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Metabolomic Characterization of Malolactic Fermentation and Fermentative Behaviors of Wine Yeasts in Grape Wine

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Wine contains a number of metabolites that are produced during alcoholic and malolactic fermentations (MLF) or aging, which are important compounds for determining wine quality. This study investigated changes in metabolites in wines to characterize malolactic fermentation (MLF) and to assess fermentative behaviors of wine yeast strains using ^{1}H nuclear magnetic resonance (NMR) spectroscopy coupled with multivariate statistics. Principal component analysis (PCA) showed clear differentiation between non- and induced-malolactic fermented wines by wine lactic acid bacteria (LAB) and between wines fermented with various wine yeast strains. Metabolites such as glycerol, lactate, 2,3-butanediol, succinate, leucine, isoleucine, alanine, valine, proline, choline, γ-aminobutyric acid (GABA), and polyphenols contributed to the differentiations. Decreased levels of malate and citrate along with increased levels of lactate were the metabolites most responsible for the differentiation of induced-MLF wines from non-MLF wines. In particular, high succinate levels provided evidence of an inhibitory effect of *Saccharomyces bayanus* against spontaneous MLF. Furthermore, dependence of metabolites on wine yeast strains was observed, demonstrating their different fermentative behaviors. This study demonstrates that wine fermentation by yeast and LAB can be characterized through global and multivariate statistical analysis of ^{1}H NMR spectral data.

KEYWORDS: Wine; NMR; PCA; metabolomics; metabolites; malolactic fermentation (MLF); yeast

INTRODUCTION

The chemical composition or metabolites in wines originate from the grapes and are also produced during alcoholic fermentation by wine yeast and during malolactic fermentation (MLF) by lactic acid bacteria (LAB). Therefore, bacterial strains affect the wine quality as do the factors of grape variety, climate, and soil, resulting in variations in the metabolites of wines (*1*−*3*). Alcoholic fermentation is the first fermentation process by wine yeast in winemaking, and the metabolic byproducts of yeast activity are important for characterizing the winemaking abilities of the wine yeasts to ensure reproducible fermentation performance and product quality. *Saccharomyces cerevisiae* is the main species of yeast in winemaking, and it strongly inhibits other yeast species as fermentation proceeds (*4*), leading winemakers to introduce specially cultured yeast strains. MLF is the second fermentation process that typically occurrs in winemaking after the completion of alcoholic fermentation; in this process, malate is converted into lactate and CO_2 to reduce the acidity of the wine. MLF is supported by different species of LAB, such as *Oenococcus*, *Pediococcus*, and *Lactobacillus*, among which *Oenococcus oeni* is most often associated with MLF in the harsh wine environment (*5*, *6*). *O. oeni* is the predominant species in red wines undergoing spontaneous MLF, accounting for 98.5% of total species isolated (*7*). In addition to reducing acidity, LAB influence the aroma compounds of wine derived from fruit and the alcoholic fermentation and confer biological stability to the final product (*8*). The growth of LAB in wine must be tightly controlled to ensure the growth of desirable LAB that produce no off-flavors and that contribute to the rapid completion of MLF to achieve early stability of the wine.

Metabolomics or metabonomics is defined as the comprehensive and quantitative analysis of all metabolites and make it possible to visualize metabolic differences and identify the metabolites contributing to the differentiation (*9*), thereby providing insight into metabolic alterations or perturbations in drug toxicity (*10*), disease status (*11*), human metabolic phenotype (*12*), and dietary interventions (*13*) by using multivariate statistical analysis of a metabolomic data set. Metabolomics highlights the comprehensive understanding of metabolic pathways or provides potential biomarkers. Recently, we showed the metabolic dependence of wines on grape varieties, production areas, and wine yeast strains, as well as the statistical time course of metabolite evolutions during alcoholic fermentation, through ^{1}H NMR-based metabolomic studies (*1*, *2*).

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Table 1. Organic Acid Contents of Oxalate, Tartarate, Malate, and Lactate in Meoru Wines (MW) after Alcoholic Fermentation with Various Yeast Strains and after Malolactic Fermentation with *O. oeni* of Lactic Acid Bacteria (LAB)

yeast strain[a]	LAB	oxalate (mg/L)	tartarate (mg/L)	malate (mg/L)	lactate (mg/L)	assay
PC	[b]	340.5 ± 22.5	1585.2 ± 250.8	6926.5 ± 159.8	741.9 ± 93.5	MW1
PC	*O. oeni*	254.6 ± 6.0	1142.1 ± 26.1	nd[c]	5199.9 ± 132.1	MW4
CDB	[b]	362.0 ± 40.0	1589.8 ± 37.6	nd	6890.4 ± 111.5	MW2
CDB	*O. oeni*	366.2 ± 31.2	1680.1 ± 231.9	nd	5565.6 ± 72.6	MW5
KUBY-501	[b]	351.5 ± 1.8	1677.4 ± 274.5	nd	6586.0 ± 94.3	MW3
KUBY-501	*O. oeni*	305.2 ± 29.5	1381.3 ± 246.8	nd	5518.4 ± 133.3	MW6

[a] PC, CDB, and KUBY-501 denote strains *S. bayanus* PC, *S. cerevisiae* CDB, and *S. cerevisiae* KUBY-501, respectively. [b] No inoculation of LAB *O. oeni*. [c] Not detected.

As part of our efforts to assess and improve the quality of wine vinified in Korea, we report here the metabolic characterization of Korean wild grape, Meoru (*Vitis coignetiae*), wine during MLF and the fermentative behaviors of wine yeast strains through multivariate statistical analysis of ^{1}H NMR spectral data.

MATERIALS AND METHODS

Yeast and Bacterial Strains. Two commercial yeast strains, *Saccharomyces bayanus* (Red Star, Premier Cuvée, PC) and *S. cerevisiae* Côte des Blancs (CDB), and one *S. cerevisiae* (KUBY-501) strain isolated from Korean wild berrry, raspberry (*Rubus coreanus*) (*2*), were used for alcoholic fermentation. Commercial lactic acid bacterium *O. oeni* (MCW) was purchased from Vinquiry (Healdsburg, Canada) and used for MLF.

Winemaking. Korean wild grapes Meoru (*V. coignetiae*) (600 kg) were harvested in October 2006 and crushed. The must was distributed into 18 25-L plastic tanks, producing six batches for each of the three yeast strains. Three batches for each yeast strain were used only for alcoholic fermentation, and the other three batches were used for MLF by inoculation of LAB, following alcoholic fermentation. Dried yeasts were first activated in YPD medium (1% w/v yeast extract, 1% w/v peptone, and 2% w/v dextrose) and then cultured in a 1:1 mixture of YPD and grape juice at 25 °C for 24 h to obtain a final cell count of 2×10^6 cells/mL. Potassium metabisulfite (100 mg/kg) and starters of the three yeast strains were added into the must. Alcoholic fermentation with the three yeast strains was carried out in the plastic tanks at 20−22 °C for 15 days. After completion of alcoholic fermentation, the musts were transferred into 18 20-L glass carboys and the grape skins were removed.

Malolactic Fermentation. The LAB *O. oeni* were purchased for MLF in wine and grown in MRS broth at 30 °C to obtain the appropriate biomass of 4×10^7 cfu/mL. *O. oeni* was then inoculated into the glass carboy at day 16 in the glass carboys after alcoholic fermentation to produce MW4 (after alcoholic fermentation with PC strain), MW5 (after alcoholic fermentation with CDB strain), and MW6 (after alcoholic fermentation with KUBY-501 strain). MLF was carried out at storage room temperature (20 ± 2 °C). At the end of MLF Meoru wines were cold settled and racked two times. After aging in each carboy for one year, the wines were bottled.

Organic Acid Analysis. All standards and wine samples were filtered through a 0.45-μm PTFE membrane filter prior to HPLC analysis. An HPLC unit (Gilson, Villiers-le-Bel, France) and a Prevail organic acid column (250 × 4.6 mm, Alltech), coupled with a heating device at 30 °C, were used. Twenty microliters of wines and standards was injected on the column. Elution was carried out at a flow rate of 1.0 mL/min with 25 mM KH_2PO_4 (pH 2.5) as the mobile phase. Components were detected with a UV detector at 210 nm.

Chemical Analysis of the Wines. Alcohol content, titratable acidity, pH, and reducing sugars were measured according to official AOAC methods.

Chemicals. All chemical reagents were of analytical grade. Standards of organic acids, D_2O (99.9%), and DSS (97%) were purchased from Sigma (St. Louis, MO).

^{1}H NMR Spectroscopic Analysis of Wines. One milliliter of must or wine was lyophilized in a 1-mL Eppendorf tube and dissolved in 99.9% deuterium oxide (400 μL, D_2O), mixed with 400 mM oxalate buffer (140 μL, pH 4.0) and 5 mM sodium 2,2-dimethyl-2-silapentane-5-sulfonate (60 μL, DSS, 97%), and then centrifuged at 13000 rpm for 10 min. Supernatants (550 μL) were transferred into 5-mm NMR tubes. D_2O and DSS provided a field frequency lock and chemical shift reference (^{1}H, δ 0.00), respectively. ^{1}H NMR spectra were acquired on a Varian 600 MHz NMR spectrometer (Varian Inc., Palo Alto, CA) operating at 599.84 MHz ^{1}H frequency and a temperature of 298 K, using a triple resonance 5-mm HCN salt-tolerant cold probe. A NOESYPRESAT pulse sequence was applied to suppress the residual water signal. For each sample, 16 transients were collected into 32K data points using a spectral width of 9615.4 Hz with a relaxation delay of 1.5 s, an acquisition time of 4.00 s, and a mixing time of 400 ms. A 0.3 Hz line-broadening function was applied to all spectra prior to Fourier transformation (FT).

Table 2. pH, Titratable Acidity, Total Phenol Content, and Alcohol Content in Meoru Wines (MW) after Alcoholic Fermentation with Various Yeast Strains and after Malolactic Fermentation with *O. oeni* of Lactic Acid Bacteria (LAB)

yeast strain[a]	LAB	pH	titratable acidity (g/L)	total phenol (mg/L)	alcohol (%)	assay
PC	[b]	3.80	8.6	5463.3	12.9	MW1
PC	*O. oeni*	4.10	6.6	4834.7	11.5	MW4
CDB	[b]	4.22	7.5	4688.9	11.7	MW2
CDB	*O. oeni*	4.16	6.1	4761.9	12.2	MW5
KUBY-501	[b]	4.09	6.3	4643.4	11.6	MW3
KUBY-501	*O. oeni*	4.19	6.3	4661.7	11.5	MW6

[a] PC, CDB, and KUBY-501 denote strains *S. bayanus* PC, *S. cerevisiae* CDB, and *S. cerevisiae* KUBY-501, respectively. [b] No inoculation of LAB *O. oeni*.

NMR Data Preprocessing and Multivariate Analysis. All NMR spectra were phased and baseline corrected by Chenomx NMR suite 4.6 software, professional edition (Chenomx Inc.). The NMR spectral data were reduced into 0.001 ppm spectral buckets, whereas the region corresponding to water (4.6−4.8 ppm) was removed. In addition, the regions for residual ethanol (1.15−1.20 and 3.59−3.72 ppm) from incomplete removal during lyophilization and for DSS (−0.5 to 0.7 ppm) were also removed. The spectra were then normalized to the total spectral area and converted to ASCII format. The ASCII format files were imported into MATLAB (R2006a, Mathworks, Inc., 2006), and all spectra were aligned using the Correlation Optimized Warping (COW) method (*14*). The resulting data sets were then imported into SIMCA-P version 12.0 (Umetrics, Umea, Sweden) for multivariate statistical analysis. Signal assignment for representative samples was facilitated via acquisition of two-dimensional (2D) total correlation spectroscopy (TOCSY), heteronuclear multiple-bond correlation (HMBC), heteronuclear single-quantum correlation (HSQC), spiking experiments, and comparisons to literature (*1*, *2*). In addition, Chenomx NMR suite 4.6 software was utilized to assign the metabolites in wine. The mean center was applied for all multivariate analyses by SIMCA-P version 12.0 (Umetrics). Principal component analysis (PCA), an unsupervised pattern recognition method, was performed to examine the intrinsic variation in the data set. Hotelling's T2 region, shown as an ellipse in the scores plots, defines the 95% confidence interval of the modeled variation (*15*). The quality of the PCA

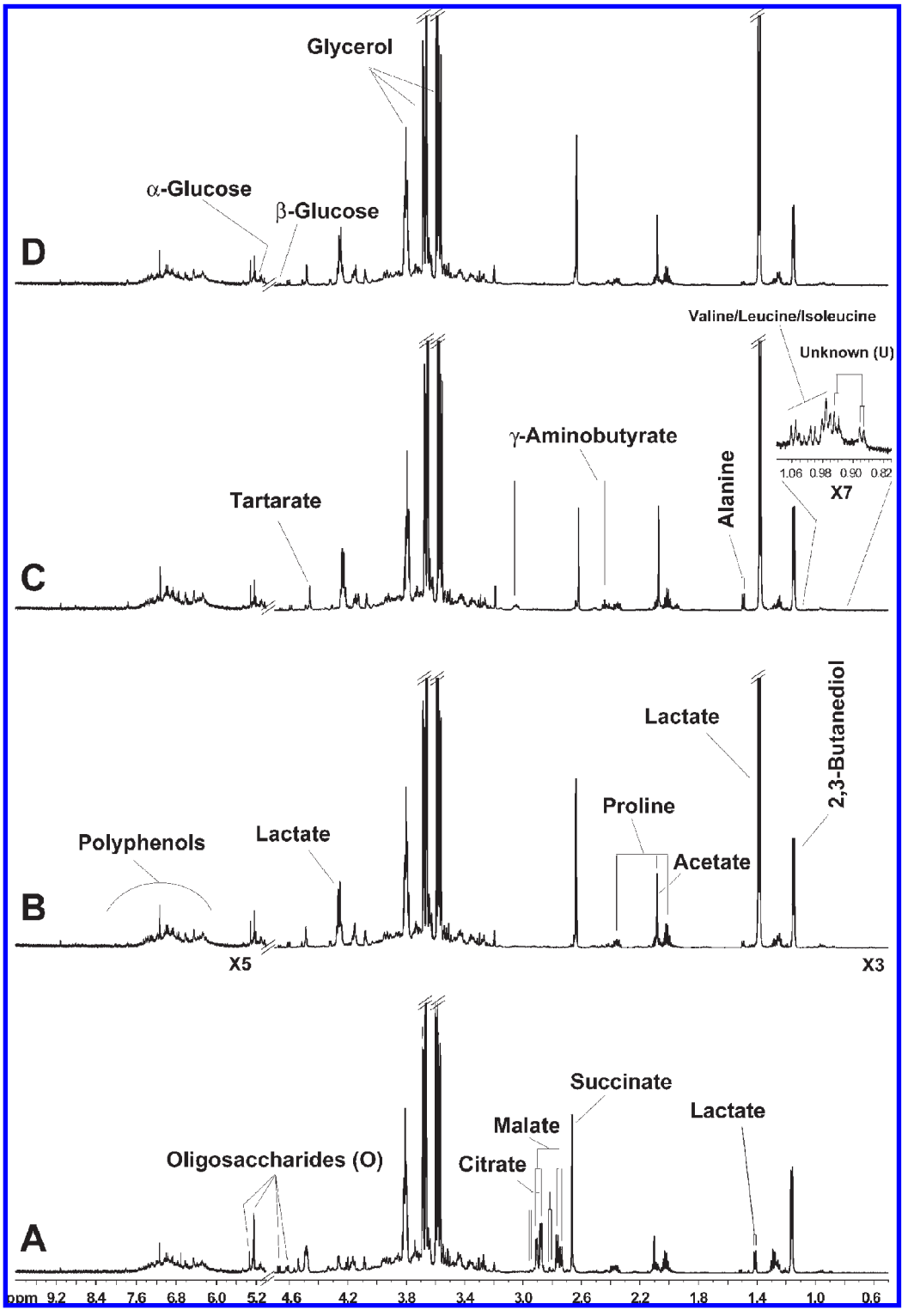

Figure 1. Representative ^{1}H NMR spectra of Meoru wines fermented only with *S. bayanus* PC (MW1) (**A**) and malolactic fermented by inoculation of *O. oeni*, following alcoholic fermentation with *S. bayanus* PC (MW4) (**B**), *S. cerevisiae* CDB (MW5) (**C**), and *S. cerevisiae* KUBY-501 (MW6) (**D**).

models is described by R^2 and Q^2 values. R^2 is defined as the proportion of variance in the data explained by the models and indicates goodness of fit, and Q^2 is defined as the proportion of variance in the data predictable by the model and indicates predictability (*16*).

RESULTS AND DISCUSSION

Development of MLF. On the basis of changes in malate and lactate contents (**Table 1**), spontaneous MLF occurred in MW2 and MW3 but not in MW1: MW1 was fermented with *S. baynaus* Premier Cuvée (PC); MW2, with *S. cerevisiae* Côte des Blancs (CDB); and MW3 with *S. cerevisiae* KUBY-501. That is, conversion of malate to lactate was not observed in MW1, whereas malate was completely converted into lactate in MW2 and MW3, showing spontaneous MLF. As expected, MW4, MW5, and MW6, which were inoculated with LAB *O. oeni* after alcoholic fermentation by each yeast strain, also showed complete conversion of malate into lactate. MW1 and MW4 were fermented with *S. bayanus* PC, and MW4, but not MW1, was inoculated with *O. oeni*, resulting in induced- and non-MLF, respectively. It has been reported that *S. bayanus* makes MLF in wine difficult by

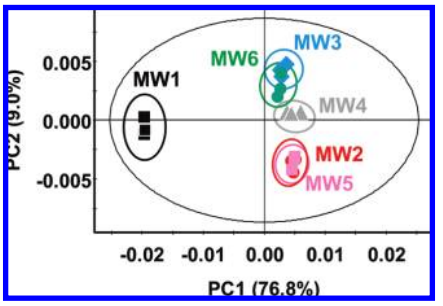

Figure 2. PCA score plot derived from the ^{1}H NMR spectra of Meoru wines with non- or induced-malolactic fermentation (MLF) with LAB *O. oeni*, following alcoholic fermentation with various wine yeast strains. MW2 and MW5 denote Meoru wines fermented with *S. cerevisiae* CDB; MLF occurred spontaneously in MW2 and was induced in MW5 by inoculation of *O. oeni*. MW3 and MW6 represent Meoru wines fermented with *S. cerevisiae* KUBY-501; MLF occurred spontaneously in MW3 and was induced in MW6 by *O. oeni*. MW1 and MW4 are Meoru wines fermented with *S. bayanus* PC; MLF was not observed in MW1 but was induced in MW4 by inoculation of *O. oeni*.

delaying the release of nutrients available for LAB (*17*). In the present study, MLF also increased the pH from 3.80 in MW1 to 4.10 in MW4, and the titratable acidity decreased from 8.6 to 6.6 g/L of tartaric acid (**Table 2**), which are typical changes in pH and titratable acidity during MLF. These results were in good agreement with the data reported during MLF with *O. oeni* in grape wines (*18*).

^{1}H NMR Wine Spectra and PCA Models. Figure 1 shows the representative ^{1}H NMR spectra of Meoru wines fermented with LAB *O. oeni*, following alcoholic fermentation with wine yeast strains of *S. bayanus* PC (MW4), *S. cerevisiae* CDB (MW5), and *S. cerevisiae* KUBY-501 (MW6). Marked high levels of malate and citrate along with low levels of lactate were observed by the 1D ^{1}H NMR spectra of MW1 (**Figure 1A**), revealing neither spontaneous nor induced MLF. PCA score plot showed clear differentiation between MW1 and other wines, indicating significant metabolic changes after MLF (**Figure 2**). MW2 and MW3 represented Meoru wines in which MLF occurred spontaneously, following alcoholic fermentation with *S. cerevisiae* CDB and KUBY5-1, respectively. In addition, MW4, MW5, and MW6 were Meoru wines in which MLF was induced by inoculation of *O. oeni*, after completion of alcoholic fermentation with *S. bayanus* PC, *S. cerevisiae* CDB, and *S. cerevisiae* KUBY-501, respectively. No differentiations between MW2 and MW5 or between MW3 and MW6 in the PCA score plots indicated that there were no metabolic differences between spontaneous and induced MLF wines when they were fermented with the same yeast strain (data not shown).

Changes in Metabolites during MLF. Panels **A** and **B** of **Figure 3** show the PCA score and loading plots, respectively, derived from the ^{1}H NMR spectra of Meoru wines with non- (MW1) and induced-MLF (MW4). The separation between MW1 and MW4 was observed by the first component (PC1) in the PCA score plot, which accounted for 98.2% of the total variance of the data set, and high goodness of fit with an R_X^2 value of 0.99 and high predictability with a Q^2 value of 0.98 for the PCA model (**Figure 3A**). The upper section of the loading plots represents metabolites that were increased in induced MLF wines (MW4), whereas the lower section reveals metabolites that were increased in non-MLF wines (MW1). Increased levels of leucine, valine, alanine, lactate, and succinate together with decreased levels of malate, citrate, and oligosaccharide (O) in induced-MLF wines (MW4) contributed to the separation as shown in PCA loading plot (**Figure 3B**). To better see the effect of metabolites that have a low PCA loading coefficient on the separation, unknown compound (U), leucine, alanine, and O were expanded from the PCA loading plot (**Figure 3C**). Panels **D** and **E** of **Figure 3** represent the PCA column loading plot with a bar at 95% confidence level and mean spectra, respectively, corresponding to the expansions of U, leucine, alanine, and O. The smaller the bar, the higher the confidence limit in the PCA loading plots (**Figure 3D**). Thus, metabolites with low loading coefficients also contributed significantly to the separation.

Malate dramatically decreased and lactate increased in MW4 with MLF induced by *O. oeni* compared to MW1 with non-MLF, as shown in 1D ^{1}H NMR spectra (**Figure 1A**,**B**) and PCA loading plot (**Figure 3B**). This result is in good agreement with the decrease of malate and increase of lactate obtained by quantitative ^{1}H NMR spectroscopy in alcoholic fermentation and MLF (*19*) and with the analytical results given in **Table 1**. During MLF, citrate also decreased as shown in the PCA loading plot (**Figure 3B**). It is well-known that citrate is metabolized to acetate, acetoin, diacetyl, and 2,3-butanediol by LAB through citrate metabolism (*20*). Valine and leucine levels were also increased during MLF as shown in **Figure 3B**. Pozo-Bayón et al. (*21*, *22*) reported increases in valine and leucine contents in all wines after MLF with *O. oeni* strains and *Lactobacillus plantarum* strains. These amino acids are synthesized from α-acetolactate by catabolic and anabolic α-acetolactate synthases. α-Acetolactate of acetoinic compounds serves as an intermediate in the biosynthesis of amino acids in LAB (*23*). Therefore, increases in valine and leucine levels during MLF indicate the activation of acetolactate synthases. Alanine levels also were increased during MLF (**Figure 3B**). Elevated levels of alanine in MLF wines induced with *O. oeni* and *L. plantarum* have been also reported (*21*). Alanine can be synthesized from pyruvate by aminotransferase. The activation of the alanine transaminase of *O. oeni* could also contribute to increases in pyruvate levels synthesized from citrate and sugar during MLF.

During MLF, LAB secrete numerous proteins, including proteases and peptidases, and glycosidases that ferment residual sugars, such as hexoses, pentose, or oligosaccharides left by yeasts, and transform numerous wine components (*20*, *24*, *25*). The consumption of residual carbon nutrients by LAB stabilizes the wine microbiologically (*26*). The reductions in O during MLF shown in **Figure 3** might therefore be due to further consumption by *O. oeni*. Because succinate is an intermediate in the tricarboxylic acid (TCA) cycle during anaerobic fermentation (*20*), it is likely that succinate increased with the growth of LAB during MLF.

Changes of Metabolites in Meoru Wines with Various Yeast Strains. Spontaneous MLF was observed in Meoru wines fermented with *S. cerevisiae* CDB (MW2) and *S. cerevisiae* KUBY-501 (MW3). However, spontaneous MLF did not occur in wines fermented with *S. bayanus* PC (MW1). It was interesting to note that no significant differences in metabolites between spontaneous and induced-MLF wines MW2 and MW4 and between MW3 and MW6, respectively, were observed in PCA score and loading plots (data not shown) because *O. oeni* is the predominant species in red wines undergoing spontaneous MLF, accounting for 98.5% of total species isolated (*7*). In fact, there were also no metabolic differences among wines in which MLF was induced by various *O. oeni* strains (unpublished data). The dependence of metabolites on yeast strains could, therefore, be comparable in MW4, MW5, and MW6 wines, in which MLF was induced by *O. oeni*, following alcoholic fermentation with the yeast strains *S. baynaus* PC, *S. cerevisiae* CDB, and *S. cerevisiae* KUBY-501, respectively, in the present study.

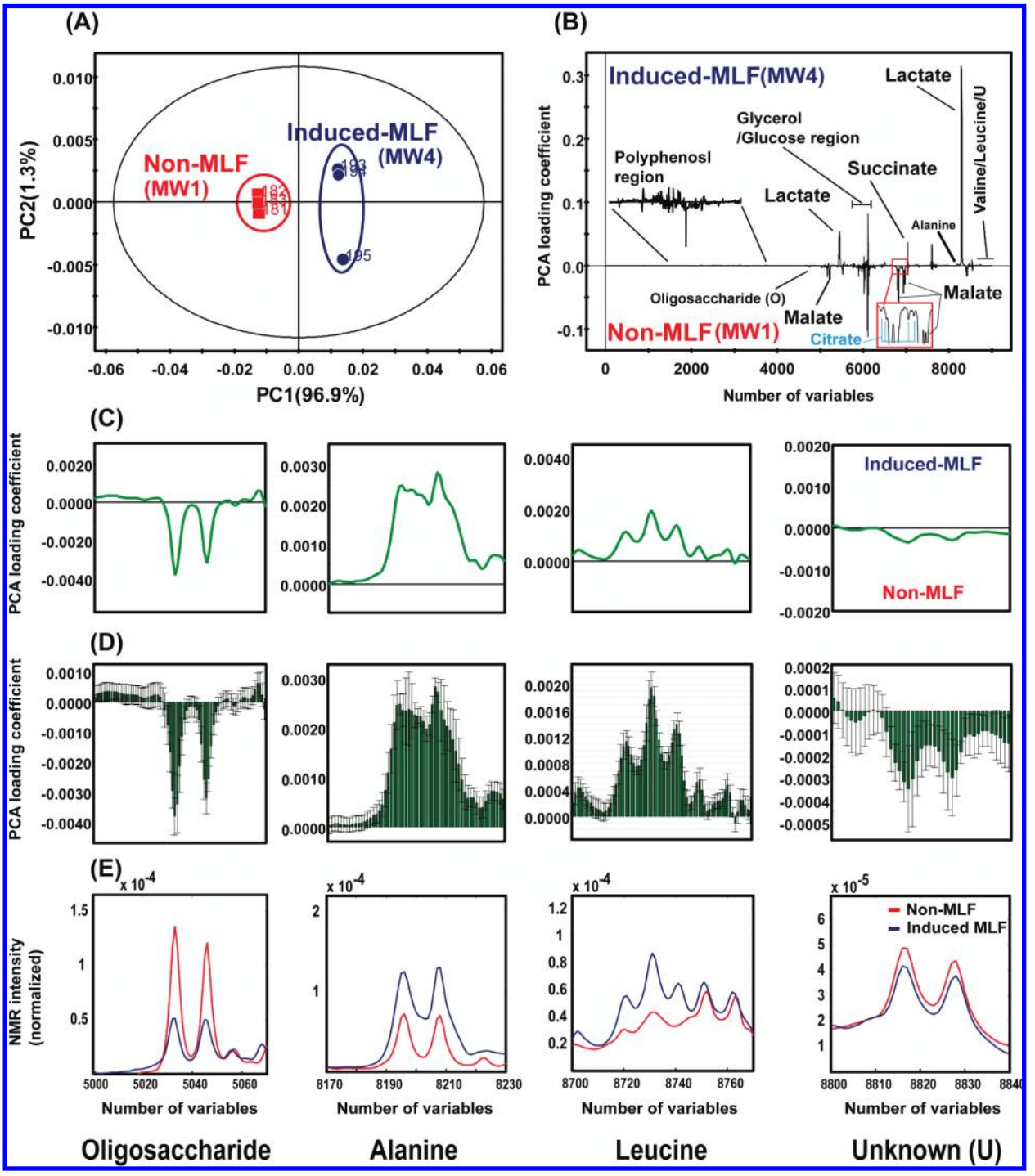

Figure 3. PCA score (**A**) and loading (**B**) plots derived from the ^{1}H NMR spectra of Meoru wines with nonmalolactic fermentation (MLF) (MW1) and induced-MLF (MW4) with *O. oeni* after completion of alcoholic fermentation by *S. bayanus* PC. PCA score plot shows clear differentiation between non- and induced-MLF wines with high goodness of fit and predictability of the model as indicated by R_X^2 of 0.99 and Q^2 of 0.98, respectively. The PCA line loading plots (**C**) highlight the expansions of typical metabolites from (**B**); from right, unknown compounds (U) at 0.88 ppm (doublet) and 0.94 ppm (doublet), leucine, alanine, and oligosaccharide (O). (**D**, **E**) PCA column loading plot with the bar and the mean spectra, respectively, corresponding to each metabolite in (**C**). The smaller the bar, the higher the confidence limit in PCA column loading plots (**D**). Although lactate, which is the main product during MLF, with its high loading coefficient, was excluded for the PCA, separation between non- and induced-MLF wines was still observed (data not shown), indicating that other metabolites with low loading coefficients also significantly contribute to the separation.

S. bayanus PC versus S. cerevisiae CDB. **Figure 4A** shows the PCA score plot obtained by ^{1}H NMR spectra of Meoru wines fermented with *O. oeni* after alcoholic fermentation with *S. bayanus* PC (MW4) and *S. cerevisiae* CDB (MW5) and shows the clear separation between MW4 and MW5 by (PC1, which accounted for 80.7% of the total variance of the data set. The PCA loading plot revealed the metabolites responsible for the separation. There were distortions in the metabolite resonances of lactate and glycerol in the loading plot (**Figure 4B**). The distortion could be observed mainly from incomplete spectral alignment. However, differences in the intensities between the distorted resonances, such as lactate and glycerol, in the loading plots were not found when we considered the confidence limit and mean spectra as described in **Figure 3**. To avoid the effect of the

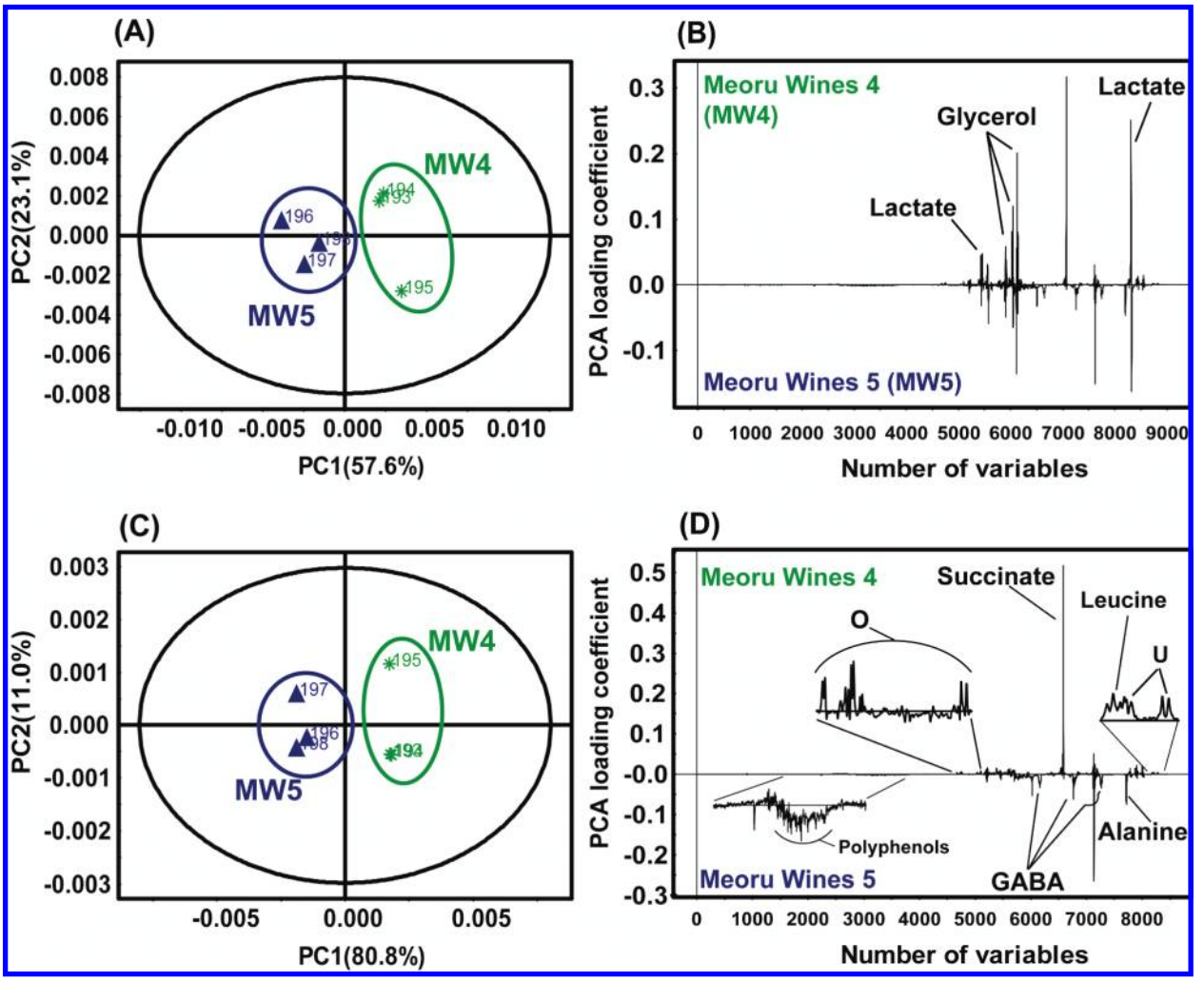

Figure 4. PCA score (**A**, **C**) and loading (**B**, **D**) plots derived from the ^{1}H NMR spectra of Meoru wines with induced-MLF, followed by alcoholic fermentation with *S. bayanus* PC (MW4) and *S. cerevisiae* CDB (MW5). Exclusion of lactate and glycerol (**D**), the resonances of which were distorted in the loading plot (**B**) mainly due to incomplete spectral alignment, provided the PCA model with increased goodness of fit (R_X^2) from 0.81 to 0.91 and predictability (Q^2) from 0.44 to 0.78. Note that there were no significant differences in the levels of the metabolites with distorted resonances between MW4 and MW5 wines, even though the metabolites had high values of PCA loading coefficients. GABA, γ-aminobutyrate; U, unknown compound at 0.88 ppm (doublet) and 0.94 ppm (doublet); O, oligosaccharides.

distorted metabolites on the PCA model, these metabolites were excluded and the PCA score plot was then regenerated as shown in **Figure 4C**. After exclusion of the distorted metabolites, the total variance of the data set increased from 80.7 to 91.9% and the variance of PC1 from 57.6 to 80.8%. Furthermore, the statistical values of R_X^2 and Q^2 also increased from 0.81 to 0.92 and from 0.44 to 0.78, respectively, demonstrating an increase in the goodness of fit and the predictability of the PCA model. It is therefore recommended that distorted metabolites with high loading coefficients, such as glycerol and lactate in this study, can be excluded in the PCA model. The PCA loading plot showed that the separation between MW4 and MW5 by PC1 was caused by increased levels in U, leucine, succinate, and glucose in MW4, together with increases in alanine, γ-aminobutyric acid (GABA), and polyphenol levels in MW5 (**Figure 4D**).

S. bayanus PC versus S. cerevisiae KUBY-501. The PCA score plot also showed clear separation between Meoru wines with MLF, following alcoholic fermentation with *S. bayanus* PC (MW4) and *S. cerevisiae* KUBY-501 (MW6), as shown in **Figure 5A**, accounting for 95.0% of the total variance of the data set with high values of R_X^2 of 0.95 and Q^2 of 0.87. The metabolites responsible for the separation between MW4 and MW6 were increased levels of leucine, valine, 2,3-butanediol, alanine, succinate, and proline in MW4 wines (**Figure 5B**).

S. cerevisiae CDB versus S. cerevisiae KUBY-501. Meoru wines fermented with *S. cerevisiae* CDB (MW5) and with *S. cerevisiae* KUBY-501 (MW6) were also clearly differentiated as shown in the PCA score plot (**Figure 5C**). On the PCA loading plot in **Figure 5D**, the metabolites responsible for the separation of MW5 from MW6 were increased levels of leucine, valine, 2,3-butanediol, alanine, GABA, and polyphenols together with decreased levels of U, succinate, glycerol, and O in MW5.

It was interesting to note that proline levels were lowest in MW6 compared to those in MW4 and MW5. This indicates that strains *S. bayanus* PC (MW4) and *S. cerevisiae* CDB (MW5) synthesize proline or that strain *S. cerevisiae* KUBY-501 (MW6) utilizes proline during the fermentation. It is well-known that proline is not taken up by wine yeast under anaerobic fermentative conditions (*27*). In addition, the proline content in wine depends on grape variety (*28*, *29*). Our previous study (*1*) showed that Australian Cabernet Sauvignon wines contain much higher levels of proline than Korean Campbell Early wines on the basis of the metabolomic study using NMR spectroscopy coupled with multivariate statistical analysis; thus, proline was not consumed by wine yeast, and its levels depended on grape variety and

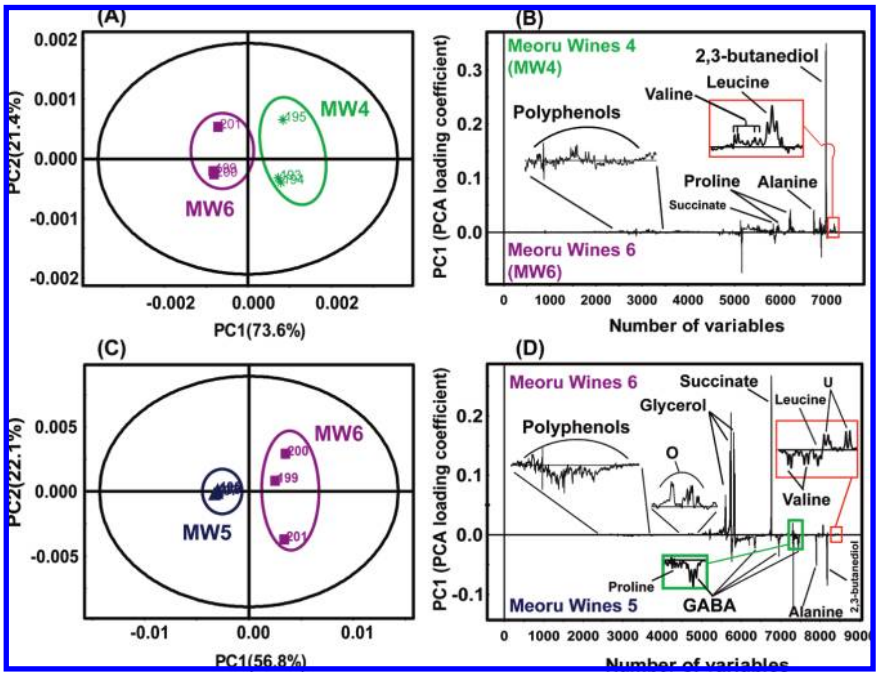

Figure 5. PCA score (**A**, **C**) and loading (**B**, **D**) plots derived from the ^{1}H NMR spectra of Meoru wines with induced-MLF, following alcoholic fermentation by *S. bayanus* PC (MW4), *S. cerevisiae* CDB (MW5), and *S. cerevisiae* KUBY-501 (MW6). Distorted resonances corresponding to lactate and glycerol were excluded in PCA loading plot **B**, whereas only lactate resonances were excluded in PCA loading plot **D**, ensuring no significant differences and improvements of goodness of fit (R_X^2) and predictability (Q^2) as described in **Figure 4**. GABA, γ-aminobutyrate; U, unknown compound at 0.88 ppm (doublet) and 0.94 ppm (doublet); O, oligosaccharides.

production area. According to recent reports (*30*), overexpression of the gene alcohol dehydrogenase II (ADH2) of *S. bayanus* yielded 10 times more L-proline from L-glutamate through de novo synthesis and increased the levels of acetate, reducing the levels of 2,3-butanediol compared to those in the control strain. In the present study, differences in the levels of 2,3-butanediol between MW5 and MW6 were not significant. In contrast to proline synthesis by ADH2, proline is converted to glutamate in mitochondria of *S. cerevisiae* by proline oxidase and pyrroline-5-carboxylate dehydrogenase (*31*). Therefore, different levels of proline among MW4 with *S. bayanus* PC, MW5 with *S. cerevisiae* CDB, and MW6 with *S. cerevisiae* KUBY-501 might be due to different activities of enzymes related to synthesis or catabolism of proline. The differences in proline levels according to yeast strain, however, seem to be negligible compared to those by grape variety or production area.

Amino acids, including leucine, valine, and alanine, and 2, 3-butanediol also contributed to separation between Meoru wines fermented with various yeast strains as shown in PCA score and loading plots in **Figures 4** and **5**. In pairwise comparison of the loading plots, higher levels of leucine and valine were observed in MW4 and MW5 compared to those in MW6, indicating accumulation of α-acetolactate of acetoinic compounds because α-acetolactates are precursors of leucine and valine (*23*). Higher levels of 2,3-butanediol in MW4 and MW5 also indicated accumulation of the acetoinic compounds by strains *S. baynaus* PC and *S. cerevisiae* CDB compared to strain *S. cerevisiae* KUBY-501. Levels of alanine, leucine, and valine were lowest in MW6. This indicated that *S. cerevisiae* KUBY-501 grew slowly compared to *S. bayanus* PC and *S. cerevisiae* CDB because amino acids are precursors or products of protein synthesis indicating bacterial growth (*32*).

Levels of succinate and U at 0.88 ppm (doublet) and 0.94 ppm (doublet), which may be an intermediate in the metabolism of valine and 2-isopropylmalate, were lowest in Meoru wines (MW5) fermented with *S. cerevisiae* CDB. The stability of succinate levels has been reported during aging of wines (*33*), and levels of U have been found to be highest in wines with active fermenting yeast strains in our previous study (*2*). Therefore, *S. cerevisiae* CDB appears to have the lowest fermentative behavior compared with *S. bayanus* PC and *S. cerevisiae* KUBY-501. In addition, levels of polyphenol compounds were highest and O levels were lowest in wines with *S. cerevisiae* CDB strain, indicative of higher activities of pectic enzymes in *S. cerevisiae* CDB than in *S. bayanus* PC and *S. cerevisiae* KUBY-501. Polygalacturonases in *S. cerevisiae* yeast catalyze the hydrolysis of pectins on grape skin, which favors polyphenol extraction (*34*).

It was also interesting to note that markedly elevated levels of GABA were significant in discriminating MW5 from MW4 and MW6 in PCA loading plots in **Figures 4** and **5**, which is also apparent on inspection of the typical spectra in **Figure 1**. Thus, the GABA contents were dependent on the yeast strain because all vinification conditions, including the grapes, were the same in this study. GABA is a four-carbon non-protein amino acid that is conserved from bacteria to plants and vertebrates and is largely and rapidly produced via GABA shunt in response to biotic and abiotic stresses in plants (*35*, *36*). In general, *S. cerevisiae* can utilize GABA through GABA transaminase and succinic semialdehyde dehydrogenase to produce α-ketoglutarate and succinate, respectively (*37*). In contrast, GABA accumulation in growing cultures of *S. cerevisiae* has been reported, and its concentration depends on the source of nitrogen available during growth, the pH, and the concentration of solutes in the medium (*38*). Accumulated GABA in the intracellular pool can be released into the extracellular environment during autolysis (*39*). However, further investigation is needed to determine the dependence of GABA accumulation on yeast strain. In the present study, spontaneous MLF by *O. oeni* was observed in wines fermented with *S. cerevisiae* CDB and KUBY-501, but not in wines fermented with *S. bayanus* PC. In general, in wine MLF occurs spontaneously weeks or several months after alcoholic fermentation by yeast strains. *O. oeni* of LAB is well adapted to

survival and growth in wine, although the wine represents a harsh environment for bacterial growth. The spontaneous growth of *O. oeni* strains in wine is related to their ability to use various energy sources such as sugars, malate, and amino acids (*5*) and to tolerate acidity, SO_2, ethanol, pH, and temperature (*7*−*9*). Cryotolerant yeast strains are used as starters in winemaking from must lacking acidity because they produce more glycerol, succinate, and β-phenylethanol and less acetate and ethanol (*40*, *41*). Succinate and β-phenylethanol exert an inhibitory effect on LAB growth (*42*). In the present study, the higher levels of succinate in wines fermented with *S. bayanus* PC compared to those in wines fermented with *S. cerevisiae* demonstrate the providence of its inhibitory effect against spontaneous MLF. In addition, metabolites, which are dependent on the fermentative behaviors of yeast strains, suggest, for example, that *S. cerevisiae* CDB has the lowest fermentative behavior, resulting in the lowest levels of succinate and U, and that *S. cerevisiae* KUBY-501 grows most slowly, resulting in the lowest levels of amino acids, including alanine, leucine, and valine. Therefore, this metabolomic study using multivariate statistical analysis of ^{1}H NMR spectral data provides insights into MLF by LAB and alcoholic fermentation by wine yeast strains.

LITERATURE CITED

(1) Son, H. S.; Kim, K. M.; van den Berg, F.; Hwang, G. S.; Park, W. M.; Lee, C. H.; Hong, Y. S. ^{1}H NMR-based metabolomic characterization of wines by grape varieties and production areas. *J. Agric. Food Chem*. **2008**, *56*, 8007–8016.

(2) Son, H. S.; Hwang, G. S.; Kim, K., M.; Kim, E. Y.; van den Berg, F.; Park, W. M.; Lee, C. H.; Hong, Y. S. ^{1}H NMR-based metabolomic approach for understanding the fermentation behaviors of wine yeast strains. *Anal. Chem*. **2009**, *81*, 1137–1145.

(3) Mateo, J. J.; Jimenez, M.; Pastor, A.; Huerta, T. Yeast starter cultures affecting wine fermentation and volatiles. *Food Res. Int*. **2001**, *34*, 307–314.

(4) Heard, G. M.; Fleet, G. H. Occurrence and growth of yeast species during the fermentation of some Australian wines. *Food Technol*. **1986**, *38*, 22–25.

(5) Wibowo, D.; Eshenbruch, R.; Davis, C. R.; Fleet, G. H.; Lee, T. H. Occurrence and growth of lactic acid bacteria in wine: a review. *Am. J. Enol. Vitic*. **1985**, *36*, 301–313.

(6) Bartowsky, E. J.; Costello, P.; Henschke, P. Management of malolactic fermentation-wine flavour manipulation. *Aust. N. Z. Grapegrower Winemaker* **2002**, *7*−*8*, 10–12.

(7) Lopez, I.; Tenorio, C.; Zarazga, M.; Dizy, M.; Torres, C.; Ruiz-larrea, F. Evidence of mixed wild populations of *Oenococcus oeni* strains during wine spontaneous malolactic fermentations. *Eur. Food Res. Technol*. **2007**, *226*, 215–223.

(8) Bou, M.; Brown, N.; Costello, P.; Degre, R.; Dieterich, W.; Gertsen-Briand, S.; Kollar, S.; Krieger, S.; Kyne, A.; Loubser, P.; Morenzoni, R.; Palacios, A.; Powell, C.; Specht, K. S.; Specht, G.; Theodore, D.; Van Zandycke, S. *Malolactic Fermentation in Wine*; Lallemand: Montreal, Canada, 2005.

(9) Nicholson, J. K.; Lindon, J. C. Systems biology: metabonomics. *Nature (London)* **2008**, *455*, 1054–1056.

(10) Coen, M.; Hong, Y. S.; Clayton, T. A.; Rohde, C. M.; Pearce, J. T.; Reily, M. D.; Robertson, D. G.; Holmes, E.; Lindon, J. C.; Nicholson, J. K. The mechanism of galactosamine toxicity revisited; a metabonomic study. *J. Proteome Res*. **2007**, *6*, 2711–2719.

(11) Brindle, J. T.; Antii, H.; Holmes, E.; Tranter, G.; Nicholson, J. K.; Bethell, H. W.; Clarke, S.; Schofield, P. M.; McKilligin, E.; Mosedale, D. E.; Grainger, D. J. Rapid and noninvasive diagnosis of the presence and severity of coronary heart disease using ^{1}H NMR-based metabonomics. *Nat. Med*. **2002**, *8*, 1439–1444.

(12) Holmes, E.; Loo, R. L.; Stamler, J.; Bictash, M.; Yap, I. K. S.; Chan, Q.; Ebbels, T.; De Iorio, M.; Brown, I. J.; Veselkov, K. A.; Daviglus, M. L.; Kesteloot, H.; Ueshima, H.; Zhao, L. C.; Nicholson, J. K.; Elliott, P. Human metabolic phenotype diversity and its association with diet and blood pressure. *Nature (London)* **2008**, *453*, 396–400.

(13) Wang, Y.; Tang, H.; Nicholson, J. K.; Hylands, P. J.; Sampson, J.; Holmes, E. A metabonomic strategy for the detection of the metabolic effects of chamomile (*Matricaria recutita* L.) ingestion. *J. Agric. Food Chem*. **2005**, *53*, 191–196.

(14) Larsen, F. H.; van den Berg, F.; Engelsen, S. B. An exploratory chemometric study of ^{1}H NMR spectra of table wines. *J. Chemometr*. **2006**, *20*, 198–208.

(15) Hotelling, H. The generalization of Student's ratio. *Ann. Math. Stat*. **1931**, *2*, 360–378.

(16) Trygg, J.; Wold, S. Orthogonal projections to latent structures (O-PLS). *J. Chemom*. **2002**, *16*, 119–128.

(17) Nygaard, M.; Prahl, C. Compatibility between strains of *Saccharomyces cerevisiae* and *Leuconostoc oenos* as an important factor for successful malolactic fermentation. In *The Fourth International Symposium on Cool-Climate Viticulture and Oenology*, Rochester, NY, 16−20 July, 1996; pp V1-103−V1-106.

(18) Ugliano, M.; Moio, L. Changes in the concentration of yeast-derived volatile compounds of red wine during malolactic fermentation with four commercial starter cultures of *Oenococcus oeni*. *J. Agric. Food Chem*. **2005**, *53*, 10134–10139.

(19) Avenzoza, A.; Busto, J. H.; Canal, N.; Peregina, J. M. Time course of the evolution of malic and lactic acids in the alochol and malolactic fermentation of grape must by quantitative ^{1}H NMR (qHNMR) spectroscopy. *J. Agric. Food Chem*. **2006**, *54*, 4715–4720.

(20) Lonvaud-Funel, A. Lactic acid bacteria in the quality improvement and depreciation of wine. *Antonie van Leeuwenhoeck* **1999**, *76*, 317–331.

(21) Pozo-Bayón, M. A.; Alegría, E. G.; Polo, M. C.; Tenorio, C.; Martín-Álvarez, P. J.; De La Banda, M. T. C.; Ruiz-Larrea, F.; Moreno-Arribas, M. V. Wine volatile and amino acid composition after malolactic fermentation: Effect of *Oenococcus oeni* and *Lactobacillus plantarum* starter cultures. *J. Agric. Food Chem*. **2005**, *53*, 8729–8735.

(22) Salema, M.; Capucho, I.; Poolman, B.; Romao, M. V. S.; Dias, M. C. L. In vitro reassembly of the malolactic fermentation pathway of *Leuconostoc oenos* (*Oenococcus oeni*). *J. Bacteriol*. **1996**, *178*, 5537–5539.

(23) Bartowsky, E. J.; Henschke, P. A. The 'buttery' attribute of wine-diacetyl-desirability, spoilage and beyond. *Int. J. Food Microbiol*. **2004**, *96*, 235–252.

(24) Poolman, B. Energy transduction in lactic acid bacteria. *FEMS Microbiol. Rev*. **1993**, *12*, 125–148.

(25) Remize, F.; Augagneur, Y.; Guilloux-Benatier, M.; Guzzo, J. Effect of nitrogen limitation and nature of the feed upon *Oenococcus oeni* metabolism and extracellular protein production. *J. Appl. Microbiol*. **2005**, *98*, 652–661.

(26) Kunkee, R. E. Some roles of malic acid in the malolactic fermentation in wine making. *FEMS Microbiol. Lett*. **1991**, *88*, 55–71.

(27) Boulton, B.; Singleton, Y. L.; Bisson, L. F.; Kunkee, R. E. Yeast and biochemistry of ethanol fermentation. In *Principles and Practices of Winemaking*; Boulton, B., Singleton, Y. L., Bisson, L. F., Kunkee, R. E., Eds.; Chapman and Hall: New York, 1996; pp 139−172.

(28) Stines, A. p.; Grubb, J.; Gockowiak, H.; Henschke, P.; Hoj, P. B.; Heewijck, R. V. Proline and arginine accumulation in developing berries of *V. vinifera* in Australian vineyards: influence of vine cultivar, berry maturity and tissue type. *Aust. J. Grape Wine Res*. **2000**, *6*, 150–158.

(29) Du Toit, P. G.; Dry, P. R.; Loveys, B. R. A preliminary investigation on partial rootzone dying (PRD) effects on grapevine performance, nitrogen assimilation and berry composition. *S. Afr. J. Enol. Vitic*. **2003**, *24*, 43–54.

(30) Maestre, O.; García-martínez, T.; Peinado, R. A.; Mauricio, J. C. Effects of ADH2 overexpression in *Saccharomyces bayanus* during alcohol fermentation. *Appl. Environ. Microbiol*. **2008**, *74*, 702–707.

(31) Salmon, J.-M.; Barre, P. Improvement of nitrogen assimilation and fermenatin kinetics under enological conditions by derepression of alternative nitrogen-assimilatory pathways in an industrial *Saccharomyces cerevisiae* strain. *Appl. Environ. Microbiol*. **1998**, *64*, 3831–3837.

(32) Zapparoli, G.; Moser, M.; Dellaglio, F.; Tourdot-Maréchal, R.; Guzzo, J. Typical metabolic traits of two *Oenococcus oeni* strains isolated from Valpolicella wines. *Lett. Appl. Microbiol*. **2004**, *39*, 48–54.
(33) Thoukis, G. M.; Ueda, M.; Wright, D. The formation of succinic acid during alcoholic fermentation. *Am. J. Enol. Vitic*. **1965**, *16*, 1–8.
(34) Blanco, P.; Sieiro, C.; Villa, T. G. Production of pectic enzymes in yeast. *FEMS Microbiol. Lett*. **1999**, *175*, 1–9.
(35) Shelp, B. J.; Bown, A. W.; McLean, M. D. Metabolism and functions of γ-aminobutyric acid. *Trends Plant Sci*. **1999**, *4*, 446–452.
(36) Bouche, N.; Fromm, H. GABA in plants: just a metabolite?. *Trends Plant Sci*. **2004**, *9*, 110–115.
(37) Pietruszko, R.; Fowden, L. γ-Aminobutyric acid metabolism in plants. 1. Metabolism in yeasts. *Ann. Bot*. **1961**, *25*, 491–511.
(38) McKelvey, J.; Rai, R.; Cooper, T. G. GABA transport in *Saccharomyces cerevisiae*. *Yeast* **1990**, *6*, 263–270.
(39) Martinez-Rodriguez, A. J.; Carrascosa, A. V.; Polo, M. C. Release of nitrogen compounds to the extracellular medium by three strains of *Saccharomyces cerevisiae* during induced autolysis in a model wine system. *J. Food Microbiol*. **2001**, *68*, 155–160.
(40) Castellari, L.; Ferruzzi, M.; Magrini, A.; Giudici, P.; Passarelli, P.; Zambonelli, C. Unbalanced wine fermentation by cryotolerant vs. non-cryotolerant *Saccharomyces* strains. *Vitis* **1994**, *33*, 49–52.
(41) Bertolini, L.; Zambonelli, C.; Giudici, P.; Castellari, L. Higher alcohol production by cryotolerant *Saccharomyces* strains. *Am. J. Enol. Vitic*. **1996**, *47*, 343–345.
(42) Caridi, A.; Corte, V. Inhibition of malolactic fermentaion by cryotolerant yeasts. *Biotechnol. Lett*. **1997**, *19*, 723–726.

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