AEM Accepts, published online ahead of print on 27 December 2013
Appl. Environ. Microbiol. doi:10.1128/AEM.03780-13

Evaluation of non-*Saccharomyces* yeast for the reduction of alcohol content in wine

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Running title: Using non-*Saccharomyces* strains to reduce alcohol content in wine

Key words: wine; yeast; non-*Saccharomyces*; low alcohol

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ABSTRACT

Over recent decades the average ethanol concentration of wine has increased, due largely 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 on wine sensory properties, reducing the perceived complexity of flavours 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.

Non-conventional yeast, in particular non-*Saccharomyces* yeast, have shown potential for producing wines with lower alcohol content. These yeast species, which are largely associated with grapes pre-harvest, 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 lower ethanol concentration when used in sequential inoculation regimes with a *S. cerevisiae* wine strain. A sequential inoculation of *Metschnikowia pulcherrima* AWRI1149 followed by a *S. cerevisiae* wine strain was the best combination able to produce wine with a lower ethanol concentration than the single-inoculum, wine yeast, control. Sequential fermentations utilising AWRI1149 produced wines with 0.9% (v/v) and 1.6% (v/v) (corresponding to 7.1 g/L and 12.6 g/L) less ethanol concentration in Chardonnay and Shiraz, respectively. In Chardonnay, 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 there was a greater total concentration of higher alcohols and reduced total concentration of volatile acids than in control *S. cerevisiae* wines, whereas the total concentration of esters was not significantly different.

INTRODUCTION

Grapes grown in warm climates have the potential to produce rich, full bodied wines with ripe fruit flavour 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% (v/v) (25). High alcohol content in wine has several important consequences: it can compromise wine quality, including increasing the perception of hotness, body and viscosity and, to a lesser extent, sweetness and acidity. In addition it can lead to a decrease in aroma and flavour intensity (2, 23, 24, 28) and costs to the consumer increase in countries where taxes are levied according to alcohol concentration (13). 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 (26, 35).

Consequently there is significant interest in the development of strategies to produce reduced alcohol wines, and this ideally is achieved in such a way that balance, flavour 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 specialised 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 (32).

63 *Saccharomyces cerevisiae* is the principal microorganism responsible for vinification, since
64 it completes fermentation of available sugars after displacing other yeast species present
65 in grape must. It achieves this through key adaptations to must/wine environments,
66 especially through its ability to produce and tolerate high concentrations of alcohol (3, 18,
67 20). *S. cerevisiae* not only completes fermentation, transforming sugar (glucose and
68 fructose) in the must into ethanol and CO_2, but also produces metabolites that have a
69 positive influence on the sensory properties of wine (19, 44, 51, 55). All currently available
70 *S. cerevisiae* wine yeasts produce similar ethanol yields, resulting in comparable alcohol
71 concentrations when fermenting the same must (39, 56). Research efforts have therefore
72 been directed to developing *S. cerevisiae* strains that produce wines with lower alcohol
73 concentrations (32).
74
75 Another promising strategy to produce reduced-alcohol wines takes advantage of wine
76 yeast that do not belong to the *S. cerevisiae* species and that metabolise sugar without
77 generating ethanol or do so with less efficiency. The natural microflora present on grapes,
78 and harvesting and winemaking equipment, includes many non-*Saccharomyces* yeasts,
79 which are present at least during the early stages of fermentation (19, 20, 42, 43).
80 However, non-*Saccharomyces* species are generally not capable of completing alcoholic
81 fermentation; co-inoculation or sequential inoculation with *S. cerevisiae* is needed to
82 achieve this (6, 10, 12, 31, 50).
83
84 Several studies have reported the effects of non-*Saccharomyces* strains on wine
85 composition, flavour and aroma (6, 12, 14-16, 21, 36, 50, 54). For example, sequential
86 inoculation of *Torulaspora delbruekii* followed by a wine strain of *S. cerevisiae* was shown
87 to decrease volatile acidity and increase aroma complexity (16). 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 (15), and non-*Saccharomyces* strains were found to increase glycerol production and affect total acidity (12, 50).

Some studies have reported moderately reduced ethanol yields when using non-*Saccharomyces* and *S. cerevisiae* strains in co- or sequential-inoculations, with decreases in ethanol concentration ranging 0.2 – 0.7% (v/v) compared to single *S. cerevisiae* inocula (12, 14, 17, 47). These studies, however, focused only on a few candidate species of non-*Saccharomyces* yeasts. In this work we systematically screened fifty 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 plates (3 g/L malt extract, 3 g/L yeast extract, 5 g/L peptone, 10 g/L glucose, 16 g/L agar) and stored at 4°C.

Defined medium used in screening and confirmation analyses consisted of 75 g/L glucose, 75 g/L fructose, 3 g/L tartaric acid and 6.76 g/L Yeast Nitrogen Base adjusted to pH 3.5. Chemically Defined Grape Juice (CDGJ) medium consisted of (per litre): 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 (48). CDGJ contained 307 mg N/L of yeast assimilable nitrogen (YAN). Nitrogen mix solution contained (per litre): ammonium hydroxide solution (28-30%, Sigma-Aldrich catalogue number 221228) 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 (1000X) contained (per litre): $MnSO_4 \cdot H2O$ 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. Vitamins stock solution (1000X) contained (per litre): 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 (100X) contained (per litre): palmitic acids 2.0 g, oleic acid 1.0 g, linoleic acid 3 g, and linolenic acid 0.5 g. Sterol stock solution (1000X) contained (per litre): 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 whereas non-*Saccharomyces* yeasts produce small green colonies on this medium (27).

Screening of non*-Saccharomyces *yeasts

Strains were screened individually in small-scale bioreactors (Medicel Oy, Finland) that enable control of sparging gas flow and composition. Starter cultures of all yeast strains were grown overnight in YM medium under aerobic conditions at 28°C, shaking at 200 rpm. These cultures were then used to inoculate 200 mL of defined medium at a final optical density of 0.1 (600 nm). 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 four days, which was enough time for *S. cerevisiae* to consume all sugar, fermentations were stopped and samples taken for analysis, including determinations 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 *S. cerevisiae* were evaluated in triplicate using small-scale bioreactors as above. After four days of fermentation, *S. cerevisiae* AWRI1631 was inoculated into each bioreactor (OD_{600} equivalent to 0.1 or 1 x 10^6 cells/mL) to ensure completion of fermentation. Samples were taken for analysis 7 days after the non-*Saccharomyces* inoculation.

Non*-Saccharomyces/S. cerevisiae *sequential fermentations in Chemically Defined Grape Juice

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

equipped with fermentation locks and containing 100 mL of CDGJ medium. Starter cultures were grown overnight in YM medium under aerobic conditions at 28°C, shaking at 200 rpm, and then inoculated at OD_{600} of 0.1 (approximately 1 x 10^6 cells/mL) into CDGJ. Ferments were incubated at 28°C (120 rpm) under initial semi-aerobic conditions; flasks fitted with air-locks ensure anaerobic conditions after all oxygen in the headspace is consumed. After 50% of sugar was consumed (7 days) *S. cerevisiae* AWRI1631 was inoculated into each ferment (OD_{600} equivalent to 0.1 or 1 x 10^6 cells/mL) to ensure 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). Chardonnay juice contained 196 g/L of sugar (equal amounts of glucose and fructose) and 263 mg N/L of yeast assimilable nitrogen (YAN). To ensure a final ethanol concentration higher than 13% (v/v) sugar concentration was adjusted to 230 g/L by adding equal amounts of glucose and fructose and then the Chardonnay must was filter sterilised (0.2 µm Millipore, USA). Shiraz must contained 240 g/L of sugar (glucose and fructose) and 160 mg N/L of YAN. In this case YAN was adjusted to 250 mg N/L by adding diammonium phosphate (DAP).

Starter cultures were prepared by growing strains in YM medium as described earlier then inoculating into 100 mL grape must, diluted 1:1 with water, in 250 mL Erlenmeyer flasks.

Flasks were incubated overnight at 28°C with shaking (120 rpm) under aerobic conditions and 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 L plastic containers containing 1 kg of de-stemmed grapes. Frozen Shiraz berries were carefully removed from stems to avoid damage. Berries were then mixed and distributed into containers. After adding potassium metabisulphite 50 mg/kg, berries were crushed using a stainless steel masher, and must pH 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 OD_{600} 0.1 (1 x 10^6 cells/mL) and incubated at 22°C. When 50% of the sugar was consumed, fermentations were inoculated with *S. cerevisiae* AWRI1631 as described earlier. Samples were taken regularly to quantify yeast cell populations and for monitoring fermentation by measurement of sugar concentration in culture supernatants.

Analytical techniques

Yeast growth was followed spectrophotometrically by measuring 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 BioRad HPX87H column as described previously (38). Volatile fermentation products were analysed 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 (49).

Statistical analysis

Differences between measurements were determined using analysis of variance (LSD test) with the statistical software Statgraphics Centurion v5.0. Differences were considered significant when *p* values were lower 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 four days of culture. Some non-*Saccharomyces* strains were unable to consume any sugar while others utilised up to 68% (Table 1). To discriminate strains with slow growth from strains diverting carbon away from ethanol production we estimated ethanol yield, which is defined as the amount of ethanol produced per gram of consumed sugar. Of the non-*Saccharomyces* strains exhibiting lower ethanol yield than AWRI1631 (0.44 g ethanol/g sugar), only 15 consumed at least approximately 20% of the initial sugar within four 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 four days *S. cerevisiae* AWRI1631 was sequentially inoculated into all fermentations. After seven days, AWRI1631 had produced 9.0 % (v/v) ethanol (the conversion factor for %

(v/v) to g/L 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 AWRI1631 alone. However, several of these contained residual sugar. Sequential fermentations using AWRI863 led to the lowest sugar consumption (approximately 50% of total sugar), while sequential fermentations using AWRI1149, AWRI1159, AWRI1656, AWRI442, AWRI1052 and AWRI258 gave high 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/L). 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).

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 four days of fermentation with non-*Saccharomyces* strains, AWRI1631 was inoculated into the culture. All fermentations were completed (>99% sugar consumed) by day 10. All sequentially inoculated fermentations generated less ethanol than the control, AWRI1631 (Table 3). Specifically, AWRI1149/AWRI1631 produced the least ethanol, 1.3% (v/v) lower than the control. Sequential cultures inoculated first with AWRI1149, AWRI1159, AWRI1656 or

AWRI442 produced more glycerol than the control. There were greater amounts of malic acid than 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 *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 yield 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 slower 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 utilisation 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 utilised. In Shiraz fermented with

AWRI1149/AWRI1631 sugar consumption rate did not increase after inoculation with AWRI1631 suggesting that indigenous strains of *S. cerevisiae* were present in the grape must, which was not sterilised prior to inoculation.

Compared to the *S. cerevisiae* control, wines made with AWRI1149/AWRI1631 had less ethanol; a reduction of 0.9% (v/v) in Chardonnay and 1.6% (v/v) 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 utilised (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 ($p > 0.05$) to 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 compared to the control. In addition, sequential inoculations produced Chardonnay with greater total concentrations of esters and higher alcohols, and a lower total concentration of volatile acids. 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. 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. 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 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 hours and reached a maximum cell density of 3 x 10^8 CFU/mL (FIG 2). In the sequential inoculation, AWRI1149 had a doubling time of 1.3 hours reaching a maximum cell density of 3.5 x 10^8 (CFU/mL). After sequential inoculation with AWRI1631, AWRI1149 cell numbers decreased rapidly as AWRI1631 population increased, reaching 4 x 10^8 CFU/mL. In the control Shiraz fermentation, AWRI1631 reached a maximum cell density of 1.6 x 10^8 CFU/mL. In the sequential fermentation, AWRI1149 cell numbers decreased prior to inoculation with AWRI1631, reaching a maximum of 1 x 10^8 CFU/mL, AWRI1631 reached

a maximum of 1 x 10^8 CFU/mL. It should be noted that, since Shiraz must was not sterilised, 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 lower ethanol concentrations than many currently available wines, however few strategies to achieve this end have been tested. The strategy that was trialled in the work described in this paper involved the use of non-*Saccharomyces* yeast to metabolise, and thereby remove, some of the sugar in grape juice. Non-*Saccharomyces* yeast are generally recognised as being either non-ethanologenic or, at least, less efficient than *Saccharomyces* spp. in converting sugar into ethanol.

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

Several studies have evaluated the oenological properties of non-*Saccharomyces* yeasts in association with *S. cerevisiae* strains (6, 8, 12, 14, 17, 36, 47, 50), but none have reported either substantial reductions in ethanol concentration or significant ethanol decrease 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/L) at the end of fermentation (6, 8, 36).

An initial screening of fifty non-*Saccharomyces* isolates was performed, 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 co-fermentations went to completion (i.e leaving less than 1.5 g/L sugar at the end of fermentation).

Whilst 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 (7-9, 11, 50), *M. pulcherrima* have been reported to increase the concentration of volatile compounds in wine (12, 31, 45, 59), and *S. malidevorans* is able to decrease wine acidity (52). However, when compared to S. cerevisiae controls none of these strains has been previously observed to produce wines with significantly lower ethanol content.

Of the above four non-*Saccharomyces* yeast strains *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. Whilst there was increased production of glycerol and some organic acids in the products of 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 inoculated fermentations, wild ferments have not shown lower ethanol concentration than inoculated wines at similar residual sugar concentration (22, 57). Similarly, the un-inoculated control included in this study showed that the indigenous microflora in the Shiraz must did not generate a fermentation product with 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 on wine composition.

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

409 *M. pulcherrima* AWRI1149 had a slower growth than several of the non-*Saccharomyces*
410 strains tested in this study, consistent with other reports (5, 12, 37). In grape must, *M.*
411 *pulcherrima* also showed slower growth than *S. cerevisiae* but reached higher maximum
412 cell numbers. This may indicate that stress conditions, such as osmotic stress, affect the
413 initial growth rate of *M. pulcherrima*. A steady but slow proliferation of non-*Saccharomyces*
414 cells during fermentation has been attributed to the osmotic stress caused by high sugar
415 concentration in other studies (40, 53). In sequential inoculations, *M. pulcherrima*
416 population declined quickly after inoculation with *S. cerevisiae*; indeed no colonies were
417 recovered on plates two days after *S. cerevisiae* inoculation. Negative effects of *S.*
418 *cerevisiae* on the growth of *M. pulcherrima* have been reported previously (12, 30, 45, 47).
419
420 For any winemaking yeast strain the impact that it has on wine flavour and aroma is of
421 critical importance. In both Chardonnay and Shiraz, the products of AWRI1149/AWRI1631
422 sequential inoculations generally had chemical profiles consistent with good quality wines.
423 Whilst there was an increased total concentration of higher alcohols, the levels were such
424 that this would be expected to contribute positively to a wine's sensory properties. At
425 moderate concentrations (<400 mg/L), higher alcohols typically contribute positively to
426 aroma complexity (4, 41). All wines that were produced using AWRI1149/AWRI1631
427 sequential inoculations had concentrations of higher alcohols below 400 mg/L.
428 AWRI1149/AWRI1631 sequential inoculations produced wines with lower total
429 concentration of volatile acids than the AWRI1631 control. Volatile acids are generally
430 associated with negative aromas in wine (4, 33). This is consistent with previous studies
431 using *M. pulcherrima* and *S. cerevisiae*, which reported wines with a significant decrease
432 in volatile acidity (acetic, lactic, propanoic and butanoic acids) (12, 30).
433

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/L). With sensory descriptors of, respectively, pear and banana, they would be expected to increase the fruity aroma in this wine.

Although non-*Saccharomyces* yeast can impart novel and desirable characters to wine, they can also produce metabolites that impact negatively on wine flavour profiles (36, 46, 58). Ethyl acetate was present in higher concentrations in both Chardonnay and Shiraz wines produced using AWRI1149/AWRI1631 in a sequential fermentation. At low levels (approximate 50 mg/L) ethyl acetate may add aroma complexity to wine, but at concentrations above 150 mg/L it is associated with negative sensory descriptors, such as nail polish remover (1, 29). 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 co-inoculation experiments have been associated with the generation of compounds such as ethyl acetate and acetic acid at concentrations considered detrimental to wine quality (15, 47).

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

ACKNOWLEDGEMENTS

The AWRI, a member of the Wine Innovation Cluster in Adelaide, is supported by Australia's grapegrowers and winemakers through their investment body the Grape and Wine Research Development Corporation with matching funds from the Australian Government. Dr Angela Contreras was supported by an 2012 Endeavour Australia Postgraduate and Postdoctoral Research Fellowships (Department of Education, Science and Training, Australian Government) and Becas Chile Fellowship 2013. Dr Claudio Hidalgo was supported by a mobility bursary from the Spanish Ministry of Science and Innovation (Spain).

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663 **FIGURE LEGENDS**

664

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

669

670 FIG 2. Cell numbers in Chardonnay (A) and Shiraz (B) fermentations. *S. cerevisiae*
671 AWRI1631 (circles) and *M. pulcherrima* AWRI1149 (squares). Cultures inoculated solely
672 with *S. cerevisiae* AWRI1631 are represented as solid lines, sequential inoculations are
673 represented as dotted lines.

674

675 **TABLE 1.** Ethanol yield and percentage sugar consumed for non-*Saccharomyces* yeasts
676 evaluated under anaerobic conditions.

Strain	Species	Ethanol yield [g ethanol/ g sugar]	Consumed sugar [%]
AWRI1631	***Saccharomyces cerevisiae***	**0.44**	**98.5**
AWRI258*	*Hanseniaspora valbyensis*	0.06	19.6
AWRI1499	*Dekkera bruxellensis*	0.09	8.5
AWRI1199	*Pichia fermentans*	0.11	2.5
AWRI1159*	*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*	*Metschnikowia pulcherrima*	0.31	22.7
AWRI1052*	*Kluyveromyces marxianus*	0.31	37.7
AWRI1045	*Williopsis mrakii*	0.32	16.2
AWRI1047*	*Wickerhamomyces subpelliculosus*	0.33	47.3
AWRI1005*	*Kluyveromyces marxianus*	0.33	33.8
AWRI863*	*Hanseniaspora uvarum*	0.33	20.5
AWRI1103*	*Dekkera bruxellensis*	0.34	32.2
AWRI1043*	*Pichia ciferrii*	0.34	46.0
AWRI1149*	*Metschnikowia pulcherrima*	0.35	42.7
AWRI747*	*Torulaspora pretoriensis*	0.35	28.9
AWRI141*	*Schizosaccharomyces pombe*	0.35	25.1
AWRI1165	*Candida diversa*	0.35	14.4
AWRI442*	*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*	*Issatchenkia orientalis*	0.40	22.0
AWRI1152*	*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*	[a]	2.4
AWRI1127	*Dekkera bruxellensis*	[a]	1.0
AWRI1095	*Pichia membranifaciens*	[a]	0.7
AWRI1051	*Pichia anomala*	[a]	0.0
AWRI1098	*Hasegawaea japonica*	[a]	0.0
AWRI1044	*Pichia holstii*	[a]	0.0
AWRI1094	*Pachysolen tannophilus*	[a]	0.0
AWRI2053	*Pichia spp*	[a]	0.0
AWRI1006	*Yamadazyma mexicanum*	[a]	0.0
AWRI743	*Rhodotorula glutinis*	[a]	0.0
AWRI69	*Sporobolomyces roseus*	[a]	0.0
AWRI950	*Dekkera custersiana*	[a]	0.0

677

678 Initial sugar concentration was 150 g/L.

679 * Candidate, 'low-ethanol', non-*Saccharomyces* yeast strains to be trialled in sequential

680 fermentation experiments.

681 [a] Ethanol produced below limit of detection

682 **TABLE 2.** Fermentation parameters and products for candidate non-*Saccharomyces* yeasts in sequential inoculation experiments to
683 reduce ethanol yields.

Strain	Ethanol yield [g ethanol/ g sugar]	Consumed sugar [%]	Ethanol [% v/v]	Glycerol [g/L]	Acetic acid [g/L]	Malic acid [g/L]	Succinic acid [g/L]
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*	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*	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*	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*	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

684

685 Initial sugar concentration was 150 g/L.

686 Values shown are means ± standard deviation of three independent replicates.

687 * Strains chosen for sequential inoculation experiments in CDGJ.

688 **TABLE 3.** Fermentation parameters and products for non-*Saccharomyces* yeasts in sequential inoculation experiments in CDGJ.

Strain	Ethanol yield [g ethanol/ g sugar]	Consumed sugar [%]	Ethanol [%v/v]	Glycerol [g/L]	Acetic acid [g/L]	Malic acid [g/L]	Succinic acid [g/L]
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

689

690 Initial sugar concentration was 200 g/L.

691 Values are means ± standard deviation of three independent replicates.

692 Shared superscript letters (a, b, c) in the same column indicate no significant difference, different superscript letters in the same

693 column indicate differences are significant (LSD test, $p < 0.05$).

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

Strain	Ethanol yield [g ethanol/ g sugar]	Consumed sugar [%]	Ethanol [%v/v]	Growth rate [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	$0.48^{a} \pm 0.02$	$76.6^{bc} \pm 10.3$	$7.3^{b} \pm 0.8$	$0.29^{b} \pm 0.01$

696
697 Growth rate was obtained as the slope of the linear part of the growth curve, log OD v/s
698 time. Growth rates for *M. pulcherrima* are given for the period prior to inoculation with
699 *S. cerevisiae* AWRI1631.
700 Initial sugar concentration was 150 g/L.
701 Values are means ± standard deviation of three independent replicates.
702 Shared superscript letters (a, b, c) in the same column indicate no significant difference,
703 different superscript letters in the same column indicate differences are significant (LSD
704 test, $p < 0.05$).

705 **TABLE 5**. Chemical profiles of wines produced using either *S. cerevisiae* AWRI1631 alone

706 (control) or *M. pulcherrima* AWRI1149/*S. cerevisiae* AWRI1631 sequential inoculation.

	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
Ethanol [%v/v]	15.1^{a} ± 0.1	14.2^{b} ± 0.2	13.8^{a} ± 0.8	12.2^{b} ± 0.1
Glycerol [g/L]	6.5^{a} ± 0.2	8.1^{b} ± 0.5	10.1^{a} ± 0.1	14.9^{b} ± 0.4
Malic acid [g/L]	2.7^{a} ± 0.7	2.0^{a} ± 0.0	0.8^{a} ± 0.0	0.7^{b} ± 0.1
Succinic acid [g/L]	2.8^{a} ± 0.3	2.1^{b} ± 0.2	2.7^{a} ± 0.1	3.2^{b} ± 0.1
Esters				
Ethyl acetate [mg/L]	31.8^{a} ± 1.0	207.6^{b} ± 33.2	19.4^{a} ± 1.8	53.9^{b} ± 18.3
2-Methylpropyl acetate [mg/L]	<LOD	<LOD	0.4^{a} ± 0.0	0.9^{b} ± 0.3
Ethyl butanoate [mg/L]	0.02^{a} ± 0.04	0.02^{a} ± 0.0	0.1^{a} ± 0.0	0.1^{a} ± 0.0
2- & 3-Methylbutyl acetate [mg/L]	7.8^{a} ± 2.3	15.9^{b} ± 2.5	7.0^{a} ± 0.7	6.1^{a} ± 1.3
Ethyl hexanoate [mg/L]	0.03^{a} ± 0.01	0.03^{a} ± 0.0	0.4^{a} ± 0.0	0.4^{a} ± 0.1
Hexyl acetate [mg/L]	0.7^{a} ± 0.4	0.5^{a} ± 0.0	0.3^{a} ± 0.1	0.1^{b} ± 0.0
Ethyl octanoate [mg/L]	0.1^{a} ± 0.01	0.05^{a} ± 0.0	0.2^{a} ± 0.0	0.1^{b} ± 0.0
Ethyl decanoate [mg/L]	0.1^{a} ± 0.01	0.03^{a} ± 0.0	0.1^{a} ± 0.0	0.1^{a} ± 0.0
Total esters [mg/L]§	8.7^{a} ± 2.7	16.6^{b} ± 2.5	8.7^{a} ± 0.8	7.8^{a} ± 1.7
Higher alcohols				
Butanol [mg/L]	2.8^{a} ± 0.4	2.4^{b} ± 0.0	1.5^{a} ± 0.2	2.0^{b} ± 0.3
2-Methyl propanol [mg/L]	79.5^{a} ± 3.6	252.5^{b} ± 20.5	60.5^{a} ± 2.3	134.6^{b} ± 1.7
2&3-Methyl butanol [mg/L]	109.2^{a} ± 5.1	122.6^{b} ± 5.6	156.5^{a} ± 7.4	219.8^{b} ± 19
Hexanol [mg/L]	1.3^{a} ± 0.1	0.7^{b} ± 0.0	1.3^{a} ± 0.1	1.5^{a} ± 0.4
Total higher alcohols [mg/L]	192.7^{a} ± 8.5	378.3^{b} ± 26.1	234.5^{a} ± 18	371.0^{b} ± 30
Volatile Acids				
Acetic acid [g/L]	0.1^{a} ± 0.0	0.1^{a} ± 0.0	0.3^{a} ± 0.0	0.3^{a} ± 0.2
Propanoic acid [mg/L]	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/L]	4.0^{a} ± 0.3	2.3^{b} ± 0.0	4.6^{a} ±0.0	3.7^{b} ± 0.1
Butanoic acid [mg/L]	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/L]	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/L]	0.6^{a} ± 0.0	0.1^{b} ± 0.0	1.7^{a} ± 0.0	1.5^{a} ± 0.3
Hexanoic acid [mg/L]	2.7^{a} ± 0.1	1.9^{b} ± 0.1	2.4^{a} ± 0.1	1.9^{b} ± 0.2
Octanoic acid [mg/]	4.3^{a} ± 0.1	3.0^{b} ± 0.2	1.7^{a} ± 0.0	1.0^{b} ± 0.0
Decanoic acid [mg/L]	1.2^{a} ± 0.2	0.8^{a} ± 0.0	0.4^{a} ± 0.0	0.3^{b} ± 0.0

Total volatile acids [mg/L] †	14.8[a] ± 0.9	9.6[b] ± 0.4	16.1[a] ± 0.2	13.8[b] ± 0.3

Values are means ± standard deviation of three independent replicates.

Shared superscript letters (a, b, c) in the same column indicate no significant difference, different superscript letters in the same column indicate differences are significant (LSD test, $p < 0.05$).

LOD indicates below Limit of Detection (0.5 µg/L).

§ total excludes ethyl acetate

† total excludes acetic acid.