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Evaluation of Non-*Saccharomyces* Yeasts for the Reduction of Alcohol Content in Wine

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Over recent decades, the average ethanol concentration of wine has increased, largely due to consumer preference for wine styles associated with increased grape maturity; sugar content increases with grape maturity, and this translates into increased alcohol content in wine. However, high ethanol content impacts wine sensory properties, reducing the perceived complexity of flavors and aromas. In addition, for health and economic reasons, the wine sector is actively seeking technologies to facilitate the production of wines with lower ethanol content. Nonconventional yeast species, in particular, non-*Saccharomyces* yeasts, have shown potential for producing wines with lower alcohol content. These yeast species, which are largely associated with grapes preharvest, are present in the early stages of fermentation but, in general, are not capable of completing alcoholic fermentation. We have evaluated 50 different non-*Saccharomyces* isolates belonging to 24 different genera for their capacity to produce wine with a lower ethanol concentration when used in sequential inoculation regimes with a *Saccharomyces cerevisiae* wine strain. A sequential inoculation of *Metschnikowia pulcherrima* AWRI1149 followed by an *S. cerevisiae* wine strain was best able to produce wine with an ethanol concentration lower than that achieved with the single-inoculum, wine yeast control. Sequential fermentations utilizing AWRI1149 produced wines with 0.9% (vol/vol) and 1.6% (vol/vol) (corresponding to 7.1 g/liter and 12.6 g/liter, respectively) lower ethanol concentrations in Chardonnay and Shiraz wines, respectively. In Chardonnay wine, the total concentration of esters and higher alcohols was higher for wines generated from sequential inoculations, whereas the total concentration of volatile acids was significantly lower. In sequentially inoculated Shiraz wines, the total concentration of higher alcohols was higher and the total concentration of volatile acids was reduced compared with those in control *S. cerevisiae* wines, whereas the total concentrations of esters were not significantly different.

Grapes grown in warm climates have the potential to produce rich, full-bodied wines with ripe fruit flavor profiles. However, a warm climate and lengthy maturation periods can lead to grapes with high sugar concentrations, and this, in turn, leads to wines with high concentrations of ethanol. Therefore, in many wine-producing regions in both Old and New World countries, it is not uncommon to find wines with ethanol concentrations exceeding 15% (vol/vol) (1). High alcohol content in wine has several important consequences: it can compromise wine quality, including increasing the perception of hotness, body, viscosity, and, to a lesser extent, sweetness and acidity. In addition, it can lead to a decrease in aroma and flavor intensity (2–5), and costs to the consumer increase in countries where taxes are levied according to alcohol concentration (6). In addition, health concerns linked to alcohol consumption increasingly lead to national and international public health recommendations to lower the alcohol content of alcoholic beverages, such as wine (7, 8).

Consequently, there is significant interest in the development of strategies to produce reduced-alcohol wines, and this is ideally achieved in such a way that balance, flavor profile, and other sensory characteristics are not compromised. For a new production process to be successful, it will have to be economically viable and, ideally, simple to apply without the need for specialized equipment; any approach that requires only minor modification of current practices would facilitate rapid uptake. Novel yeast strains that divert carbon metabolism away from ethanol production to other endpoints would be ideal for this purpose (9).

Saccharomyces cerevisiae is the principal microorganism responsible for vinification, since it completes fermentation of available sugars after displacing other yeast species present in grape must. It achieves this through key adaptations to must/wine environments, especially through its ability to produce and tolerate high concentrations of alcohol (10–12). *S. cerevisiae* not only completes fermentation, transforming sugar (glucose and fructose) in the must into ethanol and CO_2, but also produces metabolites that have a positive influence on the sensory properties of wine (13–16). All currently available *S. cerevisiae* wine yeasts produce similar ethanol yields, resulting in comparable alcohol concentrations when fermenting the same must (17, 18). Research efforts have therefore been directed to developing *S. cerevisiae* strains that produce wines with lower alcohol concentrations (9).

Another promising strategy to produce reduced-alcohol wines takes advantage of wine yeasts that do not belong to the *S. cerevisiae* species and that metabolize sugar without generating ethanol or do so with less efficiency. The natural microflora present on grapes and harvesting and winemaking equipment includes many non-*Saccharomyces* yeasts, which are present at least during the early stages of fermentation (12, 13, 19, 20). However, non-*Saccharomyces* species are generally not capable of completing alcoholic fermentation; coinoculation or sequential inoculation with *S. cerevisiae* is needed to achieve this (21–25).

Several studies have reported the effects of non-*Saccharomyces*

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strains on wine composition, flavor, and aroma (21, 23, 25–31). For example, sequential inoculation of *Torulaspora delbrueckii* and a wine strain of *S. cerevisiae* was shown to decrease volatile acidity and increase aroma complexity (28). In other research in this field, production of polysaccharides and the final concentrations of acetic acid and sensorially important volatile compounds, such as ethyl acetate and 2-phenylethanol, were affected by the ratio of non-*Saccharomyces* to *S. cerevisiae* strains (27), and non-*Saccharomyces* strains were found to increase glycerol production and affect total acidity (23, 25).

Some studies have reported moderately reduced ethanol yields when using non-*Saccharomyces* and *S. cerevisiae* strains in coinoculation or sequential inoculation, with the decreases in the ethanol concentration ranging from 0.2 to 0.7% (vol/vol) compared to the ethanol concentration achieved with a single *S. cerevisiae* inoculum (23, 26, 32, 33). These studies, however, focused only on a few candidate species of non-*Saccharomyces* yeasts. In this work, we systematically screened 50 isolates of non-*Saccharomyces* yeasts covering 40 species belonging to 24 different genera with the aim of identifying yeasts that, in sequential inoculation with *S. cerevisiae*, could be used for the reduction of alcohol content in wine.

MATERIALS AND METHODS

Microorganisms and media. Fifty non-*Saccharomyces* isolates were obtained from the Australian Wine Research Institute (AWRI) Wine Microorganism Culture Collection (WMCC) (Table 1). Cryogenically preserved (−80°C) strains were cultured and maintained on YM medium (3 g/liter malt extract, 3 g/liter yeast extract, 5 g/liter peptone, 10 g/liter glucose, 16 g/liter agar) plates and stored at 4°C.

Defined medium used in screening and confirmation analyses consisted of 75 g/liter glucose, 75 g/liter fructose, 3 g/liter tartaric acid, and 6.76 g/liter yeast nitrogen base adjusted to pH 3.5. Chemically defined grape juice (CDGJ) medium consisted of the following (per liter): glucose, 100 g; fructose, 100 g; citric acid, 0.2 g; malic acid, 3 g; potassium hydrogen tartrate, 2.5 g; K_2HPO_4, 1.1 g; $MgSO_4 \cdot 7H_2O$, 1.5 g; $CaCl_2 \cdot 2H_2O$, 0.4 g; H_3BO_3, 0.04 g; proline, 0.84 g; nitrogen mix solution, 20 ml; trace elements stock solution, 1 ml; vitamins solution, 1 ml; fatty acids stock solution, 1 ml; and sterol stock solution, 1 ml (34). CDGJ contained 307 mg N/liter of yeast-assimilable nitrogen (YAN). Nitrogen mix solution contained the following (per liter): ammonium hydroxide solution (28 to 30%; catalogue number 221228; Sigma-Aldrich), 27.7 g; alanine, 10.5 g; γ-amino butyrate, 7.2 g; arginine, 26.9 g; asparagine, 0.4 g; aspartate, 3.0 g; citrulline, 0.4 g; glutamate, 6.0 g; glutamine, 8.4 g; glycine, 0.4 g; histidine, 1.2 g; isoleucine, 1.2 g; leucine, 1.2 g; lysine, 0.4 g; methionine, 0.4 g; ornithine, 0.4 g; phenylalanine, 0.8 g; serine, 5.4 g; threonine, 6.0 g; tryptophan, 0.4 g; tyrosine, 0.4 g; valine, 2.1 g; and cysteine, 1.2 g. Trace elements stock solution (1,000×) contained the following (per liter): $MnSO_4 \cdot H_2O$, 3.5 g; $ZnCl_2$, 1.0 g; $FeSO_4 \cdot 7H_2O$, 6.0 g; $CuSO_4 \cdot 5H_2O$, 1.5 g; KIO_3, 0.01 g; $Co(NO_3)_2 \cdot 6H_2O$, 0.03 g; $Na_2MoO_4 \cdot 2H_2O$, 0.025 g; LiCl, 0.1 g; $NiSO_4 \cdot 6H_2O$, 0.05 g; and RbCl, 0.7 g. Vitamin stock solution (1,000×) contained the following (per liter): thiamine HCl, 0.5 g; riboflavin, 0.2 g; pyridoxine HCl, 1.0 g; calcium D-pantothenate, 1.0 g; nicotinic acid, 1.0 g; *myo*-inositol, 10 g; biotin, 0.05 g; folic acid, 0.05 g; and 4-amino benzoic acid, 0.05 g. Fatty acids stock solution (100×) contained the following (per liter): palmitic acids, 2.0 g; oleic acid, 1.0 g; linoleic acid, 3 g; and linolenic acid, 0.5 g. Sterol stock solution (1,000×) contained the following (per liter): β-sitosterol, 1.0 g. Non-*Saccharomyces* yeasts were distinguished from *S. cerevisiae* by growth on WL agar (Amyl Media Pty. Ltd., Dandenong, Australia); *Saccharomyces cerevisiae* produces large white colonies on this medium, whereas non-*Saccharomyces* yeasts produce small green colonies on this medium (35).

Screening of non-*Saccharomyces* yeasts. Strains were screened individually in small-scale bioreactors (Medicel Oy, Finland) that enable con-

TABLE 1 Ethanol yield and percent sugar consumed for non-*Saccharomyces* yeasts evaluated under anaerobic conditions[a]

Strain	Species	Ethanol yield (g ethanol/g sugar)	% sugar consumed
AWRI1631	*Saccharomyces cerevisiae*	0.44	98.5
AWRI258[b]	*Hanseniaspora valbyensis*	0.06	19.6
AWRI1499	*Dekkera bruxellensis*	0.09	8.5
AWRI1199	*Pichia fermentans*	0.11	2.5
AWRI1159[b]	*Candida stellata*	0.18	63.5
AWRI1101	*Dekkera anomala*	0.19	6.3
AWRI1128	*Dekkera anomala*	0.24	7.3
AWRI1032	*Schwanniomyces occidentalis*	0.29	14.4
AWRI1656[b]	*Metschnikowia pulcherrima*	0.31	22.7
AWRI1052[b]	*Kluyveromyces marxianus*	0.31	37.7
AWRI1045	*Williopsis mrakii*	0.32	16.2
AWRI1047[b]	*Wickerhamomyces subpelliculosus*	0.33	47.3
AWRI1005[b]	*Kluyveromyces marxianus*	0.33	33.8
AWRI863[b]	*Hanseniaspora uvarum*	0.33	20.5
AWRI1103[b]	*Dekkera bruxellensis*	0.34	32.2
AWRI1043[b]	*Pichia ciferrii*	0.34	46.0
AWRI1149[b]	*Metschnikowia pulcherrima*	0.35	42.7
AWRI747[b]	*Torulaspora pretoriensis*	0.35	28.9
AWRI141[b]	*Schizosaccharomyces pombe*	0.35	25.1
AWRI1165	*Candida diversa*	0.35	14.4
AWRI442[b]	*Schizosaccharomyces malidevorans*	0.36	32.4
AWRI1157	*Debaryomyces hansenii*	0.38	11.2
AWRI60	*Zygopichia* spp.	0.39	6.3
AWRI1181	*Kluyveromyces lactis*	0.40	12.8
AWRI1220[b]	*Issatchenkia orientalis*	0.40	22.0
AWRI1152[b]	*Torulaspora delbrueckii*	0.41	41.9
AWRI1046	*Cyberlindnera saturnus*	0.42	18.9
AWRI1053	*Debaryomyces vanrijiae* var. *vanrijiae*	0.42	5.9
AWRI1578	*Zygosaccharomyces bailii*	0.45	44.3
AWRI58	*Kluyveromyces marxianus*	0.45	58.5
AWRI56	*Zygosaccharomyces rouxii*	0.47	15.5
AWRI861	*Lachancea thermotolerans*	0.48	31.7
AWRI1665	*Torulaspora castellii*	0.48	68.1
AWRI1425	*Hanseniaspora uvarum*	0.48	14.9
AWRI1164	*Trigonopsis cantarellii*	0.48	9.7
AWRI1821	*Pichia kluyveri*	0.50	11.8
AWRI1124	*Issatchenkia terricola*	0.50	8.6
AWRI1158	*Hanseniaspora uvarum*	0.50	10.4
AWRI1161	*Candida sake*	0.50	5.9
AWRI1552	*Meyerozyma guilliermondii*	—[c]	2.4
AWRI1127	*Dekkera bruxellensis*	—	1.0
AWRI1095	*Pichia membranifaciens*	—	0.7
AWRI1051	*Pichia anomala*	—	0.0
AWRI1098	*Hasegawaea japonica*	—	0.0
AWRI1044	*Pichia holstii*	—	0.0
AWRI1094	*Pachysolen tannophilus*	—	0.0
AWRI2053	*Pichia* spp.	—	0.0
AWRI1006	*Yamadazyma mexicanum*	—	0.0
AWRI743	*Rhodotorula glutinis*	—	0.0
AWRI69	*Sporobolomyces roseus*	—	0.0
AWRI950	*Dekkera custersiana*	—	0.0

[a] The initial sugar concentration was 150 g/liter.
[b] Candidate low-ethanol, non-*Saccharomyces* yeast strain to be tested in trial sequential fermentation experiments.
[c] —, the amount of ethanol produced was below the limit of detection.

trol of sparging gas flow and composition. Starter cultures of all yeast strains were grown overnight in YM medium under aerobic conditions at 28°C with shaking at 200 rpm. These cultures were then used to inoculate 200 ml of defined medium at a final optical density (OD) at 600 nm (OD_{600}) of 0.1. The ferments were then incubated at 28°C (200 rpm) under anaerobic conditions in bioreactors. Anaerobic conditions were attained by sparging nitrogen into the medium at 5 ml/min. After 4 days, which was enough time for *S. cerevisiae* to consume all sugar, fermentations were stopped and samples were taken for analysis, including determination of ethanol content and sugar consumption. Fermentations inoculated with *S. cerevisiae* AWRI1631 were used as controls.

Confirmation of screening results in sequential inoculation trials. Additional *Metschnikowia pulcherrima* strains and non-*Saccharomyces* strains identified as having reduced ethanol yields relative to those for *S. cerevisiae* were evaluated in triplicate using small-scale bioreactors, as described above. After 4 days of fermentation, *S. cerevisiae* AWRI1631 was inoculated into each bioreactor (OD_{600} equivalent to 0.1, or 1×10^6 cells/ml) to ensure the completion of fermentation. Samples were taken for analysis 7 days after the non-*Saccharomyces* inoculation.

Non-*Saccharomyces*/*S. cerevisiae* sequential fermentations in CDGJ medium. Sequential inoculation combinations identified as generating lower ethanol yields than the control and consuming at least approximately 95% of the sugar were evaluated in CDGJ medium. Fermentations were performed in triplicate in 250-ml fermentation flasks that were equipped with fermentation locks and that contained 100 ml of CDGJ medium. Starter cultures were grown overnight in YM medium under aerobic conditions at 28°C with shaking at 200 rpm and then inoculated at an OD_{600} of 0.1 (approximately 1×10^6 cells/ml) into CDGJ medium. The ferments were incubated at 28°C (120 rpm) under initial semiaerobic conditions; flasks fitted with air locks ensured anaerobic conditions after all oxygen in the headspace was consumed. After 50% of the sugar was consumed (7 days), *S. cerevisiae* AWRI1631 was inoculated into each ferment (OD_{600} equivalent to 0.1, or 1×10^6 cells/ml) to ensure the completion of fermentation. Samples were taken for analysis 10 days after the non-*Saccharomyces* inoculation.

***M. pulcherrima* AWRI1149/*S. cerevisiae* AWRI1631 sequential fermentation in grape juice.** *M. pulcherrima* AWRI1149 was evaluated in sequential fermentations with *S. cerevisiae* in both Chardonnay and Shiraz musts prepared from grapes obtained from Blewitt Springs, Clarendon Hills, South Australia, Australia. Chardonnay juice contained 196 g/liter of sugar (equal amounts of glucose and fructose) and 263 mg N/liter of YAN. To ensure a final ethanol concentration higher than 13% (vol/vol), the sugar concentration was adjusted to 230 g/liter by adding equal amounts of glucose and fructose, and then the Chardonnay must was filter sterilized (pore size, 0.2 μm; Millipore). Shiraz must contained 240 g/liter of sugar (glucose and fructose) and 160 mg N/liter of YAN. In this case, YAN was adjusted to 250 mg N/liter by adding diammonium phosphate (DAP).

Starter cultures were prepared by growing strains in YM medium as described above and then inoculating the strains into 100 ml grape must, diluted 1:1 with water, in 250-ml Erlenmeyer flasks. The flasks were incubated overnight at 28°C with shaking (120 rpm) under aerobic conditions, and the contents were then used to inoculate grape musts.

Chardonnay fermentations were performed in triplicate in 250-ml fermentation flasks equipped with fermentation locks containing 200 ml juice with shaking (120 rpm). Shiraz fermentations were performed in triplicate in 2.5-liter plastic containers containing 1 kg of destemmed grapes. Frozen Shiraz berries were carefully removed from the stems to avoid damage. The berries were then mixed and distributed into containers. After adding potassium metabisulfite (50 mg/kg), the berries were crushed using a stainless steel masher, and the must pH was adjusted to 3.5 using potassium hydroxide. Shiraz fermentations were incubated statically, and the solids cap was plunged twice daily. Chardonnay and Shiraz fermentations were inoculated at an OD_{600} of 0.1 (1×10^6 cells/ml), and the mixtures were incubated at 22°C. When 50% of the sugar was consumed, the fermentations were inoculated with *S. cerevisiae* AWRI1631 as described above. Samples were taken regularly to quantify the yeast cell populations and for monitoring the fermentation by measurement of the sugar concentration in culture supernatants.

Analytical techniques. Yeast growth was followed spectrophotometrically by measuring the absorbance at 600 nm in CDGJ and Chardonnay must fermentations. Viable counts were determined by plating on WL agar. Plates were incubated at 28°C for 2 days.

Ethanol, glucose, fructose, glycerol, and organic acid were quantified by high-performance liquid chromatography (HPLC) using a Bio-Rad HPX87H column, as described previously (36). Volatile fermentation products were analyzed using headspace solid-phase microextraction coupled with gas chromatography-mass spectrometry (HS-SPME/GCMS) with polydeuterated internal standards for stable isotope dilution analysis (SIDA), as described previously (37).

Statistical analysis. Differences between measurements were determined using analysis of variance (a least-significant-difference [LSD] test) with the statistical software Statgraphics Centurion (v5.0). Differences were considered significant when P values were less than 0.05.

RESULTS

Screening of non-*Saccharomyces* yeasts. Fifty different non-*Saccharomyces* yeasts covering 40 species and belonging to 24 different genera (Table 1) were screened for their ability to ferment sugar and produce ethanol under anaerobic conditions during 4 days of culture. Some non-*Saccharomyces* strains were unable to consume any sugar, while others utilized up to 68% (Table 1). To discriminate strains with slow growth from strains diverting carbon away from ethanol production, we estimated the ethanol yield, which is defined as the amount of ethanol produced per gram of sugar consumed. Of the non-*Saccharomyces* strains exhibiting lower ethanol yields than AWRI1631 (0.44 g ethanol/g sugar), only 15 consumed at least approximately 20% of the initial sugar within 4 days and were chosen for sequential inoculation experiments (Table 1).

Sequential non-*Saccharomyces*/*S. cerevisiae* inoculations in defined medium. Non-*Saccharomyces* strains were grown individually in defined medium, and after 4 days, *S. cerevisiae* AWRI1631 was sequentially inoculated into all fermentations. After 7 days, AWRI1631 had produced 9.0% (vol/vol) ethanol (the conversion factor for percent [vol/vol] to g/liter is 7.89), with an ethanol yield of 0.46 g ethanol/g sugar (Table 2). Eleven of the sequentially inoculated fermentations gave an average ethanol yield lower than that obtained with AWRI1631 alone. However, several of these contained residual sugar. Sequential fermentations using AWRI863 led to the lowest level of sugar consumption (approximately 50% of total sugar), while sequential fermentations using AWRI1149, AWRI1159, AWRI1656, AWRI442, AWRI1052, and AWRI258 gave high levels of sugar consumption (approximately 95%). Only four of these non-*Saccharomyces* strains, AWRI1149, AWRI1159, AWRI1656, and AWRI442, in sequential inoculations produced less ethanol than AWRI1631 alone. Sequential fermentations with these four strains also produced the most glycerol (>9 g/liter). Compared to the control, AWRI1149, AWRI1656, and AWRI1159 fermentations (each sequentially inoculated with AWRI1631) produced higher concentrations of malic and succinic acids, while more acetic acid was found in fermentations with AWRI442. Interestingly, despite consuming less sugar than *S. cerevisiae* AWRI1631 alone, sequential inoculations with AWRI1047, AWRI1043, and AWRI1005 produced higher concentrations of glycerol and organic acids (Table 2).

TABLE 2 Fermentation parameters and products for candidate non-*Saccharomyces* yeasts in sequential inoculation experiments to reduce ethanol yields[a]

Strain	Ethanol yield (g ethanol/g sugar)	% sugar consumed	% (vol/vol) ethanol	Concn (g/liter)			
				Glycerol	Acetic acid	Malic acid	Succinic acid
AWRI1631	0.47 ± 0.02	99.7 ± 0.2	9.0 ± 0.3	6.2 ± 0.2	0.5 ± 0.0	0.3 ± 0.1	0.3 ± 0.1
AWRI1149[b]	0.36 ± 0.02	97.9 ± 3.1	6.9 ± 0.0	10.1 ± 1.0	0.0 ± 0.0	1.2 ± 0.2	1.0 ± 0.3
AWRI863	0.38 ± 0.03	52.3 ± 2.6	4.5 ± 0.3	5.1 ± 0.3	0.4 ± 0.0	1.1 ± 0.0	0.6 ± 0.0
AWRI1152	0.39 ± 0.04	78.9 ± 3.7	5.8 ± 0.8	7.4 ± 1.3	0.0 ± 0.0	0.6 ± 0.5	0.7 ± 0.2
AWRI1159[b]	0.42 ± 0.01	99.7 ± 0.0	7.8 ± 0.2	16.2 ± 0.7	0.0 ± 0.0	0.5 ± 0.0	0.8 ± 0.0
AWRI1656[b]	0.42 ± 0.01	93.5 ± 6.4	5.8 ± 0.6	11.5 ± 0.8	0.3 ± 0.0	1.1 ± 0.2	0.9 ± 0.1
AWRI1103	0.44 ± 0.01	64.6 ± 3.6	5.6 ± 0.2	6.0 ± 0.3	0.5 ± 0.0	0.2 ± 0.0	0.5 ± 0.0
AWRI1220	0.44 ± 0.03	74.7 ± 0.7	5.5 ± 0.7	7.2 ± 0.8	0.6 ± 0.1	0.6 ± 0.0	0.3 ± 0.1
AWRI747	0.44 ± 0.01	77.8 ± 1.1	6.4 ± 0.0	4.5 ± 0.0	0.1 ± 0.0	0.5 ± 0.0	0.0 ± 0.0
AWRI1047	0.44 ± 0.02	90.1 ± 1.5	7.7 ± 0.6	8.3 ± 1.2	0.5 ± 0.1	1.0 ± 0.2	0.5 ± 0.1
AWRI442[b]	0.46 ± 0.01	94.7 ± 0.6	8.3 ± 0.0	9.7 ± 0.3	1.4 ± 0.0	0.5 ± 0.2	0.4 ± 0.4
AWRI1043	0.46 ± 0.01	91.0 ± 1.8	8.0 ± 0.1	16.2 ± 0.7	0.0 ± 0.0	0.5 ± 0.0	0.8 ± 0.0
AWRI1005	0.47 ± 0.00	89.6 ± 6.1	7.9 ± 0.5	11.8 ± 0.1	1.1 ± 0.1	0.8 ± 0.0	0.5 ± 0.0
AWRI1052	0.47 ± 0.01	98.7 ± 0.9	8.8 ± 0.1	9.7 ± 0.2	0.9 ± 0.0	1.0 ± 0.2	0.7 ± 0.0
AWRI141	0.48 ± 0.05	73.0 ± 4.4	6.6 ± 0.0	5.9 ± 0.9	0.0 ± 0.0	0.4 ± 0.1	0.3 ± 0.1
AWRI258	0.50 ± 0.01	96.4 ± 0.1	9.6 ± 0.1	7.2 ± 0.1	0.5 ± 0.1	0.7 ± 0.1	0.5 ± 0.0

[a] The initial sugar concentration was 150 g/liter. The values shown are means ± standard deviations of three independent replicates.
[b] Strains chosen for sequential inoculation experiments in CDGJ.

Evaluation of sequential non-*Saccharomyces*/*S. cerevisiae* inoculations in CDGJ medium. AWRI1149, AWRI1159, AWRI1656, and AWRI442 were evaluated in sequential inoculation experiments with *S. cerevisiae* AWRI631 in CDGJ medium. After 4 days of fermentation with non-*Saccharomyces* strains, AWRI1631 was inoculated into the culture. All fermentations were completed (>99% of the sugar was consumed) by day 10. All sequentially inoculated fermentations generated less ethanol than the control, AWRI1631 (Table 3). Specifically, sequential inoculation with AWRI1149 and AWRI1631 produced the least ethanol: 1.3% (vol/vol) less than the amount produced by the control. Sequential cultures inoculated first with AWRI1149, AWRI1159, AWRI1656, or AWRI442 produced more glycerol than the control. There were larger amounts of malic acid than the amount for the control and no detectable acetic acid for sequential fermentations with either AWRI1149 or AWRI1656, and fermentations with AWRI1149, AWRI1656, or AWRI442 produced more succinic acid than the amount produced by the *S. cerevisiae* control.

Evaluation of additional *M. pulcherrima* strains in sequential fermentations. To determine whether the lower ethanol yield observed for *M. pulcherrima* AWRI1149 during sequential fermentation was a trait shared by other strains of this species, four additional *M. pulcherrima* strains were evaluated. Although all five *M. pulcherrima* sequential inoculations generated less ethanol than the control, only three produced lower ethanol yields than *S. cerevisiae* AWRI1631 alone. Of these fermentations, however, only AWRI1149/AWRI1631 was able to consume more than 95% of total sugar (Table 4). All *M. pulcherrima* strains showed lower growth rates than AWRI1631. Strains AWRI874 and AWRI1149 had the highest growth rates of all *M. pulcherrima* strains tested (Table 4).

Evaluation of *M. pulcherrima* AWRI1149 in sequential fermentations in grape musts. *M. pulcherrima* AWRI1149 was used to ferment Chardonnay and Shiraz musts to determine whether lower ethanol yields would be obtained when using must rather than synthetic grape juice. Sequential inoculation experiments were performed at 22°C. *S. cerevisiae* AWRI1631 was inoculated when half of the initial sugar in the must was consumed (day 16 for Chardonnay and day 8 for Shiraz). For the control, AWRI1631 fermentation, sugar utilization was rapid, finishing in 10 days for Chardonnay and 9 days for Shiraz (Fig. 1), AWRI1149 fermented more slowly than AWRI1631 in both Chardonnay and Shiraz. However, following sequential inoculation with AWRI1631 in Chardonnay, the rate of sugar consumption increased until all sugar was utilized. In Shiraz fermented with AWRI1149/AWRI1631, the sugar consumption rate did not increase after inoculation with AWRI1631, suggesting that indigenous strains of *S.*

TABLE 3 Fermentation parameters and products for non-*Saccharomyces* yeasts in sequential inoculation experiments in CDGJ[a]

Strain	Ethanol yield (g ethanol/g sugar)	% sugar consumed	% (vol/vol) ethanol	Concn (g/liter)			
				Glycerol	Acetic acid	Malic acid	Succinic acid
AWRI1631	0.51^{a} ± 0.01	100^{a} ± 0.0	12.8^{a} ± 0.2	6.9^{a} ± 0.1	0.4^{a} ± 0.0	3.1^{a} ± 0.0	1.2^{a} ± 0.0
AWRI1149	0.46^{c} ± 0.00	100^{a} ± 0.0	11.5^{d} ± 0.1	7.8^{d} ± 0.0	0.0^{b} ± 0.0	3.9^{d} ± 0.3	1.7^{b} ± 0.1
AWRI1656	0.47^{b} ± 0.01	100^{a} ± 0.0	12.1^{bc} ± 0.1	8.3^{b} ± 0.4	0.0^{b} ± 0.1	3.8^{d} ± 0.5	1.6^{b} ± 0.1
AWRI442	0.48^{b} ± 0.01	100^{a} ± 0.0	12.2^{b} ± 0.2	8.5^{b} ± 0.5	0.4^{a} ± 0.2	1.5^{b} ± 0.0	2.2^{c} ± 0.2
AWRI1159	0.49^{ab} ± 0.02	99.3^{b} ± 0.2	12.3^{b} ± 0.3	12.7^{c} ± 1.0	0.4^{a} ± 0.0	2.3^{c} ± 0.4	1.0^{a} ± 0.2

[a] The initial sugar concentration was 200 g/liter. Values are means ± standard deviations of three independent replicates. Shared superscript letters without italics in the same column indicate no significant difference; different superscript letters in the same column indicate that differences are significant (LSD test, $P < 0.05$).

TABLE 4 Fermentation parameters for different strains of *M. pulcherrima* used in sequential inoculations with *S. cerevisiae* AWRI1631[a]

Strain	Ethanol yield (g ethanol/g sugar)	% sugar consumed	% (vol/vol) ethanol	Growth rate[b] (h^{-1})
AWRI1631	$0.47^{a} \pm 0.02$	$99.7^{a} \pm 0.2$	$9.0^{a} \pm 0.3$	$1.63^{a} \pm 0.09$
AWRI1170	$0.33^{c} \pm 0.01$	$80.4^{b} \pm 1.4$	$4.3^{c} \pm 0.2$	$0.65^{d} \pm 0.02$
AWRI1149	$0.35^{b} \pm 0.00$	$99.7^{a} \pm 0.1$	$6.9^{b} \pm 0.0$	$0.78^{c} \pm 0.04$
AWRI874	$0.37^{b} \pm 0.02$	$68.0^{c} \pm 7.4$	$5.1^{c} \pm 0.5$	$0.73^{c} \pm 0.05$
AWRI1191	$0.41^{ab} \pm 0.07$	$82.5^{b} \pm 4.8$	$5.7^{bc} \pm 1.5$	$0.14^{e} \pm 0.03$
AWRI448	$0.48^{a} \pm 0.02$	$76.6^{bc} \pm 10.3$	$7.3^{b} \pm 0.8$	$0.29^{b} \pm 0.01$

[a] The initial sugar concentration was 150 g/liter. Values are means ± standard deviations of three independent replicates. Shared superscript letters without italics in the same column indicate no significant difference; different superscript letters in the same column indicate that differences are significant (LSD test, $P < 0.05$).
[b] Growth rate was obtained as the slope of the linear part of the growth curve consisting of log OD versus time. Growth rates for *M. pulcherrima* are given for the period prior to inoculation with *S. cerevisiae* AWRI1631.

cerevisiae were present in the grape must, which was not sterilized prior to inoculation.

Compared to the *S. cerevisiae* control, wines made with AWRI1149/AWRI1631 had less ethanol: a reduction of 0.9% (vol/vol) in Chardonnay and 1.6% (vol/vol) in Shiraz (Table 5). There were also significant differences in the concentrations of glycerol, succinic acid, and several volatile compounds (Table 5). Because the Shiraz must was not sterile, an additional control fermentation was included in which *M. pulcherrima* AWRI1149 was not added. Instead, the juice was allowed to undergo a spontaneous fermentation until 50% of the sugar was utilized (day 7), after which *S. cerevisiae* AWRI1631 was inoculated to finish the fermentation, as described above. The resultant wine had an alcohol content that was not significantly different ($P > 0.05$) from that for the control, in which AWRI1631 was added at the start; there was no reduction in ethanol concentration. Therefore, the lower ethanol concentration observed in wines made using *M. pulcherrima* AWRI1149 in a sequential inoculation of Shiraz must was due to the activity of this non-*Saccharomyces* species.

In the case of Chardonnay, sequential inoculations initiated with AWRI1149 led to wines with more glycerol and less succinic acid than the amounts for the control. In addition, sequential inoculations produced Chardonnay with higher total concentrations of esters and higher alcohols and a lower total concentration of volatile acids. The ethyl acetate concentration was 6.5-fold higher in Chardonnay fermented with AWRI1149 and AWRI1631, whereas 2- and 3-methylbutyl acetate were the main compounds responsible for the increased total concentration of esters. The amounts of 2-methyl propanol and 2- and 3-methyl butanol were significantly higher in wines produced by sequential inoculation, increasing the total concentration of higher alcohols. Although most volatile acids were present in lower concentrations in sequential inoculation wines, 2-methyl propanoic and hexanoic acids were the main compounds responsible for the lower total concentration of volatile acids in these wines.

Compared to the control (single AWRI1631 inoculum) in Shiraz, sequential inoculation initiated with AWRI1149 produced wines with higher concentrations of glycerol, succinic and malic acids, and ethyl acetate. The total ester concentration was not significantly different between the two. However, similar to what was found for Chardonnay, the sequential inoculation produced wine with increased amounts of higher alcohols and a lower total concentration of volatile acids (Table 5); again, 2-methyl propanol and 2- and 3-methyl butanol were the main compounds responsible for the increased total concentration of higher alcohols in wines generated from sequential inoculation, whereas 2-methyl propanoic and hexanoic acids were the major drivers for the lower total concentration of volatile acids.

Relative growth kinetics of the two yeast strains in sequential and control fermentations clearly showed that *M. pulcherrima* is less fit than *S. cerevisiae* in wine fermentations. In the Chardonnay control fermentation, AWRI1631 had an average doubling time of 0.9 h and reached a maximum cell density of 3×10^8 CFU/ml (Fig. 2). In the sequential inoculation, AWRI1149 had a doubling time of 1.3 h and reached a maximum cell density of 3.5×10^8 (CFU/ml). After sequential inoculation with AWRI1631, AWRI1149 cell numbers decreased rapidly as the AWRI1631 population increased, reaching 4×10^8 CFU/ml. In the control Shiraz fermentation, AWRI1631 reached a maximum cell density of 1.6×10^8 CFU/ml. In the sequential fermentation, AWRI1149 cell numbers decreased prior to inoculation with AWRI1631, reaching a maximum of 1×10^8 CFU/ml; AWRI1631 reached a maximum of 1×10^8 CFU/ml. It should be noted that, since Shiraz must was not sterilized, it is possible these viable counts overestimate the *M. pulcherrima* AWRI1149 and *S. cerevisiae* AWRI1631 populations.

DISCUSSION

Quality, health, and economic drivers have increased winemakers' interests in accessing technologies that enable the production of wines with ethanol concentrations lower than those of many currently available wines; however, few strategies to achieve this end have been tested. The strategy that was evaluated in experimental

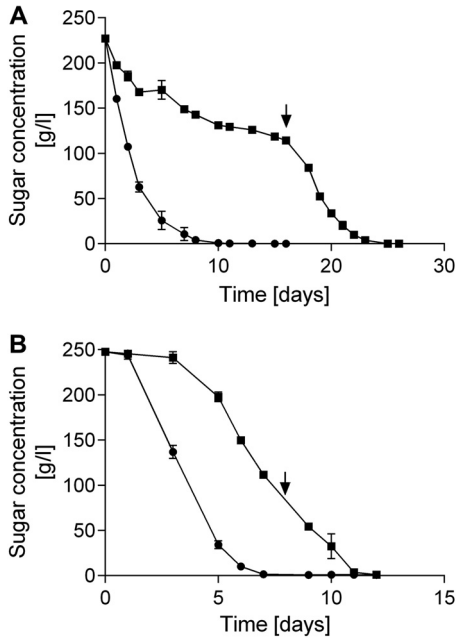

FIG 1 Sugar utilization in cultures inoculated solely with *S. cerevisiae* AWRI1631 (circles) and sequentially with *M. pulcherrima* AWRI1149 followed by AWRI1631 (squares) in Chardonnay (A) and Shiraz (B) fermentations. The time of sequential inoculation with AWRI1631 is indicated with an arrow.

TABLE 5 Chemical profiles of wines produced using either *S. cerevisiae* AWRI1631 alone (control) or *M. pulcherrima* AWRI1149/*S. cerevisiae* AWRI1631 sequential inoculation[a]

Characteristic	Chardonnay		Shiraz	
	AWRI1631 (control)	AWRI1149/AWRI1631	AWRI1631 (control)	AWRI1149/AWRI1631
% sugar consumed	100[a] ± 0.0	100[a] ± 0.0	99.6[a] ± 0.0	99.8[b] ± 0.0
% (vol/vol) ethanol	15.1[a] ± 0.1	14.2[b] ± 0.2	13.8[a] ± 0.8	12.2[b] ± 0.1
Concn (g/liter)				
Glycerol	6.5[a] ± 0.2	8.1[b] ± 0.5	10.1[a] ± 0.1	14.9[b] ± 0.4
Malic acid	2.7[a] ± 0.7	2.0[a] ± 0.0	0.8[a] ± 0.0	0.7[b] ± 0.1
Succinic acid	2.8[a] ± 0.3	2.1[b] ± 0.2	2.7[a] ± 0.1	3.2[b] ± 0.1
Ester concn (mg/liter)				
Ethyl acetate	31.8[a] ± 1.0	207.6[b] ± 33.2	19.4[a] ± 1.8	53.9[b] ± 18.3
2-Methylpropyl acetate	<LOD[b]	<LOD	0.4[a] ± 0.0	0.9[b] ± 0.3
Ethyl butanoate	0.02[a] ± 0.04	0.02[a] ± 0.0	0.1[a] ± 0.0	0.1[a] ± 0.0
2- and 3-methylbutyl acetate	7.8[a] ± 2.3	15.9[b] ± 2.5	7.0[a] ± 0.7	6.1[a] ± 1.3
Ethyl hexanoate	0.03[a] ± 0.01	0.03[a] ± 0.0	0.4[a] ± 0.0	0.4[a] ± 0.1
Hexyl acetate	0.7[a] ± 0.4	0.5[a] ± 0.0	0.3[a] ± 0.1	0.1[b] ± 0.0
Ethyl octanoate	0.1[a] ± 0.01	0.05[a] ± 0.0	0.2[a] ± 0.0	0.1[b] ± 0.0
Ethyl decanoate	0.1[a] ± 0.01	0.03[a] ± 0.0	0.1[a] ± 0.0	0.1[a] ± 0.0
Total esters[c]	8.7[a] ± 2.7	16.6[b] ± 2.5	8.7[a] ± 0.8	7.8[a] ± 1.7
Higher alcohol concn (mg/liter)				
Butanol	2.8[a] ± 0.4	2.4[b] ± 0.0	1.5[a] ± 0.2	2.0[b] ± 0.3
2-Methyl propanol	79.5[a] ± 3.6	252.5[b] ± 20.5	60.5[a] ± 2.3	134.6[b] ± 1.7
2- and 3-methyl butanol	109.2[a] ± 5.1	122.6[b] ± 5.6	156.5[a] ± 7.4	219.8[b] ± 19
Hexanol	1.3[a] ± 0.1	0.7[b] ± 0.0	1.3[a] ± 0.1	1.5[a] ± 0.4
Total higher alcohols	192.7[a] ± 8.5	378.3[b] ± 26.1	234.5[a] ± 18	371.0[b] ± 30
Volatile acid concn				
Acetic acid (g/liter)	0.1[a] ± 0.0	0.1[a] ± 0.0	0.3[a] ± 0.0	0.3[a] ± 0.2
Propanoic acid (mg/liter)	1.0[a] ± 0.1	0.8[b] ± 0.0	2.6[a] ± 0.0	3.0[b] ± 0.2
2-Methyl propanoic acid (mg/liter)	4.0[a] ± 0.3	2.3[b] ± 0.0	4.6[a] ±0.0	3.7[b] ± 0.1
Butanoic acid (mg/liter)	0.6[a] ± 0.0	0.7[a] ± 0.0	1.1[a] ± 0.1	1.0[b] ± 0.1
2-Methyl butanoic acid (mg/liter)	0.4[a] ± 0.0	0.1[b] ± 0.0	1.4[a] ± 0.1	1.4[a] ± 0.2
3-Methyl butanoic acid (mg/liter)	0.6[a] ± 0.0	0.1[b] ± 0.0	1.7[a] ± 0.0	1.5[a] ± 0.3
Hexanoic acid (mg/liter)	2.7[a] ± 0.1	1.9[b] ± 0.1	2.4[a] ± 0.1	1.9[b] ± 0.2
Octanoic acid (mg/liter)	4.3[a] ± 0.1	3.0[b] ± 0.2	1.7[a] ± 0.0	1.0[b] ± 0.0
Decanoic acid (mg/liter)	1.2[a] ± 0.2	0.8[a] ± 0.0	0.4[a] ± 0.0	0.3[b] ± 0.0
Total volatile acids (mg/liter)[d]	14.8[a] ± 0.9	9.6[b] ± 0.4	16.1[a] ± 0.2	13.8[b] ± 0.3

[a] Values are means ± standard deviations of three independent replicates. Shared superscript letters without italics in the same column indicate no significant difference; different superscript letters in the same column indicate that differences are significant (LSD test, $P < 0.05$).
[b] <LOD, below the limit of detection (0.5 μg/liter).
[c] The total excludes ethyl acetate.
[d] The total excludes acetic acid.

trials in the work described in this paper involved the use of non-*Saccharomyces* yeasts to metabolize, and thereby remove, some of the sugar in grape juice. Non-*Saccharomyces* yeasts are generally recognized as being either nonethanologenic or, at least, less efficient than *Saccharomyces* spp. in converting sugar into ethanol.

Although they are naturally occurring during wine fermentations, non-*Saccharomyces* yeasts are not able to consume all sugar present in grape must. Therefore, sequential strategies utilizing *S. cerevisiae* strains were needed to ensure the completion of fermentation. Ideally, an effective low-ethanol, non-*Saccharomyces* strain should exhibit a low ethanol yield but consume enough sugar so that a real impact on the ethanol concentration is achieved and be compatible with *S. cerevisiae* to ensure the completion of fermentation.

Several studies have evaluated the oenological properties of non-*Saccharomyces* yeasts in association with *S. cerevisiae* strains (21, 23, 25, 26, 30, 32, 33, 38), but none have reported either substantial reductions in ethanol concentration or a significant decrease in the amount of ethanol without detrimental impacts on wine quality. For example, where lower ethanol yields have been reported, they are usually associated with high residual sugar (>5 g/liter) at the end of fermentation (21, 30, 38).

An initial screening of 50 non-*Saccharomyces* isolates was performed with each as a single inoculum, followed by confirmation experiments using a sequential inoculation regime. This led to the identification of four non-*Saccharomyces* yeast strains (two strains identified as *Metschnikowia pulcherrima* and one strain each of *Schizosaccharomyces malidevorans* and *Candida stellata*) which, in sequential inoculations with *S. cerevisiae* AWRI1631, produced less ethanol than *S. cerevisiae* alone. All four cofermentations went

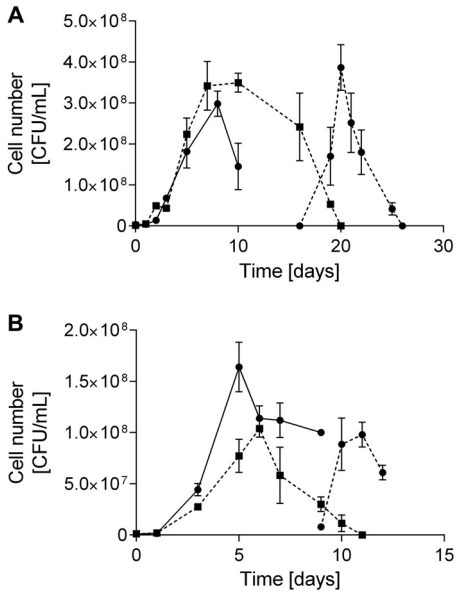

FIG 2 Cell numbers in Chardonnay (A) and Shiraz (B) fermentations. Circles, *S. cerevisiae* AWRI1631; squares, *M. pulcherrima* AWRI1149. Cultures inoculated solely with *S. cerevisiae* AWRI1631 are represented as solid lines; sequential inoculations are represented as dashed lines.

to completion (i.e., they left less than 1.5 g/liter sugar at the end of fermentation).

While there is limited information in the literature on the impacts of these yeasts on wine fermentation outcomes, *Candida stellata* has been observed to produce substantial amounts of glycerol during winemaking (25, 38–41), *M. pulcherrima* has been reported to increase the concentration of volatile compounds in wine (23, 24, 42, 43), and *S. malidevorans* is able to decrease wine acidity (44). However, compared to the *S. cerevisiae* controls, none of these strains has been previously observed to produce wines with a significantly lower ethanol content.

Of the four non-*Saccharomyces* yeast strains described above, *M. pulcherrima* AWRI1149, in sequential inoculations with *S. cerevisiae* AWRI1631, consistently gave the lowest ethanol yield and produced lower ethanol yields than the control in Chardonnay and Shiraz wines. While there was increased production of glycerol and some organic acids in the products of the AWRI1149/AWRI1631 fermentations, these increases were not sufficient to explain the observed decrease in ethanol concentration. Therefore, it seems likely that additional carbon sinks are used by AWRI1149.

Although the indigenous flora present in grape must may contribute to a reduced ethanol concentration in finished wine compared to that in inoculated fermentations, wild ferments have not shown an ethanol concentration lower than that in inoculated wines at similar residual sugar concentrations (45, 46). Similarly, the uninoculated control included in this study showed that the indigenous microflora in the Shiraz must did not generate a fermentation product with a reduced ethanol concentration. Therefore, the lower ethanol concentration observed in wines produced using *M. pulcherrima* AWRI1149 sequentially inoculated Shiraz must is attributable to addition of this non-*Saccharomyces* species. Future research will determine the succession of the indigenous microflora following sequential inoculation and how interactions between AWRI1149 and the indigenous microflora impact the wine composition.

Besides AWRI1149 and AWRI1656, we tested four additional strains of *M. pulcherrima* in sequential inoculation trials with *S. cerevisiae* AWRI1631 to determine whether the reduction in ethanol yield was a common trait within this species. While some of these sequential inoculations produced less ethanol than the *S. cerevisiae* control, this was largely due to the reduced sugar consumption associated, in most cases, with lower growth rates. Two sequential fermentations, AWRI1149/AWRI1631 and AWRI1656/AWRI1631, were able to utilize all sugar and deliver an ethanol yield less than that for the control. This indicates that the reduced ethanol yield associated with these strains in wine fermentations is not a characteristic shared by all members of the *M. pulcherrima* species.

M. pulcherrima AWRI1149 had a lower growth rate than several of the non-*Saccharomyces* strains tested in this study, consistent with other reports (23, 47, 48). In grape must, *M. pulcherrima* also showed slower growth than *S. cerevisiae* but reached higher maximum cell numbers. This may indicate that stress conditions, such as osmotic stress, affect the initial growth rate of *M. pulcherrima*. A steady but slow proliferation of non-*Saccharomyces* cells during fermentation has been attributed to the osmotic stress caused by high sugar concentrations in other studies (49, 50). In sequential inoculations, the *M. pulcherrima* population declined quickly after inoculation with *S. cerevisiae*; indeed, no colonies were recovered on plates 2 days after *S. cerevisiae* inoculation. Negative effects of *S. cerevisiae* on the growth of *M. pulcherrima* have been reported previously (23, 33, 42, 51).

For any winemaking yeast strain, the impact that it has on wine flavor and aroma is of critical importance. In both Chardonnay and Shiraz, the products of AWRI1149/AWRI1631 sequential inoculations generally had chemical profiles consistent with good-quality wines. While there was an increased total concentration of higher alcohols, the levels were such that this would be expected to contribute positively to a wine's sensory properties. At moderate concentrations (<400 mg/liter), higher alcohols typically contribute positively to aroma complexity (52, 53). All wines that were produced using AWRI1149/AWRI1631 sequential inoculations had concentrations of higher alcohols below 400 mg/liter. AWRI1149/AWRI1631 sequential inoculations produced wines with total concentrations of volatile acids less than those for the AWRI1631 control. Volatile acids are generally associated with negative aromas in wine (52, 54). This is consistent with previous studies using *M. pulcherrima* and *S. cerevisiae*, which reported wines with a significant decrease in volatile acidity (acetic, lactic, propanoic, and butanoic acids) (23, 51).

Relative to the control, *S. cerevisiae* AWRI1631, there was a higher total concentration of esters in Chardonnay wines produced using an AWRI1149/AWRI1631 sequential inoculation. The main compounds responsible for this were 2- and 3-methylbutyl acetate, which were present at concentrations above their sensory threshold (190 μg/liter). With sensory descriptors of, respectively, pear and banana, they would be expected to increase the fruity aroma in this wine.

Although non-*Saccharomyces* yeasts can impart novel and desirable characters to wine, they can also produce metabolites that negatively impact wine flavor profiles (30, 55, 56). Ethyl acetate was present in higher concentrations in both Chardonnay and Shiraz wines produced using AWRI1149/AWRI1631 in a sequen-

tial fermentation. At low levels (approximately 50 mg/liter), ethyl acetate may add aroma complexity to wine, but at concentrations above 150 mg/liter, it is associated with negative sensory descriptors, such as nail polish remover (57, 58). Therefore, it is likely that Chardonnay wines produced using *M. pulcherrima* in sequential fermentations will have some negative sensory attributes as a result of the high concentration of this compound. High cell density ratios between non-*Saccharomyces* and *S. cerevisiae* yeasts in coinoculation experiments have been associated with the generation of compounds, such as ethyl acetate and acetic acid, at concentrations considered detrimental to wine quality (27, 33).

In contrast, Shiraz wine produced using the same sequential inoculation had considerably less ethyl acetate. In fact, ethyl acetate was present at a level that would be expected to bring desirable complexity to wine. This difference between Chardonnay and Shiraz is consistent with the work of Lilly et al. (59), in which it was found that the level of production of ethyl acetate in wine fermentations is determined, at least in part, by grape variety.

In conclusion, one very simple and economic strategy to help winemakers reduce alcohol levels in their wines is first to inoculate grape must with *M. pulcherrima* AWRI1149 and then to finish the fermentation by adding a wine strain of *S. cerevisiae*. While this strategy carries some risk of compromising wine quality in some grape musts, as for the Chardonnay used in this study, it might improve sensory attributes in Shiraz and reduce the alcohol level by up to 1.6% (vol/vol). A decrease in ethanol concentration of this magnitude will be a significant advantage to winemakers sourcing grapes from warm, sunny regions; reducing the ethanol content from, for example, 15% (vol/vol) ethanol to 13.4% (vol/vol) is a significant development in the generation of reduced-alcohol wines.

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