




Article

Chemical and Sensory Profiles of Merlot Wines Produced by Sequential Inoculation of *Metschnikowia pulcherrima* or *Meyerozyma guilliermondii*

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Abstract: Inoculation of selected non-*Saccharomyces* yeasts with *Saccharomyces cerevisiae* as means to produce Merlot wines with reduced ethanol contents was investigated. Fermentations of grape musts (25.4° Brix, pH 3.50, and 4.23 g/L titratable acidity) were conducted in stainless steel tanks inoculated with *Metschnikowia pulcherrima* strains P01A016 or NS-MP or *Meyerozyma guilliermondii* P40D002 with *S. cerevisiae* Syrah added after three days. After fermentation, wines with *Mt. pulcherrima* contained 13.8% (P01A016) or 13.9% (NS-MP) *v/v* ethanol, respectively, amounts which were lower than in wines with *S. cerevisiae* alone (14.9% *v/v*). Delayed inoculation of must with *S. cerevisiae* (day 3) or musts with *My. guilliermondii* contained elevated concentrations of ethyl acetate (145 and 148 mg/L, respectively), concentrations significantly higher than those with *S. cerevisiae* inoculated on day 0 or with either strain of *Mt. pulcherrima*. Descriptive sensory analysis revealed a significant effect due to panelist but not due to *Mt. pulcherrima* or *My. guilliermondii*. This research indicates the potential for commercial application of these yeasts towards the production of reduced alcohol wines but without imparting negative sensory attributes.

Keywords: *Metschnikowia pulcherrima*; *Meyerozyma guilliermondii*; fermentation; ethanol



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1. Introduction

The use of non-*Saccharomyces* yeasts, in particular species of *Metschnikowia*, have gained popularity within the wine industry globally as a means to alter sensory profiles [1,2]. These yeasts can positively contribute to wine quality and complexity [3–5] through synthesis of compounds responsible for ‘fruity’ and/or ‘floral’ aromas [6–8]. In addition, many synthesize glycosidases and/or pectinases that can release varietal grape aromas as well as hydrolyze polysaccharides [9,10]. Conversely, however, wine quality can also be decreased through production of ethyl acetate, acetic acid, acetaldehyde, and/or other undesirable aroma or flavor compounds [6,11]. Growth of these yeasts in grape musts may also consume key nutrients important for *Saccharomyces cerevisiae*, resulting in increased risks associated with sluggish or stuck fermentations [12–14]. Several commercial preparations of *Metschnikowia* are now available to winemakers [2].

More recently, a number of studies have identified certain species of non-*Saccharomyces* yeasts, in particular, *Metschnikowia pulcherrima*, whose early growth can produce wines with less alcohol [2,5,8,15–21]. Canonico et al. [16] noted that these yeasts consume sugar through respiro-fermentative metabolisms (Crabtree negative) whereas metabolized carbon from sugars can be diverted to by-products other than ethanol. However, differences in response exist as reported by García et al. [22] and Hranilovic et al. [23] who found that $\leq 50\%$ of *Mt. pulcherrima* strains examined yielded lower ethanol concentrations. Evaluating a number of different species isolated from Washington State vineyards [24], Aplin et al. [20] and Aplin and Edwards [21] reported that *Mt. pulcherrima* as well as a

strain of *Meyerozyma guilliermondii* produced lower alcohol wines without large amounts of residual sugar (≤ 0.6 g/L) or acetic acid (≤ 0.25 g/L). Subsequent inoculation of *S. cerevisiae* is still required to complete fermentation because non-*Saccharomyces* yeasts are typically incapable of consuming all the available sugar in a grape must [1,9,20].

As many studies regarding use of *Mt. pulcherrima* were conducted in smaller laboratory-scale fermentations, often <1 L [5,15,19,25,26], few studies have been conducted under larger, pilot winery-scale conditions. Furthermore, while the sensory effects of different strains of *Mt. pulcherrima* have been reported [3–5,27], impacts of *My. guilliermondii* on wine quality have not been described. Thus, the objectives of this research were to evaluate the influence of *Mt. pulcherrima* and *My. guilliermondii* on ethanol production and quality of wines produced under pilot-scale winery conditions.

2. Materials and Methods

2.1. Yeast Strains

Mt. pulcherrima P01A016 and *My. guilliermondii* P40D002 were previously isolated from vineyards located at the Irrigated Research and Extension Center (Prosser, WA, USA) as described by Bourret et al. [24]. Additional strains of *Mt. pulcherrima* (NS-MP) and *S. cerevisiae* (Enoferm Syrah) were acquired from Lallemand Inc. (Montréal, QC, Canada). All yeasts were maintained on yeast peptone dextrose (YPD) agar plates containing 10 g/L yeast extract (Becton, Dickinson, and Company, Sparks, MD, USA), 20 g/L peptone (Becton, Dickinson, and Company, Sparks, MD, USA), 20 g/L glucose (Thermo Fisher Scientific, Waltham, MA, USA), and 15 g/L agar (Acros Organics, Morris, NJ, USA).

Non-*Saccharomyces* yeast starter cultures were prepared by inoculating yeast/mold (YM) broth (Becton, Dickinson, and Company, Sparks, MD) from single colonies grown on YPD agar. Upon reaching the late exponential phase, cells were harvested by centrifugation at $2000 \times g$ for 20 min, washed twice with 0.2 M Na_2HPO_4 (pH 7.0) buffer, then resuspended in buffer prior to inoculation. Active dry yeast cultures of *S. cerevisiae* were rehydrated according to manufacturer's instructions.

2.2. Merlot Grape Must

Merlot grapes were hand-harvested 22 September 2016 from vineyards located at the Irrigated Agriculture Research and Extension Center (Washington State University, Prosser, WA, USA) and processed using a stainless steel crusher-destemmer (approximately 1300 kg/h). Immediately after crushing, 20 mg/L total SO_2 were added as $\text{K}_2\text{S}_2\text{O}_5$ to 37.5 kg of must which had been placed in 300 L stainless steel, jacketed tanks. The must contained 264 g/L glucose and fructose, pH 3.50, 4.23 g/L titratable acidity (as tartaric acid), 198 mg N/L (yeast assimilable nitrogen or YAN). Diammonium phosphate was added at a rate of 0.2 g/kg to increase available nitrogen to 250 mg N/L. Musts were then either uninoculated or inoculated at 10^6 cfu/mL with *Mt. pulcherrima* P01A016, *Mt. pulcherrima* NS-MP, *My. guilliermondii* P40D002, or *S. cerevisiae*, in triplicate. After three days, *S. cerevisiae* (10^6 cfu/mL) into those treatments not previously inoculated with this yeast. Fermentations were conducted at cellar temperature (approximately 20 °C), without heating or chilling, with 1-min daily punch-downs using a stainless-steel punch-down tool. At approximately 18° Brix, 0.2 g/kg Fermid-K (Lallemand Inc.) was added as a nitrogen supplement to all tanks while fermentations were pressed at approximately 0° Brix using a hydraulic bladder press (Speidel, Ofterdingen, Germany). Once dry (<2 g/L reducing sugars), wines were racked and moved to 3–4 °C after addition of 50 mg/L total SO_2 . After 9 months of storage, enough $\text{K}_2\text{S}_2\text{O}_5$ was added to achieve 0.8 mg/L molecular SO_2 prior to bottling without filtering. Bottled wines were kept at 4 °C to limit potential microbial spoilage for at least 4 months before analyses.

2.3. Chemical and Microbiological Analyses

Yeast culturabilities were monitored by spiral plating using an Autoplate 4000 (Spiral Biotech, Bethesda, MD, USA) and both Wallenstein Laboratory nutrient agar for total yeast

populations (Becton, Dickinson, and Company, Franklin Lakes, NJ, USA) and lysine agar for non-*Saccharomyces* yeasts (Oxoid, Hampshire, UK). Plates were incubated at 28 °C for two days prior to enumeration. *S. cerevisiae* populations were estimated as differences between culturabilities on the two media.

Fermentations were monitored by measuring soluble solids with an Anton Paar DMA35 density meter (Anton Paar USA Inc., Ashland, VA, USA). Volatile acidity (Cash still), titratable acidity, residual sugars (Clinitest® method), and free and total SO₂ (aeration/oxidation) were measured while molecular SO₂ was calculated following standard methods [28]. Yeast assimilable nitrogen (YAN) was calculated as the sum of primary amino nitrogen according to Dukes and Butzke [29] and ammonium by an ion-selective probe (Denver Instruments, Orville, NY, USA).

Concentrations of glucose, fructose, glycerol, and organic acids were quantified by an Agilent 1100 HPLC system equipped with an Aminex HPX-87H column (300 × 7.8 mm, BIO-RAD, Hercules, CA, USA) equilibrated at 60 °C with 0.005M H₂SO₄ as mobile phase flowing at 0.6 mL/min.

Volatile aroma compounds were analyzed by headspace solid-phase microextraction coupled with gas chromatography-mass spectroscopy as previously described [20]. Briefly, compounds were analyzed with an Agilent HP 6890 gas chromatograph fitted with a DB-1MS column (0.32 mm × 60.0 m, 1.0 µm thickness, Phenomenex, Torrance, CA, USA) linked to an HP 5973 Mass Selective Detector. Fibers were desorbed for 3 min at 250 °C with the injection inlet operating in splitless mode and a carrier gas (He) flow of 0.7 mL/min. The temperature program was as follows: 33 °C for 5 min, increase to 50 °C at 2.0 °C/min, increase to 225 °C as 5.0 °C/min and hold for 5 min. The mass spectrometer was operating in electron impact mode at 70 eV (150 °C ion source). Compounds were identified using the Wiley/NIST library while quantification was accomplished using 4-methyl-2-pentanol and 2-octanol as internal standards.

2.4. Sensory Analyses

Merlot wines were evaluated by a trained panel ($n = 10$, four males and six females aged 24 to 40) consisting of regular wine drinkers recruited from the Washington State University community. Panelists received 12 h of training across six weeks using feedback calibration through Compusense Cloud ver. 8.8 sensory acquisition software (Guelph, ON, Canada). All sensory attributes were illustrated with appropriate reference standards for aroma or flavor ('estery,' 'pungency,' 'fruity,' 'dried fruit,' 'green,' 'yeasty,' 'ethanol,' 'berry,' 'earthy,' 'woody,' 'sulfur,' 'sweaty,' 'chemical,' 'vegetal' or 'barn'), taste ('sweet,' 'bitter' or 'sour'), and mouthfeel ('viscosity,' 'hot,' 'astringent' or 'roughness') as described by Aplin [30]. Samples (40 mL) were presented to panelist in covered three-digit coded ISO standard wine glasses at room temperature in individual tasting booths under white light at the Washington State University Sensory Evaluation Facility. For the final evaluations, ten wines were assessed in duplicate in a randomized order over four evaluation sessions. Five wines were evaluated each session and fresh bottles were opened each day to prevent oxidative changes to wines. To avoid fatigue, panelists were required to take a five-minute break between samples and instructed to rinse their palate with water and an unsalted cracker. Data was collected on a 15 cm unstructured line scale with anchor points 'low' (10% of the scale) and 'high' (90% of the scale) using Compusense software.

2.5. Statistical Analyses

Statistical analyses of chemical analyses were performed by ANOVA while means separations were accomplished by Fisher's LSD using XLSTAT software (Addinsoft, New York, NY, USA). For sensory data, three-way ANOVA was performed to analyze panelist, treatment, and replicate interactions while means were separated using Fisher's Least Significant Difference (LSD) post-hoc test. Differences were considered significant when $p \leq 0.05$.

3. Results

Merlot grape musts were inoculated with *S. cerevisiae* alone at days 0 or 3 (Figure 1) or with *Mt. pulcherrima* P01A016 (Figure 2A), *Mt. pulcherrima* NS-MP (Figure 2B), or *My. guilliermondii* P40D002 (Figure 2C) at day 0 with *S. cerevisiae* added on day 3. Populations of *S. cerevisiae* nearly reached $\approx 10^8$ cfu/mL by day 3 in ferments inoculated with this yeast alone on day 0 (Figure 1A) or by day 7 when inoculation was delayed (Figure 1B). Native non-*Saccharomyces* present in these ferments did not exceed 10^6 cfu/mL with inoculation of *S. cerevisiae* on day 0 (Figure 1A). However, when *S. cerevisiae* was inoculated on day 3, unidentified non-*Saccharomyces* present in the must reached populations approaching 10^7 cfu/mL before entering decline to undetectable levels by day 10 (Figure 1B). In comparison, total populations of non-*Saccharomyces* yeasts in musts inoculated with *Mt. pulcherrima* P01A016, *Mt. pulcherrima* NS-MP, or *My. guilliermondii* P40D002 approached one log higher, 10^8 cfu/mL, by day 3 prior to decreases to $\approx 10^3$ cfu/mL by day 10 (Figure 2). In these fermentations, *S. cerevisiae* approached 10^8 cfu/mL by day 7 and remained culturable at day 16.

Chemical compositions of final wines depended on the yeast species present and populations achieved. While ferments inoculated on day 0 with *S. cerevisiae* alone achieved dryness by day 13 (≤ 2 g/L reducing sugar), wines inoculated with non-*Saccharomyces* yeasts or *S. cerevisiae* added on day 3 required approximately three additional days (data not shown). In support, residual concentrations of glucose and fructose reached ≤ 0.13 g/L and were not significantly different between wines (Table 1). Furthermore, amounts of glycerol (10.0 to 10.1 g/L) and succinic acid (1.73 to 1.91 g/L) did not vary depending on the yeast present. While wines inoculated with non-*Saccharomyces* yeasts contained slightly higher titratable acidities and decreased pH, both strains of *Mt. pulcherrima* metabolized some of the malic acid present, approximately 36 to 40%. Concentrations of volatile acidity were similar (*Mt. pulcherrima* P01A016 and *My. guilliermondii* P40D002) or less (*Mt. pulcherrima* NS-MP) than wines inoculated with *S. cerevisiae* alone. In fact, wines inoculated on day 3 with *S. cerevisiae* alone contained the highest concentrations of volatile acidity (0.45 g/L), potentially due to the high populations of unidentified non-*Saccharomyces* yeasts naturally present (Figure 1B). However, wines inoculated with either strain of *Mt. pulcherrima* contained 1.0 to 1.1% *v/v* less ethanol than ferments inoculated with *S. cerevisiae* alone (14.9% *v/v*). The addition of *My. guilliermondii* to grape musts did not affect final alcohol concentration (15.0% *v/v*).

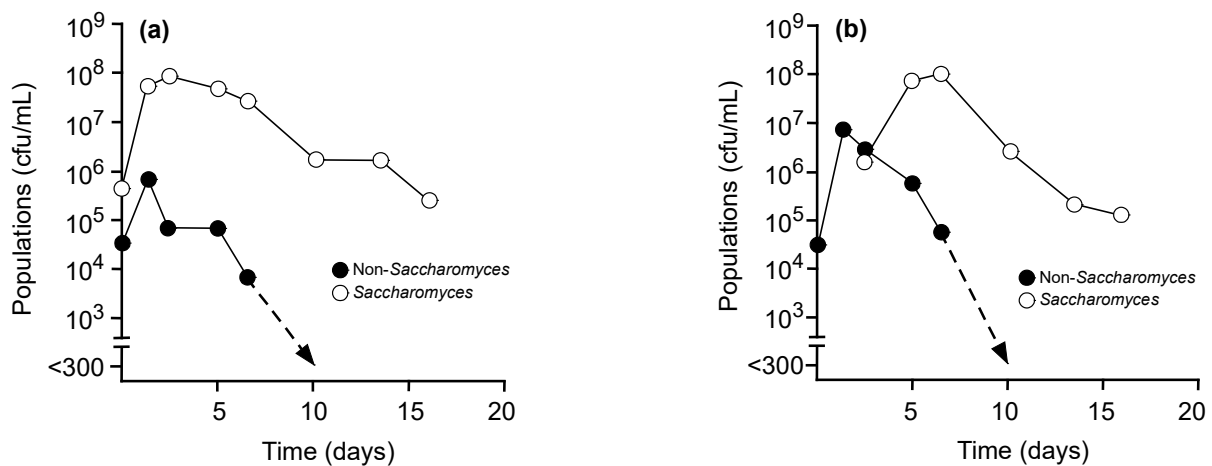


Figure 1. Culturable populations of uninoculated with non-*Saccharomyces* yeasts (●) but with *Saccharomyces cerevisiae* (○) inoculated either on day 0 (a) or day 3 (b) in Merlot grape musts.

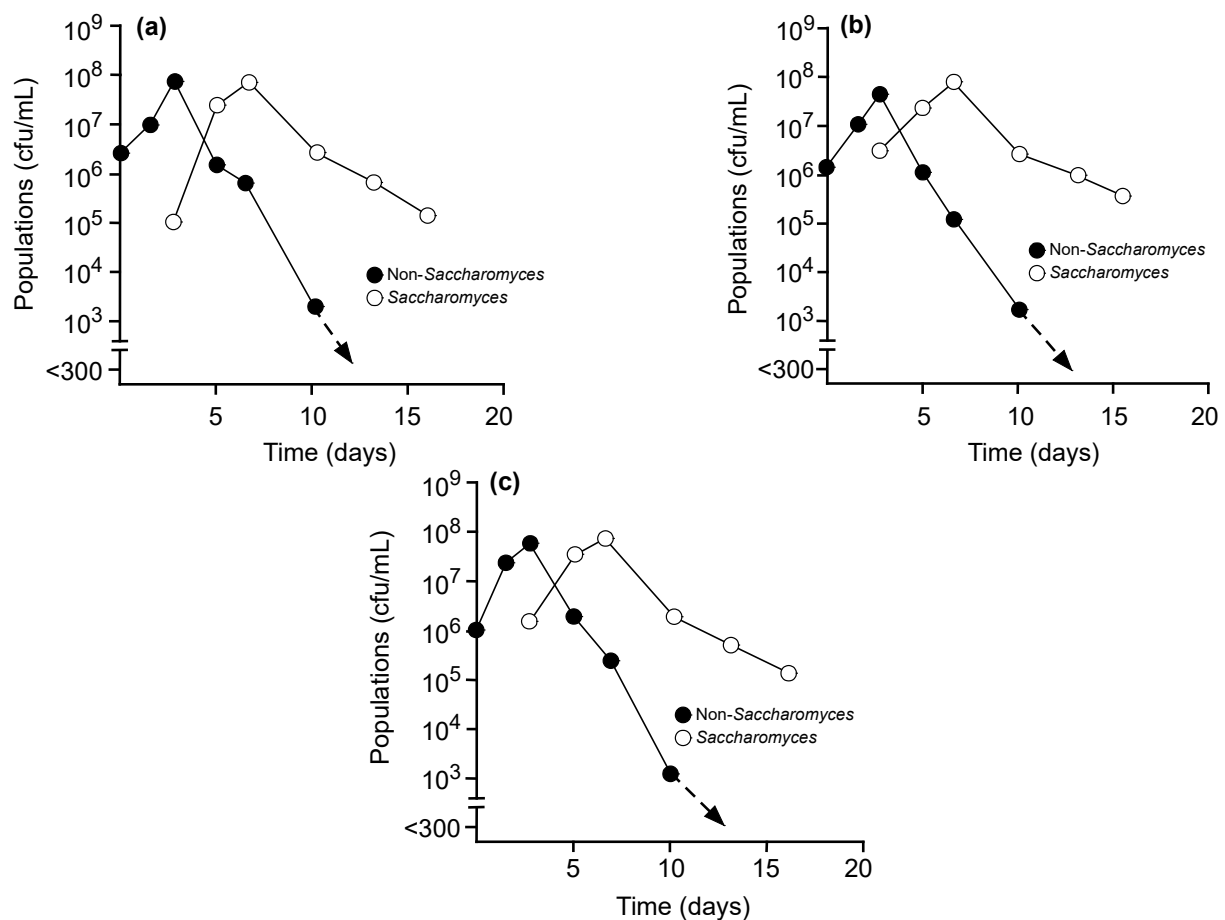


Figure 2. Culturable populations of total non-*Saccharomyces* yeasts (●) or *Saccharomyces cerevisiae* (○) in Merlot grape musts where *Mt. pulcherrima* P01A016 (a), *Mt. pulcherrima* NS-MP (b), or *My. guilliermondii* P40D002 (c) were inoculated on day 0 with *Saccharomyces cerevisiae* added on day 3.

Besides changes to composition previously described, Merlot wines inoculated with non-*Saccharomyces* yeasts exhibited different concentrations of various aroma and/or flavor compounds (Table 1). In general, higher alcohols were more abundant in wines inoculated with non-*Saccharomyces* yeasts, primarily due to increased levels of 2-methyl-1-propanol (61.4 to 70.4 mg/L) and 2- and 3-methyl-1-butanols (137 to 165 mg/L). Wines only inoculated with *S. cerevisiae* at day 0 contained 179 mg/L total higher alcohols, compared to wines with *Mt. pulcherrima* NS-MP (272 mg/L) or *My. guilliermondii* (266 mg/L). Higher concentrations of ethyl acetate were noted where addition of *S. cerevisiae* was delayed until day 3 (145 mg/L) or in the presence of *My. guilliermondii* (145 mg/L) in contrast other wines (52.3 to 73.1 mg/L). Significant differences were not observed regarding concentrations of some esters and medium-chain fatty acids (e.g., hexyl acetate, ethyl butanoate, ethyl hexanoate, and octanoic acid) while a limited number of differences were noted for others (e.g., 2- and 3-methylbutyl acetates, diethyl succinate, 2-phenylethyl acetate, ethyl octanoate, and octanoic acid).

Analysis of variance results for sensory attributes revealed no significant differences attributed to yeast but primarily to panelists (Table 2). In fact, significant panelist effects ($p < 0.05$) were noted for all attributes except 'green,' 'earthy,' 'sweaty,' and 'vegetal' (aromas) or for 'earthy' and 'vegetal' (flavors) with very few differences between aroma, taste, mouthfeel, or flavor attributes (data not shown).

Table 1. Concentration of volatile compounds in Merlot wines inoculated with or without non-*Saccharomyces* yeasts followed by *S. cerevisiae*.

Constituent	Inoculated Yeasts				
	<i>S. cerevisiae</i>	<i>S. cerevisiae</i> *	P01A016 *,†	NS-MP *,†	P40D002 *,†
Ethanol (% v/v)	14.9 ^b	14.9 ^b	13.8 ^a	13.9 ^a	15.0 ^b
Glucose + fructose (g/L)	0.109 ^a	0.114 ^a	0.119 ^a	0.112 ^a	0.130 ^a
pH	3.31 ^{c,d}	3.29 ^c	3.23 ^b	3.21 ^a	3.29 ^{c,d}
Titrate acidity (g/L)	5.56 ^a	5.61 ^a	6.09 ^b	6.09 ^b	6.81 ^c
Volatile acidity (g/L)	0.37 ^{b,c}	0.45 ^d	0.35 ^{a,b}	0.33 ^a	0.40 ^c
Malic acid (g/L)	2.22 ^b	2.18 ^b	1.38 ^a	1.36 ^a	2.29 ^b
Succinic acid (g/L)	1.75 ^a	1.73 ^a	1.91 ^a	1.74 ^a	1.82 ^a
Glycerol (g/L)	10.0 ^a	10.1 ^a	10.1 ^a	10.1 ^a	10.1 ^a
Higher Alcohols (mg/L)					
1-Propanol	2.52 ^a	3.98 ^a	5.33 ^a	3.36 ^a	4.60 ^a
2-Methyl-1-propanol	29.0 ^a	64.5 ^b	61.4 ^b	64.5 ^b	70.4 ^b
2&3-Methyl-1-butanols	108 ^a	112 ^a	137 ^b	165 ^b	144 ^b
1-Hexanol	1.18 ^a	1.20 ^a	0.975 ^a	0.949 ^a	1.18 ^a
1-Octanol	1.84 ^a	0.662 ^a	1.52 ^a	0.735 ^a	1.23 ^a
2-Phenylethanol	36.5 ^a	37.3 ^a	37.9 ^a	37.8 ^a	45.3 ^a
Esters (mg/L)					
Ethyl acetate	52.3 ^a	145 ^b	73.1 ^a	64.1 ^a	148 ^b
2- and 3-Methyl-butyl acetates	0.712 ^a	0.912 ^{a,b}	0.808 ^{a,b}	0.858 ^{a,b}	1.02 ^b
Hexyl acetate	0.007 ^a	0.007 ^a	0.012 ^a	0.009 ^a	0.009 ^a
Diethyl succinate	1.24 ^b	0.787 ^a	0.992 ^{a,b}	1.00 ^{a,b}	1.05 ^{a,b}
2-Phenylethyl acetate	0.016 ^a	0.058 ^{b,c}	0.043 ^{a,b}	0.054 ^{b,c}	0.073 ^c
Ethyl butanoate	0.223 ^a	0.166 ^a	0.193 ^a	0.190 ^a	0.187 ^a
Ethyl hexanoate	0.089 ^a	0.024 ^a	0.073 ^a	0.038 ^a	0.037 ^a
Ethyl octanoate	0.541 ^b	0.353 ^a	0.388 ^{a,b}	0.348 ^a	0.355 ^a
Acids (mg/L)					
Hexanoic acid	4.05 ^c	2.39 ^a	2.79 ^{a,b}	3.90 ^{b,c}	3.09 ^{a,b,c}
Octanoic acid	4.15 ^a	3.13 ^a	3.79 ^a	3.69 ^a	3.63 ^a

* *S. cerevisiae* inoculated on day 3. a–d Means within rows with different superscripts are significant ($p \leq 0.05$). † Strains utilized were P01A016 (*Mt. pulcherrima*), NS-MP (*Mt. pulcherrima*) and P40D002 (*My. guilliermondii*).

Table 2. Significance and F ratios from analysis of variance of trained panel evaluations of aroma, taste, mouthfeel, and flavor sensory attributes in Merlot wines inoculated with or without non-*Saccharomyces* yeasts followed by *S. cerevisiae*.

Source of Variation	Pr > F (p)	Panelist (F)	Yeast (F)	Replicate (F)	Panelist * Yeast (F)
Degrees Freedom		9	4	1	36
Aroma					
‘Estery’	0.108	5.21 ***	0.895	0.709	0.567
‘Pungency’	0.350	3.13 **	1.96	0.716	0.532
‘Fruity’	0.0004 †	7.81 ***	0.695	0.038	1.70
‘Dried fruit’	0.056	5.69 ***	1.48	0.393	0.597
‘Green’	0.709	1.58	0.306	0.005	0.759
‘Yeasty’	0.095	4.56 **	0.861	0.685	0.768
‘Ethanol’	<0.0001 †	17.5 ***	2.10	2.92	0.707
‘Berry’	0.018 †	4.99 ***	1.39	0.043	1.15
‘Earthy’	0.778	0.852	0.847	1.33	0.772
‘Woody’	0.096	4.59 **	0.952	3.88	0.659
‘Sulfur’	0.496	3.97 **	0.374	4.94 *	0.221
‘Sweaty’	0.054	1.91	0.957	0.590	1.61
‘Chemical’	<0.0001 †	12.2 ***	0.911	0.756	1.12
‘Vegetal’	0.190	1.66	2.01	0.017	1.15
‘Barn’	0.044 †	4.72 **	0.949	1.06	0.952

Table 2. Cont.

Source of Variation	Pr > F (p)	Panelist (F)	Yeast (F)	Replicate (F)	Panelist * Yeast (F)
Degrees Freedom		9	4	1	36
Taste					
‘Sweet’	0.014 †	7.03 ***	0.356	0.041	0.815
‘Bitter’	0.0002 †	12.5 ***	0.934	0.478	0.678
‘Sour’	0.001 †	9.02 ***	1.37	0.527	1.057
Mouthfeel					
‘Viscosity’	0.041 †	4.23 **	1.46	0.177	1.06
‘Hot’	0.008 †	7.06 ***	1.62	0.824	0.803
‘Astringent’	<0.0001 †	16.6 ***	1.01	0.857	1.05
‘Roughness’	<0.0001 †	18.5 ***	1.55	0.482	1.25
Flavor					
‘Estery’	0.094	5.25 ***	0.740	2.99	0.550
‘Pungency’	0.001 †	9.88 ***	0.857	0.157	0.958
‘Fruity’	0.222	3.72 **	0.235	0.872	0.749
‘Dried fruit’	0.001 †	8.09 ***	1.72	2.06	1.12
‘Green’	0.007 †	7.26 ***	0.635	0.022	0.949
‘Yeasty’	0.0003 †	8.26 ***	1.29	2.35	1.57
‘Ethanol’	<0.0001 †	16.5 ***	1.71	6.72 *	1.67 *
‘Berry’	0.013 †	4.29 **	1.64	3.40	1.29
‘Earthy’	0.017 †	1.96	1.33	0.21	1.90 *
‘Woody’	0.287	3.01 **	0.740	0.961	0.771
‘Sulfur’	0.088	4.12 **	0.114	8.66 **	0.764
‘Sweaty’	0.020 †	2.41 *	1.43	2.41	1.69 *
‘Chemical’	<0.0001 †	22.3 ***	0.676	0.097	1.31
‘Vegetal’	0.802	0.753	0.645	0.122	0.827
‘Barn’	0.118	4.32 **	1.20	1.27	0.703

† Differences are significant ($p < 0.05$). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$.

4. Discussion

In sequentially inoculated grape musts, the presence of *Mt. pulcherrima* or *My. guilliermondii* did not adversely affect alcoholic fermentation by *S. cerevisiae*. While observations related to the growth of *Mt. pulcherrima* were similar to small volume fermentations [8,20,25], this is the first report describing unsterilized musts inoculated with either *Mt. pulcherrima* or *My. guilliermondii* under larger-scale vinification conditions. Of concern was that initial growth of non-*Saccharomyces* yeasts may metabolize key nutrients present in grape must [12,13], thereby yielding incomplete alcoholic fermentations [8,17,19,31]. In fact, wines with inoculated non-*Saccharomyces* yeasts reached dryness within three days of those musts inoculated with only *S. cerevisiae*. However, an apparent suppression effect against unidentified, native non-*Saccharomyces* yeasts was observed by inoculation of *S. cerevisiae* on day 0. Here, inoculation of *S. cerevisiae* upon preparation of the must resulted in total populations of non-*Saccharomyces* yeasts a log lower compared to ferments with delayed inoculation. Antagonistic effects between yeasts have been thought to be due to competition for nutrients or the production of toxic metabolites [4,8,9,26,27].

Sequential inoculation of *S. cerevisiae* three days after addition of *Mt. pulcherrima* strain P01A016 or NS-MP produced Merlot wines which contained less ethanol (13.8% and 13.9% *v/v*, respectively) compared to wines inoculated only with *S. cerevisiae* (14.9% *v/v*). In agreement, other studies have reported reductions of 0.9 to 2.5% *v/v* by addition of *Mt. pulcherrima*, the amount depending on strain and inoculation protocol [5,8,16,19,23]. Studying six strains of *Mt. pulcherrima*, García et al. [22] noted that only two resulted in wines with lower ethanol concentrations, in agreement with Hranilovic et al. [23] who reported three of six strains yielded lower amounts of ethanol. The study by Hranilovic et al. [23] also highlighted the impact of inoculation timing where addition of *S. cerevisiae* two days after *Mt. pulcherrima* yielded the greatest decrease in ethanol yield, approximately

2.5% *v/v*, compared to additions up to seven days. Earlier work by Contreras et al. [8] studied inoculation of *S. cerevisiae* once 50% of the sugar in Shiraz and Chardonnay grape musts had been consumed by *Mt. pulcherrima*, a protocol which delayed addition until day 9 or 17, respectively. Of concern is that long delays before *S. cerevisiae* inoculation may be difficult for wineries to manage because extensive growth of other non-*Saccharomyces* could decrease important nutrients or produce by-products which decrease quality [13,32].

Unlike *Mt. pulcherrima*, presence of *My. guilliermondii* did not affect the final ethanol content of wines. This finding was in contrast to Aplin et al. [20] who had conducted smaller volume fermentations (3 L) using a sterile-filtered, grape juice made from concentrate (Merlot). Later work by Aplin and Edwards [21] utilizing a high sugar must (310 g/L) also reported a decrease in ethanol production. Here, addition of *My. guilliermondii* resulted in wines containing 15.5% *v/v* ethanol compared to control wines that contained 16.1% *v/v*. Despite reaching total populations of non-*Saccharomyces* yeasts of almost 10^7 cfu/mL, the metabolism of *My. guilliermondii* may have been adversely affected by naturally-present microflora. Though studies on *My. guilliermondii* are lacking, Contreras et al. [25] noted that growth of *Mt. pulcherrima* was inhibited due to the presence of *Hanseniaspora uvarum*, *Pichia kluyveri*, and *Torulaspora delbrueckii*.

To date, the fate of carbon from metabolized sugar by *Mt. pulcherrima* remains unclear. For example, Merlot wines produced with either strain of *Mt. pulcherrima* P01A016 and NS-MP contained similar concentrations of glycerol and succinic acid as those produced by *S. cerevisiae* alone, in agreement with some reports [19] but in disagreement to others [5,23]. In fact, succinic acid can be formed by at least three additional pathways besides tricarboxylic acid cycle such as the γ -amino butyric acid bypass (shunt), glyoxylic acid bypass, and/or the methylcitric acid cycle [33] which may or may not be active in *Mt. pulcherrima* under vinification conditions. Besides glycerol and succinic acid, Hranilovic et al. [23] suggested that another metabolic product of the tricarboxylic acid cycle, fumaric acid, may also partially explain the fate of metabolized sugar.

Few reports are available regarding the impacts of *My. guilliermondii* on chemical composition or wine sensory properties. Contreras et al. [26] noted their strain consumed more sugar than *Mt. pulcherrima* (40.5% vs. 21.2%) yet expressed similar ethanol yield (0.38 vs. 0.36 g ethanol/g sugar). Despite high concentrations of ethyl acetate (148 mg/L), these wines were not sensorily different from those produced with *S. cerevisiae* alone (52.3 mg/L ethyl acetate). Ethyl acetate is associated with the off-aroma of 'nail-polish remover' when present at concentrations ≥ 150 mg/L [34]. In addition, wines with *My. guilliermondii* were characterized by increased concentrations of 2- and 3-methyl-butyl acetates, 2-phenylethyl acetate, and/or higher alcohols associated with 'fruity' and/or 'floral' descriptors [34]. In one of the few studies involving *My. guilliermondii* (synonym *Pichia guilliermondii*), Benito et al. [35] noted elevated amounts of vinylphenolic pyranoanthocyanins, highly stable wine pigments which increase color stability, as well as increased ethyl acetate and higher alcohols due to this yeast.

Descriptive sensory analysis revealed that wines produced with non-*Saccharomyces* and *S. cerevisiae* yeasts exhibited few significant differences in aroma, mouthfeel, taste and flavor characteristics. While positive sensory characteristics were not imparted, this observation further suggests that use of these yeasts does not hamper wine quality. In other studies, presence of different strains of *Mt. pulcherrima* were shown to increase 'fruity' and 'floral' aromas [5,27,36] as well as overall quality scores in Chenin blanc and Sauvignon blanc wines [3,4]. Although differences due to yeast were not noted, panelist responses were highly variable, as evidenced by the significant panelist effect for most sensory attributes ($p < 0.05$). Panelists are known to be a significant source of variation in descriptive analysis through differences in use of scale and attribute definition [37–39].

5. Conclusions

This work described Merlot wines produced with *Mt. pulcherrima* and *My. guilliermondii* yeasts under pilot-scale winery conditions. Inoculation of *Mt. pulcherrima* P01A016

and NS-MP generated wines did not impact sensory characteristics but did yield less alcohol in the final wines. Future research concerning other processing conditions (i.e., must temperature) is needed to optimize ethanol reduction in fermentations with these non-*Saccharomyces* yeasts.

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