta MLB, capable of cleaving 131 total glycosides and 62 smoke-taint associated compounds (**Table 4**). Each MLB's glycosidase activity varied with different yeast strain combinations. The least effective combination, QA23 and Alpha, only hydrolyzed a total of 8 glycosides, with 6 of them being smoke-tainted (**Table 4**). All the smoke-taint compound lists hydrolyzed by different MLBs are available upon request.

Subsequently, we conducted a principal component analysis (PCA) to assess the correlation between yeast strains and glycosidase activities. The PCA plot indicates that in smoke-exposed wines, EC1118 and QA23 exhibit a very similar activity, whereas 58W3 and GRE are correlated on different glycoside activity, and D254 was not consistently associated with either of these two groups **(Figure S4**). With non-smoke-exposed grapes, all the strains demonstrated similar effectiveness without any noticeable differences.

Similarly, we conducted a principal component analysis (PCA) to evaluate the relationship between bacteria strains and glycosidase activities (**Figure S5-S9**). Throughout the malolactic fermentation process, all bacterial strains exhibited comparable effectiveness without any apparent variations. However, we anticipate that the effectiveness may become distinguishable when conducting larger-scale malolactic fermentation.

Table 3. The number of total and smoke-taint glycosides hydrolyzed by yeasts during bucket fermentation

Yeast strains	**EC1118**	**58W3**	**GRE**	**D254**	**QA23**
Number of total glycosides	47	14	94	33	120
Number of Smoke-tainted compounds	21	8	41	17	56

Table 4. The number of total and smoke-taint glycosides hydrolyzed by MLB during MLF

Yeast		**Lalvin 31**	**O-MEGA**	**BETA**	**Alpha**	**K45H**
EC1118	Total	13	13	47	33	12
	Smoke-taint	6	6	25	18	5
58W3	Total	64	85	26	24	74
	Smoke-taint	26	34	13	11	33
GRE	Total	4	3	131	36	17
	Smoke-taint	2	1	62	20	6
D254	Total	44	33	11	49	42
	Smoke-taint	18	13	2	23	11
QA23	Total	20	9	13	8	67
	Smoke-taint	8	3	9	6	31

6.3. Summary of Scale-up Fermentations

6.3.1. Scale up fermentation using naturally smoke-exposed grapes

To evaluate glycosidase activity on flavor compounds, including the smoke-trained glycosides in the standard winemaking setting, we fermented red wines using wildfire-exposed Cabernet Sauvignon grapes in 200 L fermenters. We conducted alcoholic fermentation with selected 3 commercial yeast strains (i.e., EC1118, GRE, and 58W3) in triplicates. All three yeast strains performed alcoholic fermentation at a similar rate and were completed in 7 days. The fermentation temperatures in each of these fermentations were kept consistent with an automatic temperature control system.

After alcoholic fermentation, the free run wine from each tank of wine was divided into three 20 L stainless steel kegs, and each keg of wine was inoculated with one of the bacteria strains tested (Lalvin 31, BETA, ALPHA). In total, we conducted 9 different yeast and bacteria combinations of malolactic fermentation, and each combination was done into triplicates. We also checked acetic acid content to monitor potential spoilage microbial growth. The malolactic fermentation was completed between 15 to 22 days. The Lalvin31 and ALPHA strains conducted malolactic fermentations faster than the BETA strain.

6.3.2. Glycoside analysis

We conducted metabolite analysis using LC-MS to evaluate glycoside compounds hydrolyzed by the yeast or bacteria glycosidase enzymes. We analyzed 135 samples for metabolite analysis, including juice samples for control.

We identified about 3,700 compounds through qualitative and quantitative analysis, including samples from juice, alcoholic fermentation, and malolactic fermentations. We utilized an in-house glycoside database to determine further glycoside compounds released by yeast or bacteria. We identified about 70-80 glycoside compounds in the juice samples while discovering three times more after yeast fermentation or bacteria malolactic fermentation. Insight analysis, 58W3 hydrolyzed 26 total glycosides (**Table 5**) which was more compounds compared to bucket fermentation (14 glycosides, **Table 3**). Surprisingly, fewer total glycosides were hydrolyzed by EC1118 and GRE compared to bucket fermentation. This might result from yeast activity expression at different fermentation conditions (bucket vs tank). All the smoke-tainted compound lists hydrolyzed by different yeast strains are available upon request.

Following MLF, the three MLB strains showed variable activity in different yeast-fermented wines; however, the degree of variation is less significant compared to bucket fermentation. Even though, the GRE yeast and Alpha MLB combination were most effectively hydrolyzed total glycosides (62, **Table 6**), EC1118 yeast and Alpha MLB were least effective for the total glycosides (38, Table 6). For the smoke-tainted compounds, 58W3 yeast and Beta MLB were the most effective, 25 smoke-tainted glycosides were cleaved (Table 6). Unlike bucket fermentation, glycosidase activity in MLB surpasses yeast. This result further suggests fermentation conditions could affect MLB glycosidase activity. All the smoke-tainted compound lists hydrolyzed by different yeast-MLB combinations are available upon request.

We conducted a principal component analysis (PCA) to assess the correlation between wine strains and glycosidase activities (**Figure S10**). The PCA plot indicates that juice samples are distinguishable from fermented wines. After alcoholic fermentation, all yeast strains exhibited comparable effectiveness without any apparent variation, while GRE and EC1118 demonstrated noticeable differences throughout the malolactic fermentation process. These results suggest that yeast and bacteria glycosidase have some degree of interaction on the flavor glycoside hydrolysis.

Table 5. Number of total and smoke-taint glycosides hydrolyzed by yeasts during full-scale fermentation

Yeast strains	**EC1118**	**GRE**	**58W3**
Number of total glycosides	22	22	26
Number of Smoke-tainted compounds	9	11	12

Table 6. The number of total and smoke-taint glycosides hydrolyzed by MLB during full-scale fermentation

Yeast strains		**Lalvin 31**	**BETA**	**Alpha**
EC1118	Total	43	42	38
	Smoke-taint	18	16	17
58W3	Total	47	59	55
	Smoke-taint	15	25	24
GRE	Total	59	41	62
	Smoke-taint	22	11	17

7. **Outreach and Education Efforts - Presentations of Research**

Preliminary results of this project were presented to the Washington wine industry event, WineVit Industry Research Viewing, on February 7, 2023, in Kennewick, WA. Now the project has been successfully accomplished, and all the data analysis have been completed. We are planning more presentations at WineVit, WAVE, and other extension meetings organized by the Washington Wine Commission, the Washington Wine Technical Group, and WSU Viticulture & Enology. We are preparing three manuscripts with all findings for publication in peer-reviewed journals.

8. **Research Success Statements**:

These research findings show the wide range of glycosidic activities among wine yeast and bacteria and role they play in forming smoke taint in wines made from smoke-exposed grapes. With these insights, we might be able to select yeast and bacteria that suppress the formation of smoke taint defects either by not producing the volatiles or by releasing desirable aromas that cover up a smoke taint. Selective hydrolysis of smoke-taint-associated glycosides by selected yeast and bacteria would allow us to produce wines that can be treated to free them of the taint compounds without losing too much-desired glycoside aroma compounds. Current methods for removing potential taint glycosides also lose too much of the desired glycosides that we would like to retain in the wine.

Throughout these 3 years of research, performing fermentation at different scales and conditions, all the yeast and bacteria strains showed a wide range of activity on the aroma glycoside precursors, including smoke taint and other desirable compounds. The impact of wildfire smoke on the wine industry is a big problem that will stay with us year after year. It results in undesirable smoky flavors and aromas in the resulting wines and can result in significant economic losses from unmarketable wines. These studies can help winemakers choose a specific yeast and bacteria strain combination to mediate smoke taint in wildfire-exposed grapes. Additional research is needed to link aroma compounds with specific yeast and sensory profiles as that work was not conducted.

9. **Funds Status**:
All funds allocated for this project have been spent on salary costs. The WSU Viticulture & Enology Program supplemented costs for materials and equipment with discretional funds.

Supplemental Material:

Table S1. Selected yeast strains

Yeast strain ID	Genus	Species	Strain Designation	Source
UCD506	*Saccharomyces*	*cerevisiae*	Bordeaux	wine
UCD509	*Saccharomyces*	*cerevisiae*	Burgundy II (new)	wine
UCD512	*Saccharomyces*	*cerevisiae*	Cognac	cognac, fermentation
UCD514	*Saccharomyces*	*cerevisiae*	Geisenheim	wine
UCD522	*Saccharomyces*	*cerevisiae*	Montrachet	Montrachet wine
UCD525	*Saccharomyces*	*cerevisiae*	Port	Port wine
UCD557	*Saccharomyces*	*cerevisiae*	J1934-3A	Sherry wine
UCD932	*Saccharomyces*	*cerevisiae*	Ba2 Lambrusco	grapes, vineyard, Italy
UCD2784	*Saccharomyces*	*cerevisiae*	RM12-1a [Bb33(3)]	grapes, vineyard, California
UCD2790	*Saccharomyces*	*cerevisiae*	AWRI 1631	Wine yeast

Table S2. Selected bacteria strains

Bacteria Strain ID	Genus	Species	Strain Designation	Source
UCD139	Oenococcus	oeni	Lco 23	wine, Switzerland
UCD141	Oenococcus	oeni	NCFB 1674	wine, France
UCD146	Oenococcus	oeni	MCW	wine, California
UCD148	Oenococcus	oeni	Kli	wine, Switzerland
UCD167	Oenococcus	oeni	1081	wine, Pinot Noir
UCD176	Oenococcus	oeni	IS-1	wine, Spain
UCD199	Oenococcus	oeni	1N:F1	wine, Merlot
UCD224	Oenococcus	oeni		wine, Chile
UCD261	Oenococcus	oeni	IOEB 9306	Cider, France
UCD445	Oenococcus	oeni	PSU-1	wine

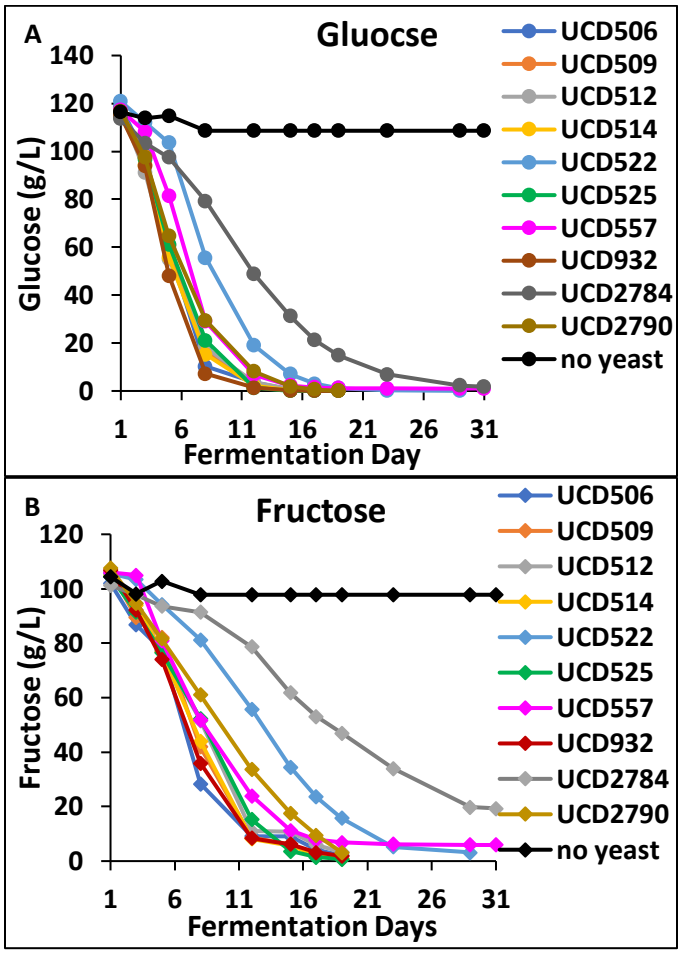

Figure S1. Mini-scale alcoholic fermentation with 10 selected yeast strains in synthetic must.

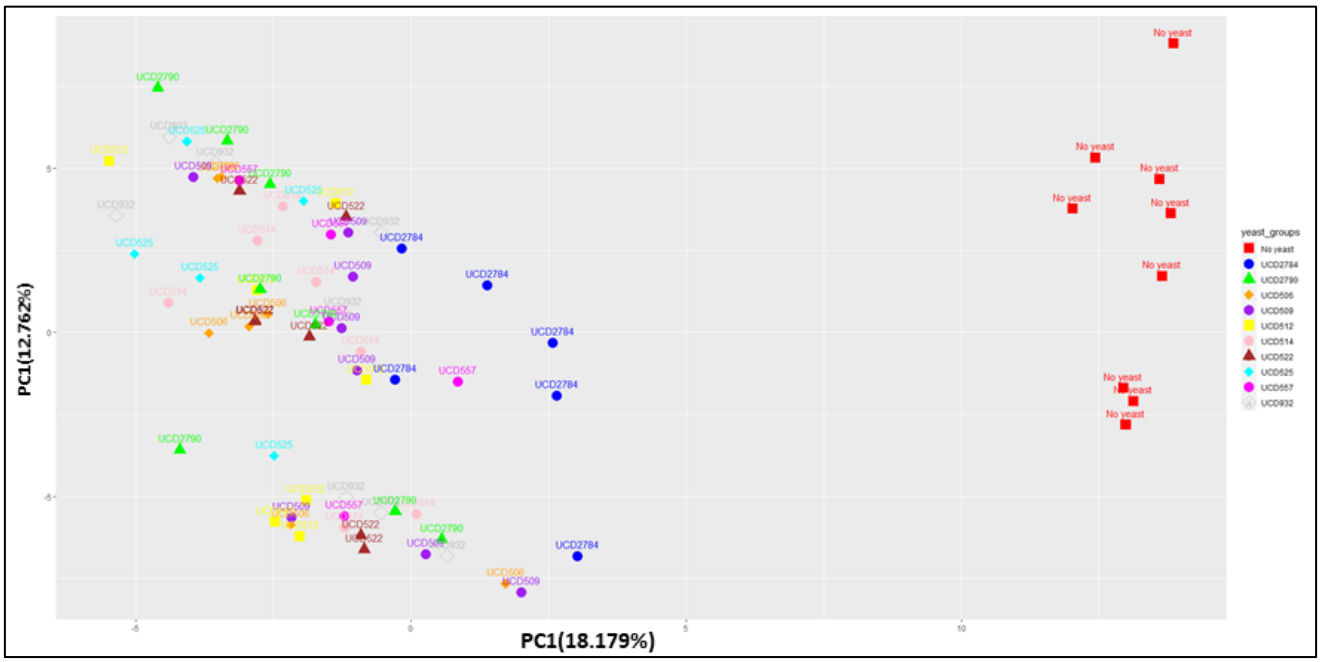

Figure S2. PCA plot for the mini-scale fermentation in the synthetic grape juice with 10 yeast strains and without yeast control.

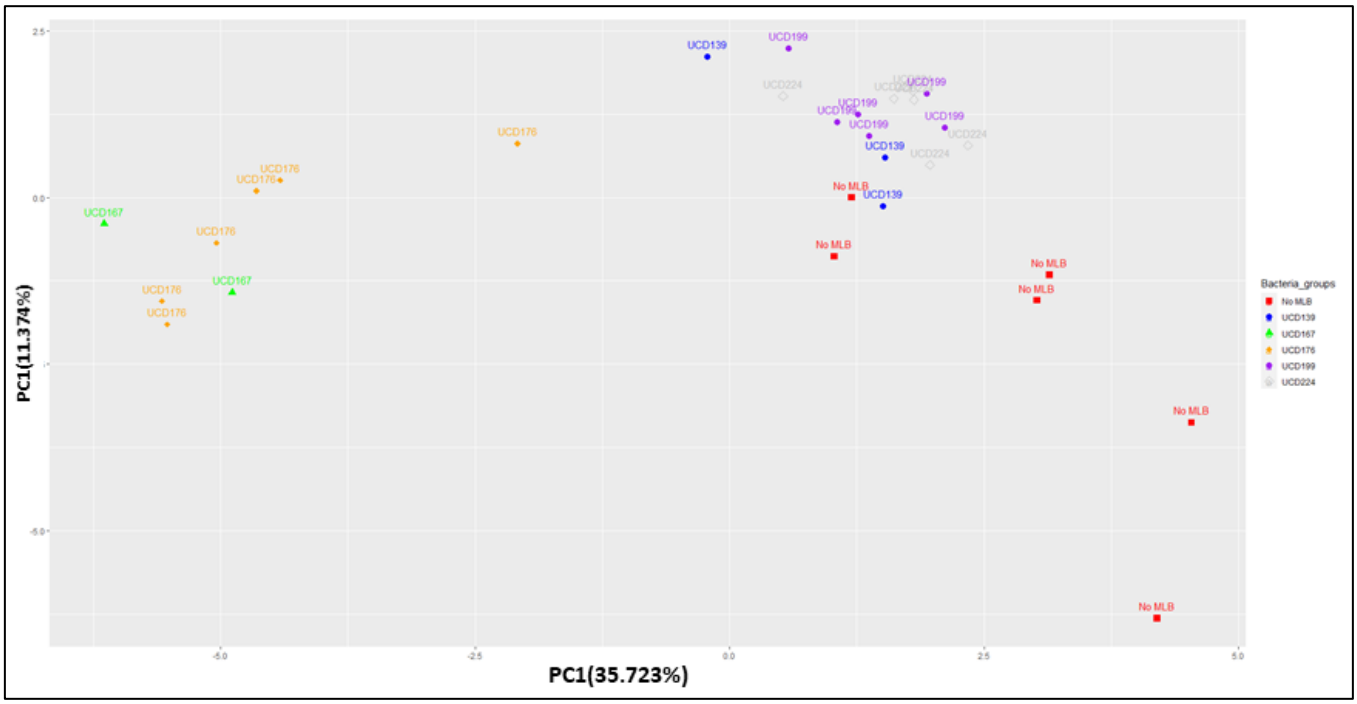

Figure S3. PCA plot for the mini-scale malolactic fermentation in the synthetic wine with 5 bacteria strains and without bacteria control

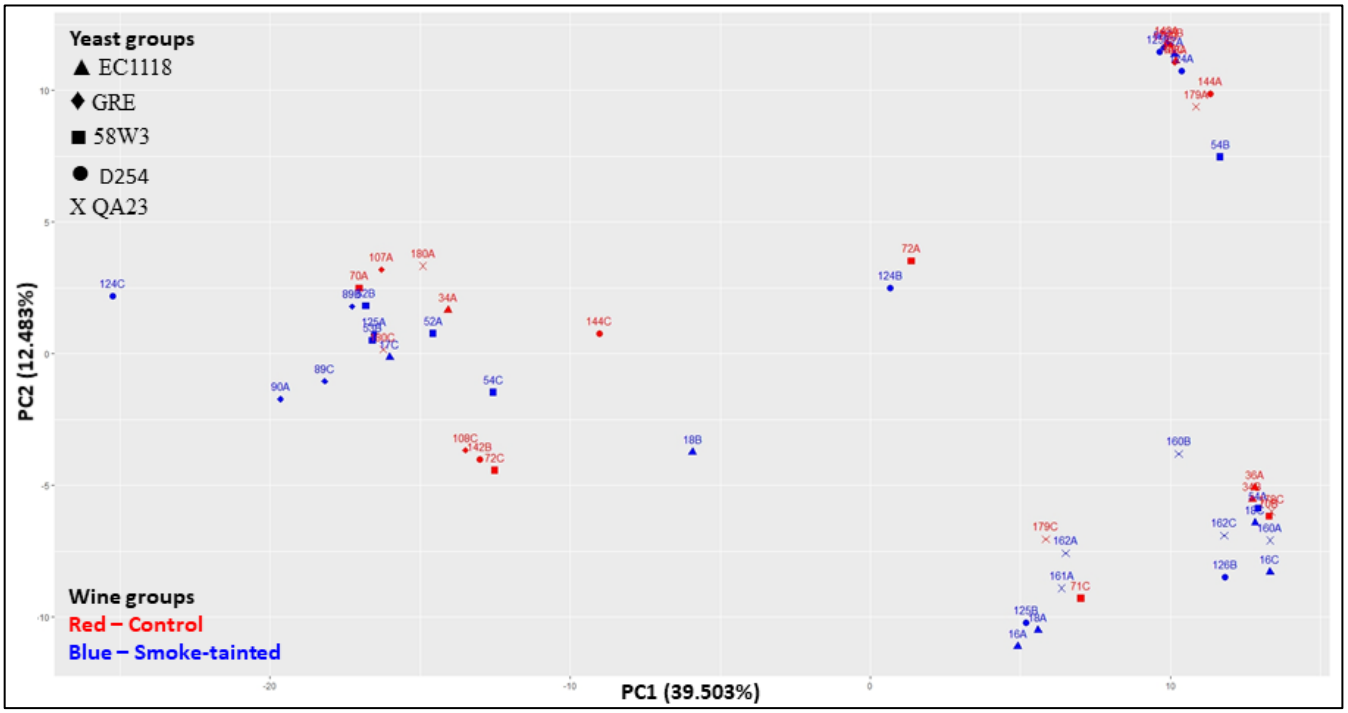

Figure S4. PCA plot for the yeast bucket fermentation with smoke-exposed and non-smoke-exposed grapes.

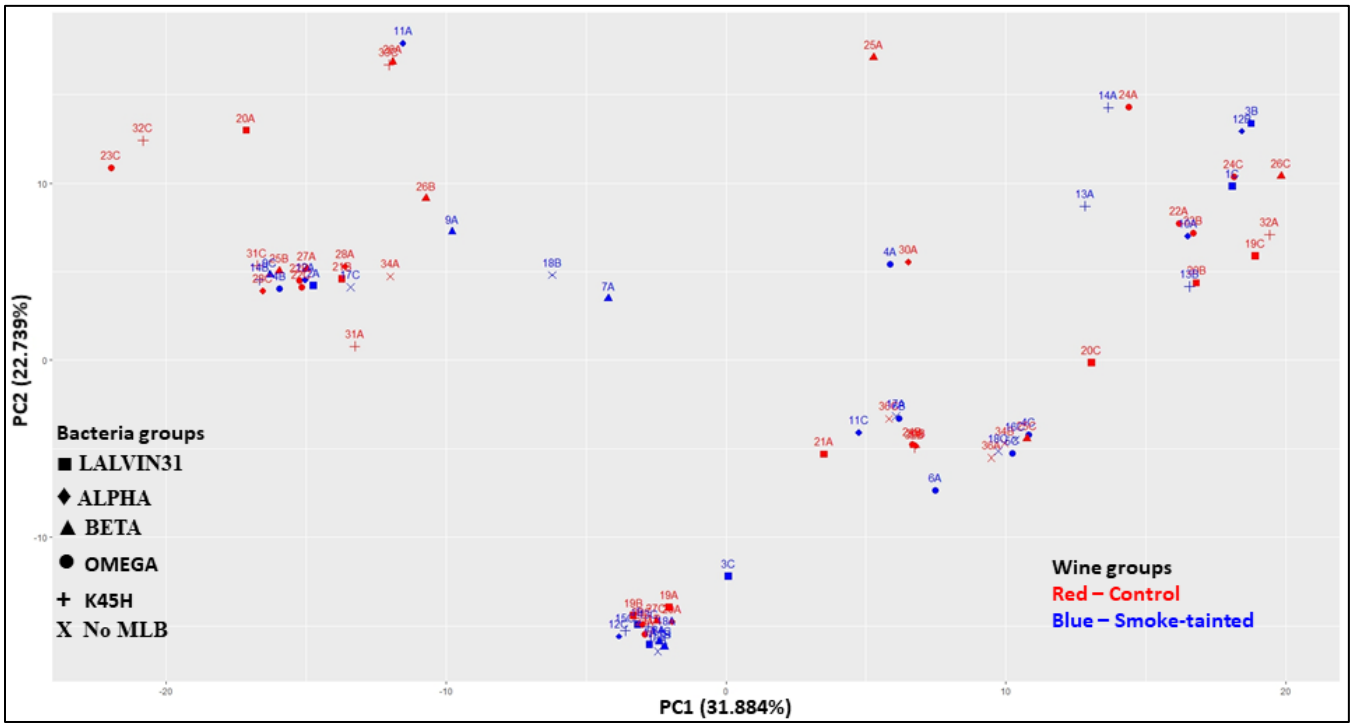

Figure S5. PCA plot for the bacteria malolactic fermentation wines processed alcoholic fermentation with yeast strain EC1118.

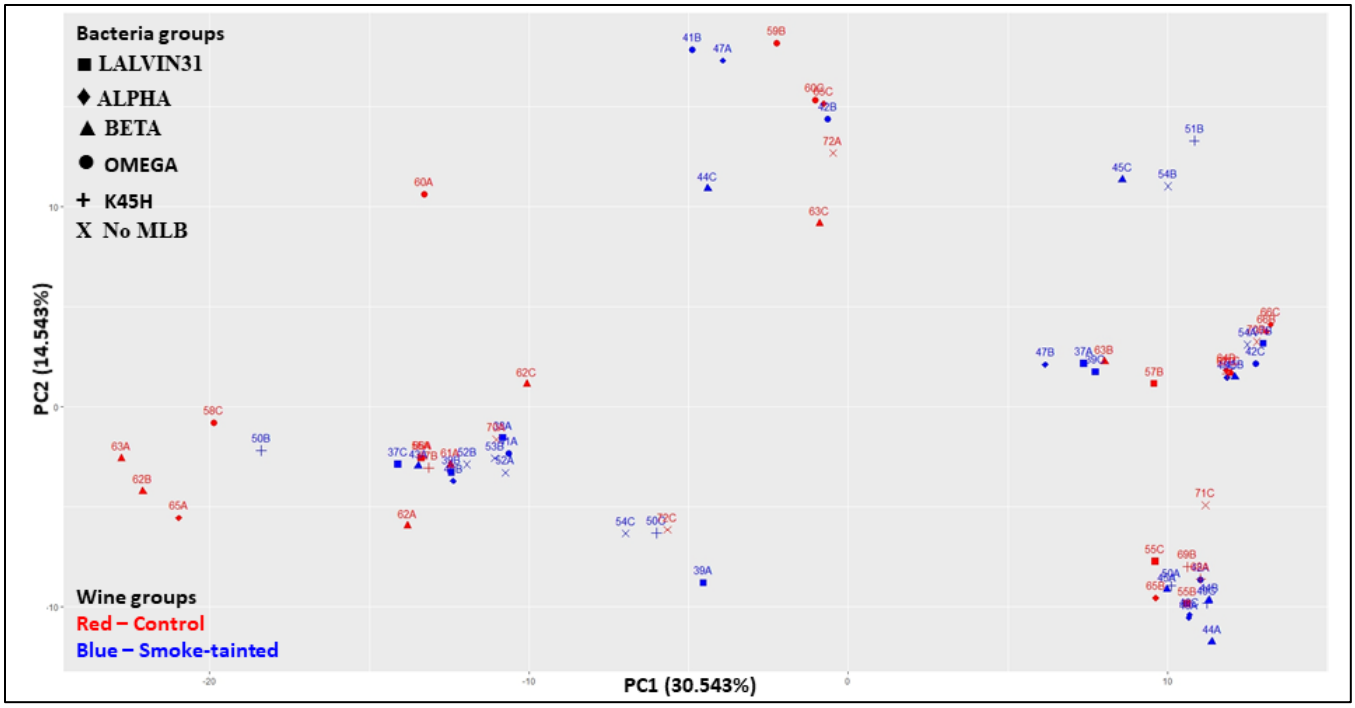

Figure S6. PCA plot for the bacteria malolactic fermentation wines processed alcoholic fermentation with yeast strain 58W3.

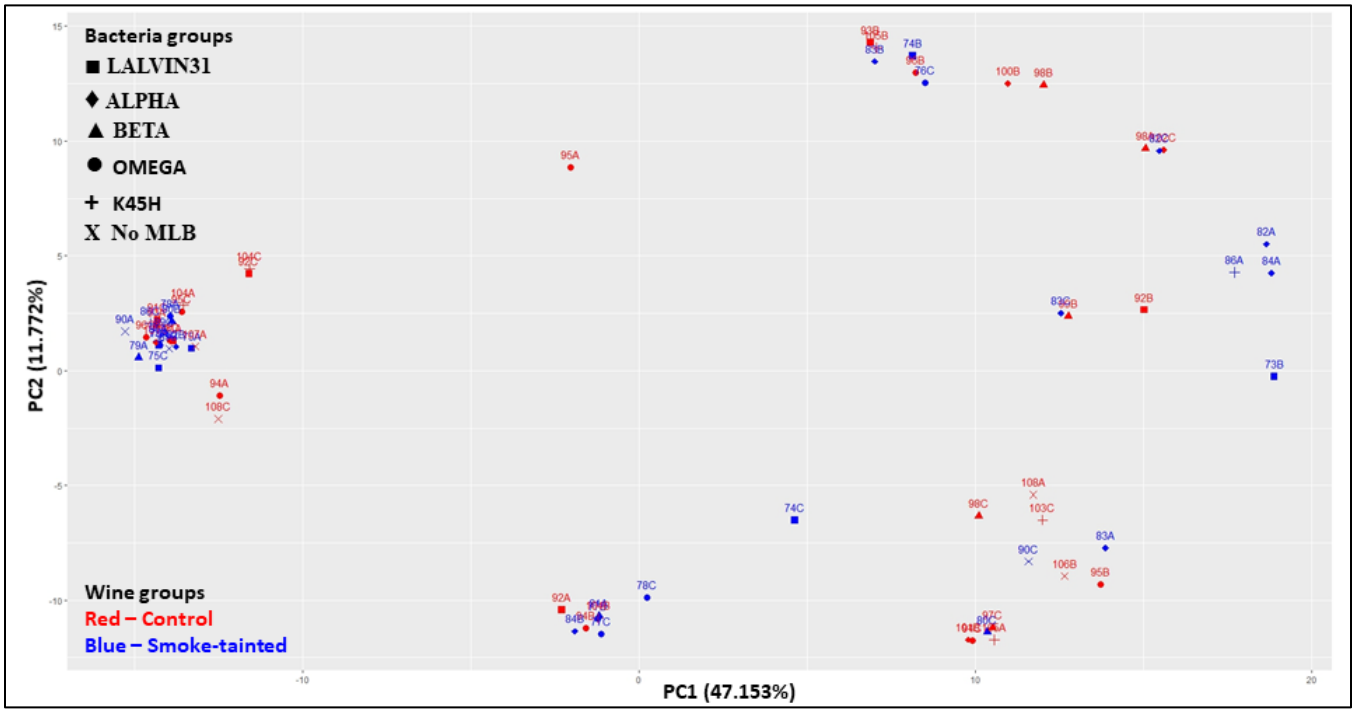

Figure S7. PCA plot for the bacteria malolactic fermentation wines processed alcoholic fermentation with yeast strain GRE.

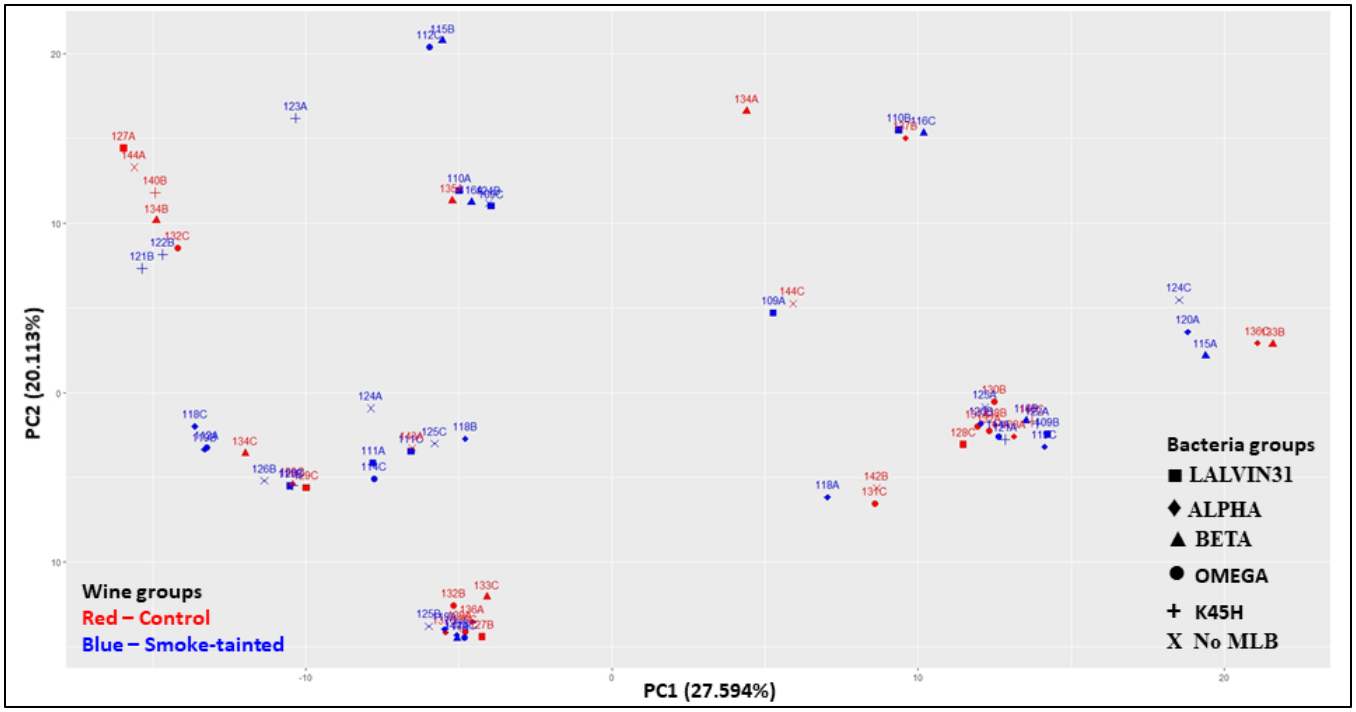

Figure S8. PCA plot for the bacteria malolactic fermentation wines processed alcoholic fermentation with yeast strain D254.

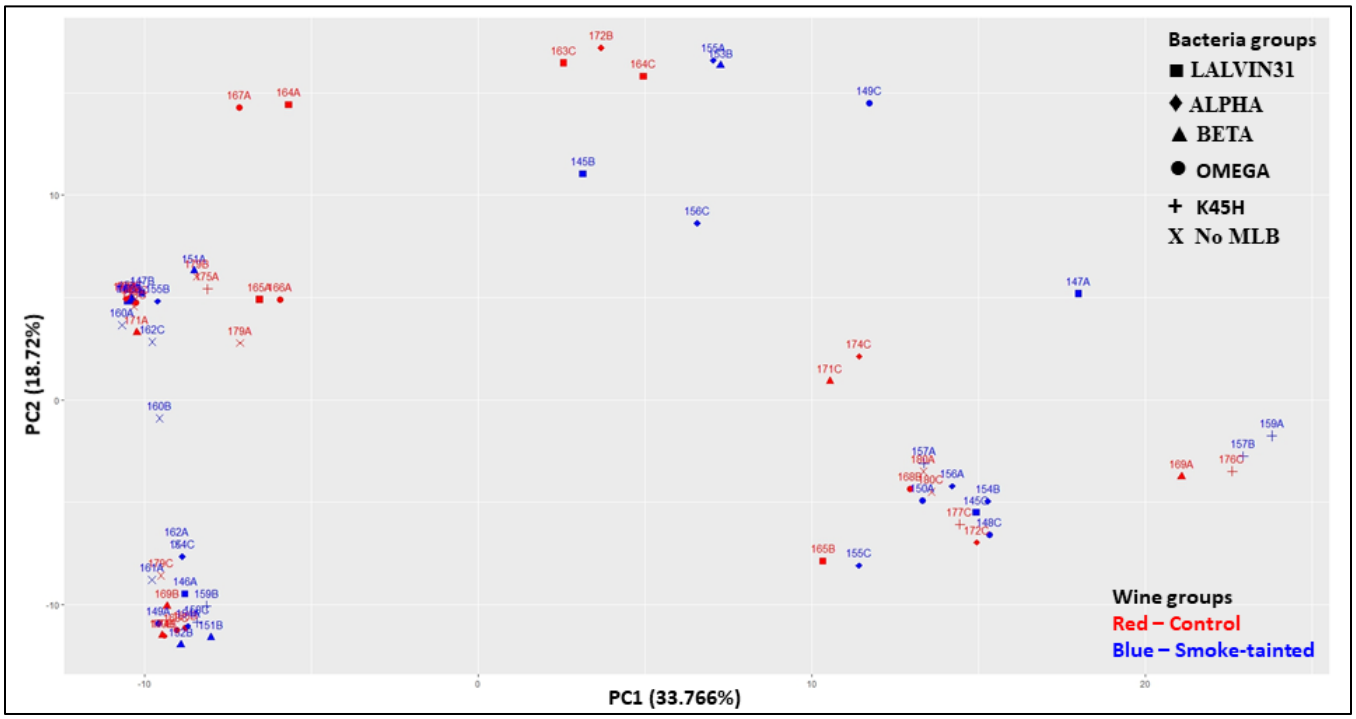

Figure S9. PCA plot for the bacteria malolactic fermentation wines processed alcoholic fermentation with yeast strain QA23.

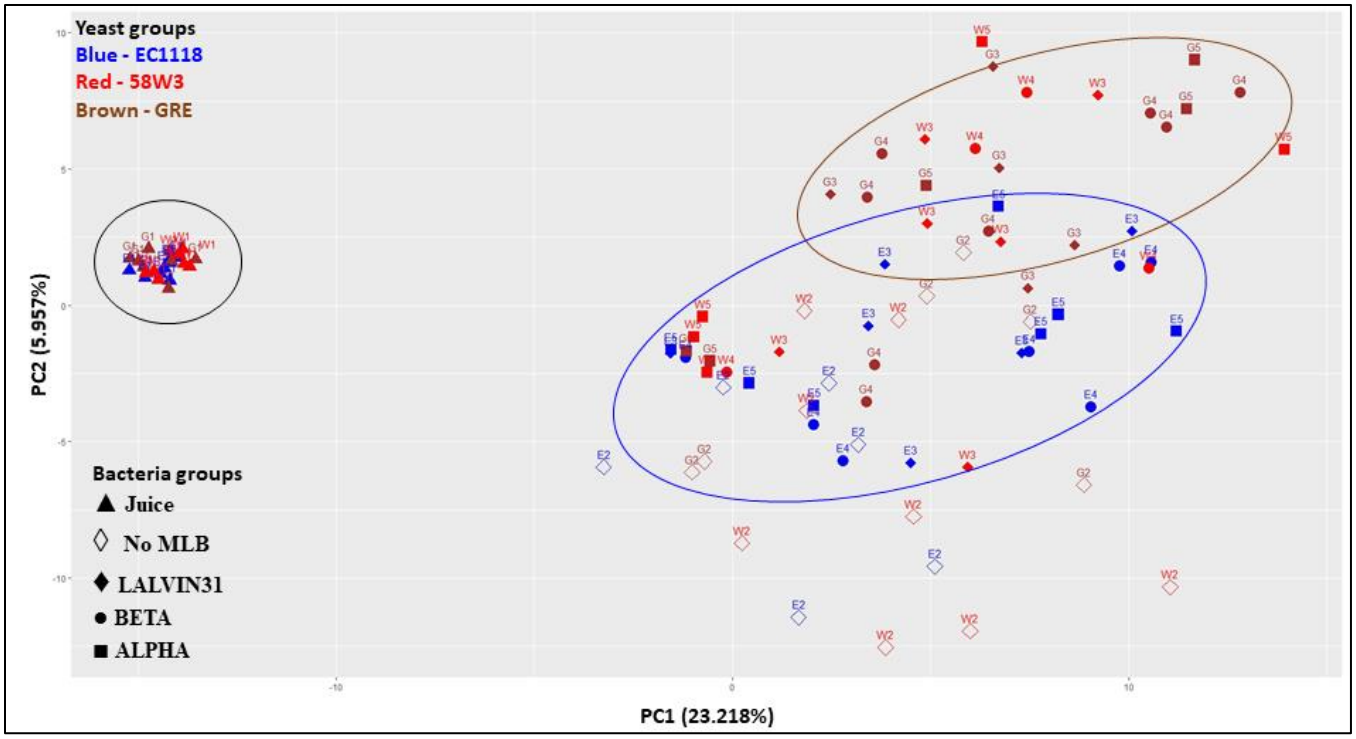

Figure S10. PCA plot for the bin fermentation. The glycosidic activity by the malolactic bacteria is grouped according to the yeast strain that conducted the alcoholic fermentation