

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

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14 **ABSTRACT**

15 Over recent decades the average ethanol concentration of wine has increased, due largely
16 to consumer preference for wine styles associated with increased grape maturity; sugar
17 content increases with grape maturity and this translates into increased alcohol content in
18 wine. However, high ethanol content impacts on wine sensory properties, reducing the
19 perceived complexity of flavours and aromas. In addition, for health and economic
20 reasons, the wine sector is actively seeking technologies to facilitate the production of
21 wines with lower ethanol content.

22 Non-conventional yeast, in particular non-*Saccharomyces* yeast, have shown potential for
23 producing wines with lower alcohol content. These yeast species, which are largely
24 associated with grapes pre-harvest, are present in the early stages of fermentation, but in
25 general are not capable of completing alcoholic fermentation. We have evaluated 50
26 different non-*Saccharomyces* isolates, belonging to 24 different genera, for their capacity
27 to produce wine with lower ethanol concentration when used in sequential inoculation
28 regimes with a *S. cerevisiae* wine strain. A sequential inoculation of *Metschnikowia*
29 *pulcherrima* AWRI1149 followed by a *S. cerevisiae* wine strain was the best combination
30 able to produce wine with a lower ethanol concentration than the single-inoculum, wine
31 yeast, control. Sequential fermentations utilising AWRI1149 produced wines with 0.9%
32 (v/v) and 1.6% (v/v) (corresponding to 7.1 g/L and 12.6 g/L) less ethanol concentration in
33 Chardonnay and Shiraz, respectively. In Chardonnay, the total concentration of esters and
34 higher alcohols was higher for wines generated from sequential inoculations, whereas the
35 total concentration of volatile acids was significantly lower. In sequentially inoculated
36 Shiraz wines there was a greater total concentration of higher alcohols and reduced total
37 concentration of volatile acids than in control *S. cerevisiae* wines, whereas the total
38 concentration of esters was not significantly different.

39 **INTRODUCTION**

40 Grapes grown in warm climates have the potential to produce rich, full bodied wines with
41 ripe fruit flavour profiles. However, a warm climate and lengthy maturation periods can
42 lead to grapes with high sugar concentrations and this, in turn, leads to wines with high
43 concentrations of ethanol. Therefore, in many wine producing regions in both old and new
44 world countries, it is not uncommon to find wines with ethanol concentrations exceeding
45 15% (v/v) (25). High alcohol content in wine has several important consequences: it can
46 compromise wine quality, including increasing the perception of hotness, body and
47 viscosity and, to a lesser extent, sweetness and acidity. In addition it can lead to a
48 decrease in aroma and flavour intensity (2, 23, 24, 28) and costs to the consumer increase
49 in countries where taxes are levied according to alcohol concentration (13). In addition,
50 health concerns linked to alcohol consumption increasingly lead to national and
51 international public health recommendations to lower the alcohol content of alcoholic
52 beverages such as wine (26, 35).

53

54 Consequently there is significant interest in the development of strategies to produce
55 reduced alcohol wines, and this ideally is achieved in such a way that balance, flavour
56 profile and other sensory characteristics are not compromised. For a new production
57 process to be successful it will have to be economically viable and, ideally, simple to apply
58 without the need for specialised equipment; any approach that requires only minor
59 modification of current practices would facilitate rapid uptake. Novel yeast strains that
60 divert carbon metabolism away from ethanol production to other endpoints would be ideal
61 for this purpose (32).

62

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 CO₂, 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 *Torulaspota 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

88 field, production of polysaccharides and the final concentrations of acetic acid and
89 sensorially important volatile compounds such as ethyl acetate and 2-phenylethanol were
90 affected by the ratio of non-*Saccharomyces* to *S. cerevisiae* strains (15), and non-
91 *Saccharomyces* strains were found to increase glycerol production and affect total acidity
92 (12, 50).

93
94 Some studies have reported moderately reduced ethanol yields when using non-
95 *Saccharomyces* and *S. cerevisiae* strains in co- or sequential-inoculations, with decreases
96 in ethanol concentration ranging 0.2 – 0.7% (v/v) compared to single *S. cerevisiae* inocula
97 (12, 14, 17, 47). These studies, however, focused only on a few candidate species of non-
98 *Saccharomyces* yeasts. In this work we systematically screened fifty isolates of non-
99 *Saccharomyces* yeasts, covering 40 species belonging to 24 different genera, with the aim
100 of identifying yeasts that, in sequential inoculation with *S. cerevisiae*, could be used for the
101 reduction of alcohol content in wine.

102

103 **MATERIALS AND METHODS**

104 ***Microorganisms and media***

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

109

110 Defined medium used in screening and confirmation analyses consisted of 75 g/L glucose,
111 75 g/L fructose, 3 g/L tartaric acid and 6.76 g/L Yeast Nitrogen Base adjusted to pH 3.5.
112 Chemically Defined Grape Juice (CDGJ) medium consisted of (per litre): glucose 100 g,

113 fructose 100 g, citric acid 0.2 g, malic acid 3 g, potassium hydrogen tartrate 2.5 g, K_2HPO_4
114 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
115 solution 20 mL, trace elements stock solution 1 mL, vitamins solution 1 mL, fatty acids
116 stock solution 1 mL, and sterol stock solution 1 mL (48). CDGJ contained 307 mg N/L of
117 yeast assimilable nitrogen (YAN). Nitrogen mix solution contained (per litre): ammonium
118 hydroxide solution (28-30%, Sigma-Aldrich catalogue number 221228) 27.7 g, alanine
119 10.5 g, γ -amino butyrate 7.2 g, arginine 26.9 g, asparagine 0.4 g, aspartate 3.0 g, citrulline
120 0.4 g, glutamate 6.0 g, glutamine 8.4 g, glycine 0.4 g, histidine 1.2 g, isoleucine 1.2 g,
121 leucine 1.2 g, lysine 0.4 g, methionine 0.4 g, ornithine 0.4 g, phenylalanine 0.8 g, serine
122 5.4 g, threonine 6.0 g, tryptophan 0.4 g, tyrosine 0.4 g, valine 2.1 g, and cysteine 1.2 g.
123 Trace elements stock solution (1000X) contained (per litre): $MnSO_4 \cdot H_2O$ 3.5 g, $ZnCl_2$ 1.0
124 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,
125 $Na_2MoO_4 \cdot 2H_2O$ 0.025 g, LiCl 0.1 g, $NiSO_4 \cdot 6H_2O$ 0.05 g, and RbCl 0.7 g. Vitamins stock
126 solution (1000X) contained (per litre): thiamine HCl 0.5 g, riboflavin 0.2 g, pyridoxine HCl
127 1.0 g, calcium D-pantothenate 1.0 g, nicotinic acid 1.0 g, myo-inositol 10 g, biotin 0.05 g,
128 folic acid 0.05 g, and 4-amino benzoic acid 0.05 g. fatty acids stock solution (100X)
129 contained (per litre): palmitic acids 2.0 g, oleic acid 1.0 g, linoleic acid 3 g, and linolenic
130 acid 0.5 g. Sterol stock solution (1000X) contained (per litre): β -sitosterol 1.0 g. Non-
131 *Saccharomyces* yeasts were distinguished from *S. cerevisiae* by growth on WL agar (Amyl
132 Media Pty Ltd, Dandenong, Australia); *Saccharomyces cerevisiae* produces large white
133 colonies whereas non-*Saccharomyces* yeasts produce small green colonies on this
134 medium (27).
135

136 **Screening of non-Saccharomyces yeasts**

137 Strains were screened individually in small-scale bioreactors (Medicel Oy, Finland) that
138 enable control of sparging gas flow and composition. Starter cultures of all yeast strains
139 were grown overnight in YM medium under aerobic conditions at 28°C, shaking at 200
140 rpm. These cultures were then used to inoculate 200 mL of defined medium at a final
141 optical density of 0.1 (600 nm). Ferments were then incubated at 28°C (200 rpm) under
142 anaerobic conditions in bioreactors. Anaerobic conditions were attained by sparging
143 nitrogen into the medium at 5 mL/min. After four days, which was enough time for *S.*
144 *cerevisiae* to consume all sugar, fermentations were stopped and samples taken for
145 analysis, including determinations of ethanol content and sugar consumption.
146 Fermentations inoculated with *S. cerevisiae* AWRI1631 were used as controls.

147

148 **Confirmation of screening results in sequential inoculation trials**

149 Additional *Metschnikowia pulcherrima* strains and non-*Saccharomyces* strains identified as
150 having reduced ethanol yields relative to *S. cerevisiae* were evaluated in triplicate using
151 small-scale bioreactors as above. After four days of fermentation, *S. cerevisiae* AWRI1631
152 was inoculated into each bioreactor (OD₆₀₀ equivalent to 0.1 or 1 x 10⁶ cells/mL) to ensure
153 completion of fermentation. Samples were taken for analysis 7 days after the non-
154 *Saccharomyces* inoculation.

155

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

157 **Grape Juice**

158 Sequential inoculation combinations identified as generating lower ethanol yields than the
159 control and consuming at least approximately 95% of the sugar were evaluated in CDGJ
160 medium. Fermentations were performed in triplicate in 250 mL fermentation flasks

161 equipped with fermentation locks and containing 100 mL of CDGJ medium. Starter
162 cultures were grown overnight in YM medium under aerobic conditions at 28°C, shaking at
163 200 rpm, and then inoculated at OD₆₀₀ of 0.1 (approximately 1 x 10⁶ cells/mL) into CDGJ.
164 Ferments were incubated at 28°C (120 rpm) under initial semi-aerobic conditions; flasks
165 fitted with air-locks ensure anaerobic conditions after all oxygen in the headspace is
166 consumed. After 50% of sugar was consumed (7 days) *S. cerevisiae* AWRI1631 was
167 inoculated into each ferment (OD₆₀₀ equivalent to 0.1 or 1 x 10⁶ cells/mL) to ensure
168 completion of fermentation. Samples were taken for analysis 10 days after the non-
169 *Saccharomyces* inoculation.

170

171 ***M. pulcherrima* AWRI1149/*S. cerevisiae* AWRI1631 sequential fermentation in grape**
172 ***juice***

173 *M. pulcherrima* AWRI1149 was evaluated in sequential fermentations with *S. cerevisiae* in
174 both Chardonnay and Shiraz musts prepared from grapes obtained from Blewitt Springs,
175 Clarendon Hills (South Australia). Chardonnay juice contained 196 g/L of sugar (equal
176 amounts of glucose and fructose) and 263 mg N/L of yeast assimilable nitrogen (YAN). To
177 ensure a final ethanol concentration higher than 13% (v/v) sugar concentration was
178 adjusted to 230 g/L by adding equal amounts of glucose and fructose and then the
179 Chardonnay must was filter sterilised (0.2 µm Millipore, USA). Shiraz must contained 240
180 g/L of sugar (glucose and fructose) and 160 mg N/L of YAN. In this case YAN was
181 adjusted to 250 mg N/L by adding diammonium phosphate (DAP).

182

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

185 Flasks were incubated overnight at 28°C with shaking (120 rpm) under aerobic conditions
186 and then used to inoculate grape musts.

187

188 Chardonnay fermentations were performed in triplicate in 250 mL fermentation flasks
189 equipped with fermentation locks containing 200 mL juice with shaking (120 rpm). Shiraz
190 fermentations were performed in triplicate in 2.5 L plastic containers containing 1 kg of de-
191 stemmed grapes. Frozen Shiraz berries were carefully removed from stems to avoid
192 damage. Berries were then mixed and distributed into containers. After adding potassium
193 metabisulphite 50 mg/kg, berries were crushed using a stainless steel masher, and must
194 pH adjusted to 3.5 using potassium hydroxide. Shiraz fermentations were incubated
195 statically and the solids cap was plunged twice daily. Chardonnay and Shiraz
196 fermentations were inoculated at OD₆₀₀ 0.1 (1 x 10⁶ cells/mL) and incubated at 22°C.
197 When 50% of the sugar was consumed, fermentations were inoculated with *S. cerevisiae*
198 AWRI1631 as described earlier. Samples were taken regularly to quantify yeast cell
199 populations and for monitoring fermentation by measurement of sugar concentration in
200 culture supernatants.

201

202 **Analytical techniques**

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

206 Ethanol, glucose, fructose, glycerol and organic acid were quantified by high-performance
207 liquid chromatography (HPLC) using a BioRad HPX87H column as described previously
208 (38). Volatile fermentation products were analysed using headspace solid-phase
209 microextraction coupled with gas chromatography–mass spectrometry (HS-SPME/GCMS),

210 with polydeuterated internal standards for stable isotope dilution analysis (SIDA) as
211 described previously (49).

212

213 **Statistical analysis**

214 Differences between measurements were determined using analysis of variance (LSD
215 test) with the statistical software Statgraphics Centurion v5.0. Differences were considered
216 significant when p values were lower than 0.05.

217

218 **RESULTS**

219 **Screening of non-Saccharomyces yeasts**

220 Fifty different non-*Saccharomyces* yeasts, covering 40 species and belonging to 24
221 different genera (Table 1), were screened for their ability to ferment sugar and produce
222 ethanol under anaerobic conditions during four days of culture. Some non-*Saccharomyces*
223 strains were unable to consume any sugar while others utilised up to 68% (Table 1). To
224 discriminate strains with slow growth from strains diverting carbon away from ethanol
225 production we estimated ethanol yield, which is defined as the amount of ethanol produced
226 per gram of consumed sugar. Of the non-*Saccharomyces* strains exhibiting lower ethanol
227 yield than AWRI1631 (0.44 g ethanol/g sugar), only 15 consumed at least approximately
228 20% of the initial sugar within four days and were chosen for sequential inoculation
229 experiments (Table 1).

230

231 **Sequential non-Saccharomyces/*S. cerevisiae* inoculations in defined medium**

232 Non-*Saccharomyces* strains were grown individually in defined medium, and after four
233 days *S. cerevisiae* AWRI1631 was sequentially inoculated into all fermentations. After
234 seven days, AWRI1631 had produced 9.0 % (v/v) ethanol (the conversion factor for %

235 (v/v) to g/L is 7.89) with an ethanol yield of 0.46 g ethanol/g sugar (Table 2). Eleven of the
236 sequentially inoculated fermentations gave an average ethanol yield lower than AWRI1631
237 alone. However, several of these contained residual sugar. Sequential fermentations using
238 AWRI863 led to the lowest sugar consumption (approximately 50% of total sugar), while
239 sequential fermentations using AWRI1149, AWRI1159, AWRI1656, AWRI442, AWRI1052
240 and AWRI258 gave high sugar consumption (approximately 95%). Only four of these non-
241 *Saccharomyces* strains, AWRI1149, AWRI1159, AWRI1656 and AWRI442, in sequential
242 inoculations, produced less ethanol than AWRI1631 alone. Sequential fermentations with
243 these four strains also produced the most glycerol (>9 g/L). Compared to the control,
244 AWRI1149, AWRI1656 and AWRI1159 fermentations (each sequentially inoculated with
245 AWRI1631) produced higher concentrations of malic and succinic acids, while more acetic
246 acid was found in fermentations with AWRI442. Interestingly, despite consuming less
247 sugar than *S. cerevisiae* AWRI1631 alone, sequential inoculations with AWRI1047,
248 AWRI1043 and AWRI1005 produced higher concentrations of glycerol and organic acids
249 (Table 2).

250

251 ***Evaluation of sequential non-Saccharomyces/S. cerevisiae inoculations in CDGJ***
252 ***medium***

253 AWRI1149, AWRI1159, AWRI1656 and AWRI442 were evaluated in sequential inoculation
254 experiments with *S. cerevisiae* AWRI631 in CDGJ medium. After four days of fermentation
255 with non-*Saccharomyces* strains, AWRI1631 was inoculated into the culture. All
256 fermentations were completed (>99% sugar consumed) by day 10. All sequentially
257 inoculated fermentations generated less ethanol than the control, AWRI1631 (Table 3).
258 Specifically, AWRI1149/AWRI1631 produced the least ethanol, 1.3% (v/v) lower than the
259 control. Sequential cultures inoculated first with AWRI1149, AWRI1159, AWRI1656 or

260 AWRI442 produced more glycerol than the control. There were greater amounts of malic
261 acid than the control and no detectable acetic acid for sequential fermentations with either
262 AWRI1149 or AWRI1656, and fermentations with AWRI1149, AWRI1656 or AWRI442
263 produced more succinic acid than the *S. cerevisiae* control.

264

265 ***Evaluation of additional M. pulcherrima strains in sequential fermentations***

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

274

275 ***Evaluation of M. pulcherrima AWRI1149 in sequential fermentations in grape musts***

276 *M. pulcherrima* AWRI1149 was used to ferment Chardonnay and Shiraz musts to
277 determine whether lower ethanol yields would be obtained when using must rather than
278 synthetic grape juice. Sequential inoculation experiments were performed at 22°C. *S.*
279 *cerevisiae* AWRI1631 was inoculated when half of the initial sugar in the must was
280 consumed (day 16 for Chardonnay and day 8 for Shiraz). For the control, AWRI1631
281 fermentation, sugar utilisation was rapid, finishing in 10 days for Chardonnay and 9 days
282 for Shiraz (FIG 1), AWRI1149 fermented more slowly than AWRI1631 in both Chardonnay
283 and Shiraz. However, following sequential inoculation with AWRI1631 in Chardonnay the
284 rate of sugar consumption increased until all sugar was utilised. In Shiraz fermented with

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

288

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

301

302 In the case of Chardonnay, sequential inoculations initiated with AWRI1149 led to wines
303 with more glycerol and less succinic acid compared to the control. In addition, sequential
304 inoculations produced Chardonnay with greater total concentrations of esters and higher
305 alcohols, and a lower total concentration of volatile acids. Ethyl acetate concentration was
306 6.5-fold higher in Chardonnay fermented with AWRI1149 and AWRI1631, whereas 2- and
307 3-methylbutyl acetate were the main compounds responsible for the increased total
308 concentration of esters. 2-Methyl propanol and 2- and 3-methyl butanol were significantly
309 higher in wines produced by sequential inoculation, increasing the total concentration of

310 higher alcohols. Although most volatile acids were present in lower concentrations in
311 sequential inoculation wines, 2-methyl propanoic and hexanoic acids were the main
312 compounds responsible for the lower total concentration of volatile acids in these wines.

313

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

324

325 Relative growth kinetics of the two yeast strains in sequential and control fermentations
326 clearly showed that *M. pulcherrima* is less fit than *S. cerevisiae* in wine fermentations. In
327 the Chardonnay control fermentation, AWRI1631 had an average doubling time of 0.9
328 hours and reached a maximum cell density of 3×10^8 CFU/mL (FIG 2). In the sequential
329 inoculation, AWRI1149 had a doubling time of 1.3 hours reaching a maximum cell density
330 of 3.5×10^8 (CFU/mL). After sequential inoculation with AWRI1631, AWRI1149 cell
331 numbers decreased rapidly as AWRI1631 population increased, reaching 4×10^8 CFU/mL.
332 In the control Shiraz fermentation, AWRI1631 reached a maximum cell density of 1.6×10^8
333 CFU/mL. In the sequential fermentation, AWRI1149 cell numbers decreased prior to
334 inoculation with AWRI1631, reaching a maximum of 1×10^8 CFU/mL, AWRI1631 reached

335 a maximum of 1×10^8 CFU/mL. It should be noted that, since Shiraz must was not
336 sterilised, it is possible these viable counts overestimate the *M. pulcherrima* AWRI1149
337 and *S. cerevisiae* AWRI1631 populations.

338

339 **DISCUSSION**

340 Quality, health and economic drivers have increased winemakers' interests in accessing
341 technologies that enable the production of wines with lower ethanol concentrations than
342 many currently available wines, however few strategies to achieve this end have been
343 tested. The strategy that was trialled in the work described in this paper involved the use of
344 non-*Saccharomyces* yeast to metabolise, and thereby remove, some of the sugar in grape
345 juice. Non-*Saccharomyces* yeast are generally recognised as being either non-
346 ethanologenic or, at least, less efficient than *Saccharomyces* spp. in converting sugar into
347 ethanol.

348

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

355

356 Several studies have evaluated the oenological properties of non-*Saccharomyces* yeasts
357 in association with *S. cerevisiae* strains (6, 8, 12, 14, 17, 36, 47, 50), but none have
358 reported either substantial reductions in ethanol concentration or significant ethanol
359 decrease without detrimental impacts on wine quality. For example, where lower ethanol

360 yields have been reported they are usually associated with high residual sugar (>5 g/L) at
361 the end of fermentation (6, 8, 36).

362

363 An initial screening of fifty non-*Saccharomyces* isolates was performed, each as a single
364 inoculum, followed by confirmation experiments using a sequential inoculation regime.
365 This led to the identification of four non-*Saccharomyces* yeast strains (two strains
366 identified as *Metschnikowia pulcherrima*, and one strain each of *Schizosaccharomyces*
367 *malidevorans* and *Candida stellata*) which, in sequential inoculations with *S. cerevisiae*
368 AWRI1631, produced less ethanol than *S. cerevisiae* alone. All four co-fermentations went
369 to completion (i.e leaving less than 1.5 g/L sugar at the end of fermentation).

370

371 Whilst there is limited information in the literature on the impacts of these yeasts on wine
372 fermentation outcomes, *Candida stellata* has been observed to produce substantial
373 amounts of glycerol during winemaking (7-9, 11, 50), *M. pulcherrima* have been reported
374 to increase the concentration of volatile compounds in wine (12, 31, 45, 59), and *S.*
375 *malidevorans* is able to decrease wine acidity (52). However, when compared to *S.*
376 *cerevisiae* controls none of these strains has been previously observed to produce wines
377 with significantly lower ethanol content.

378

379 Of the above four non-*Saccharomyces* yeast strains *M. pulcherrima* AWRI1149, in
380 sequential inoculations with *S. cerevisiae* AWRI1631, consistently gave the lowest ethanol
381 yield and produced lower ethanol yields than the control in Chardonnay and Shiraz wines.
382 Whilst there was increased production of glycerol and some organic acids in the products
383 of AWRI1149/AWRI1631 fermentations, these increases were not sufficient to explain the

384 observed decrease in ethanol concentration. Therefore, it seems likely that additional
385 carbon sinks are used by AWRI1149.

386

387 Although the indigenous flora present in grape must may contribute to a reduced ethanol
388 concentration in finished wine compared to inoculated fermentations, wild ferments have
389 not shown lower ethanol concentration than inoculated wines at similar residual sugar
390 concentration (22, 57). Similarly, the un-inoculated control included in this study showed
391 that the indigenous microflora in the Shiraz must did not generate a fermentation product
392 with reduced ethanol concentration. Therefore, the lower ethanol concentration observed
393 in wines produced using *M. pulcherrima* AWRI1149 sequentially inoculated Shiraz must is
394 attributable to addition of this non-*Saccharomyces* species. Future research will determine
395 the succession of the indigenous microflora following sequential inoculation and how
396 interactions between AWRI1149 and the indigenous microflora impact on wine
397 composition.

398

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

408

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

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

440

441 Although non-*Saccharomyces* yeast can impart novel and desirable characters to wine,
442 they can also produce metabolites that impact negatively on wine flavour profiles (36, 46,
443 58). Ethyl acetate was present in higher concentrations in both Chardonnay and Shiraz
444 wines produced using AWRI1149/AWRI1631 in a sequential fermentation. At low levels
445 (approximate 50 mg/L) ethyl acetate may add aroma complexity to wine, but at
446 concentrations above 150 mg/L it is associated with negative sensory descriptors, such as
447 nail polish remover (1, 29). Therefore, it is likely that Chardonnay wines produced using *M.*
448 *pulcherrima* in sequential fermentations will have some negative sensory attributes as a
449 result of the high concentration of this compound. High cell density ratios between non-
450 *Saccharomyces* and *S. cerevisiae* yeasts in co-inoculation experiments have been
451 associated with the generation of compounds such as ethyl acetate and acetic acid at
452 concentrations considered detrimental to wine quality (15, 47).

453

454 In contrast, Shiraz wine produced using the same sequential inoculation had considerably
455 less ethyl acetate. In fact, ethyl acetate was present at a level that would be expected to
456 bring desirable complexity to wine. This difference between Chardonnay and Shiraz is
457 consistent with the work of Lilly *et al.* (34), in which it was found that the level of production
458 of ethyl acetate in wine fermentations is determined, at least in part, by grape variety.

459

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

469

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479 REFERENCES

- 480 1. **Amerine, M. A., and E. B. Roessler.** 1983. Wines, their sensory evaluation. W.H.
481 Freeman, New York.
- 482 2. **Athes, V., M. P. Y. Lillo, C. Bernard, R. Perez-Correa, and I. Souchon.** 2004.
483 Comparison of experimental methods for measuring infinite dilution volatilities of
484 aroma compounds in water/ethanol mixtures. Journal of Agricultural and Food
485 Chemistry **52**:2021-2027.
- 486 3. **Bauer, F., and I. Pretorius.** 2000. Yeast stress response and fermentation
487 efficiency: how to survive the making of wine - a review. South African Journal of
488 Enology and Viticulture **21**:27-51.
- 489 4. **Carrascosa, A., R. Muñoz, and R. Gonzalez.** 2011. Molecular Wine Microbiology.
490 Academic Press, London.
- 491 5. **Caruso, M., C. Fiore, M. Contursi, G. Salzano, A. Paparella, and P. Romano.**
492 2002. Formation of biogenic amines as criteria for the selection of wine yeasts.
493 World Journal of Microbiology and Biotechnology **18**:159-163.
- 494 6. **Ciani, M., L. Beco, and F. Comitini.** 2006. Fermentation behaviour and metabolic
495 interactions of multistarter wine yeast fermentations. International Journal of Food
496 Microbiology **108**:239-245.
- 497 7. **Ciani, M., and L. Ferraro.** 1998. Combined use of immobilized *Candida stellata*
498 cells and *Saccharomyces cerevisiae* to improve the quality of wines. Journal of
499 Applied Microbiology **85**:247-254.
- 500 8. **Ciani, M., and L. Ferraro.** 1996. Enhanced glycerol content in wines made with
501 immobilized *Candida stellata* cells. Applied and environmental microbiology
502 **62**:128-132.

- 503 9. **Ciani, M., L. Ferraro, and F. Fatichenti.** 2000. Influence of glycerol production on
504 the aerobic and anaerobic growth of the wine yeast *Candida stellata*. Enzyme and
505 Microbial Technology **27**:698-703.
- 506 10. **Ciani, M., and F. Maccarelli.** 1998. Oenological properties of non-*Saccharomyces*
507 yeasts associated with wine-making. World Journal of Microbiology and
508 Biotechnology **14**:199-203.
- 509 11. **Clemente-Jimenez, J. M., L. Mingorance-Cazorla, S. Martinez-Rodriguez, F. J.**
510 **L. Heras-Vazquez, and F. Rodriguez-Vico.** 2004. Molecular characterization and
511 oenological properties of wine yeasts isolated during spontaneous fermentation of
512 six varieties of grape must. Food Microbiology **21**:149-155.
- 513 12. **Comitini, F., M. Gobbi, P. Domizio, C. Romani, L. Lencioni, I. Mannazzu, and**
514 **M. Ciani.** 2011. Selected non-*Saccharomyces* wine yeasts in controlled multistarter
515 fermentations with *Saccharomyces cerevisiae*. Food Microbiology **28**:873-882.
- 516 13. **de Barros-Lopes, M., J. M. Eglinton, P. A. Henschke, P. B. Hoj, and I. S.**
517 **Pretorius.** 2003. The connection between yeast and alcohol production in wine:
518 Managing the double edged sword of bottled sunshine. Australian and New
519 Zealand Wine Industry Journal **18**:27-31.
- 520 14. **Di Maio, S., G. Genna, V. Gandolfo, G. Amore, M. Ciaccio, and D. Oliva.** 2012.
521 Presence of *Candida zemplinina* in Sicilian musts and selection of a strain for wine
522 mixed fermentations. South African Journal of Enology and Viticulture **33**:80-87.
- 523 15. **Domizio, P., C. Romani, F. Comitini, M. Gobbi, L. Lencioni, I. Mannazzu, and**
524 **M. Ciani.** 2011. Potential spoilage non-*Saccharomyces* yeasts in mixed cultures
525 with *Saccharomyces cerevisiae*. Annals of Microbiology **61**:137-144.
- 526 16. **Ehsani, M., P. Renault, and V. Puente-López.** 2012. L'impact organoleptique de
527 *Torulaspota delbrueckii* en conditions oenologiques lors d'une inoculation

- 528 séquentielle avec *Saccharomyces cerevisiae*. Revue des oenologues et des
529 techniques vitivinicoles et oenologicques **39**:23-26.
- 530 17. **Ferraro, L., F. Fatichenti, and M. Ciani.** 2000. Pilot scale vinification process
531 using immobilized *Candida stellata* cells and *Saccharomyces cerevisiae*. Process
532 Biochemistry **35**:1125-1129.
- 533 18. **Fleet, G.** 2003. Yeast interactions and wine flavour. International Journal of Food
534 Microbiology **86**:11-22.
- 535 19. **Fleet, G., and G. Heard.** 1993. Yeasts - Growth during fermentation. Taylor &
536 Francis Inc, New York.
- 537 20. **Fleet, G. H.** 1993. The microorganisms of winemaking— isolation enumeration and
538 identification, p. 1-27, Wine Microbiology and Biotechnology. Harwood Academic
539 Publishers, Chur, Switzerland.
- 540 21. **Garcia, A., C. Carcel, L. Dulau, A. Samson, E. Aguera, E. Agosin, and Z.**
541 **Gunata.** 2002. Influence of a mixed culture with *Debaryomyces hansenii* and
542 *Saccharomyces cerevisiae* on the volatiles of a Muscat wine. Journal of Food
543 Science **67**:1138-1143.
- 544 22. **Garde-Cerdan, T., and C. Ancin-Azpilicueta.** 2006. Contribution of wild yeasts to
545 the formation of volatile compounds in inoculated wine fermentations. European
546 Food Research and Technology **222**:15-25.
- 547 23. **Gawel, R., L. Francis, and E. J. Waters.** 2007. Statistical correlations between the
548 in-mouth textural characteristics and the chemical composition of Shiraz wines.
549 Journal of Agricultural and Food Chemistry **55**:2683-2687.
- 550 24. **Gawel, R., S. Van Sluyter, and E. J. Waters.** 2007. The effects of ethanol and
551 glycerol on the body and other sensory characteristics of Riesling wines. Australian
552 Journal of Grape and Wine Research **13**:38-45.

- 553 25. **Godden, P. W., and R. Muhlack.** 2010. Trends in the composition of Australian
554 wine 1984-2008. *Australian and New Zealand Grapegrower and Winemaker* **5**:47-
555 61.
- 556 26. **Grant, M.** 2010. Who is listening? . p. 25-27. *In* A. W. I. T. C. Inc. (ed.),
557 Proceedings of Fourteenth Australian Wine Industry Technical Conference
- 558 27. **Green, S. R., and P. P. Gray.** 1950. A differential procedure applicable to
559 bacteriological investigation in brewing. *Wallerstein commentaries* **13**:357-366.
- 560 28. **Guth, H., and A. Sies.** 2001. Flavour of wines: towards and understanding by
561 reconstitution experiments and an analysis of ethanol's effect on odour activity of
562 key compounds. p. *In* A. W. I. T. C. Inc (ed.), Proceedings of Eleventh Australian
563 Wine Industry Technical Conference
- 564 29. **Jackson, R. S.** 2009. *Wine testing – A professional handbook*, 2nd ed. Academic
565 Press, San Diego, USA.
- 566 30. **Jolly, N., O. Augustyn, and I. S. Pretorius.** 2003. The occurrence of non-
567 *Saccharomyces cerevisiae* yeast species over three vintages in four vineyards and
568 grape musts from four production regions of the Western Cape, South Africa.
569 *South African Journal of Enology and Viticulture* **24**:35-42.
- 570 31. **Jolly, N. P., O. P. H. Augustyn, and I. S. Pretorius.** 2006. The role and use of
571 non-*Saccharomyces* yeasts in wine production. *South African Journal Enology and*
572 *Viticulture* **27**:15-39.
- 573 32. **Kutyna, D. R., C. Varela, P. A. Henschke, P. J. Chambers, and G. A. Stanley.**
574 2010. Microbiological approaches to lowering ethanol concentration in wine.
575 *Trends in Food Science & Technology* **21**:293-302.
- 576 33. **Lambrechts, M. G., and I. S. Pretorius.** 2000. Yeast and its importance to wine
577 aroma – a review. *South African Journal Enology and Viticulture* **21**:97-129.

- 578 34. **Lilly, M., M. G. Lambrechts, and I. S. Pretorius.** 2000. Effect of increased yeast
579 alcohol acetyltransferase activity on flavor profiles of wine and distillates. *Applied*
580 *and environmental microbiology* **66**:744-753.
- 581 35. **MacAvoy, M. G.** 2010. Wine – harmful or healthy? What is being considered in
582 Australia and New Zealand? . p. *In* A. W. I. T. C. Inc (ed.), Proceedings of
583 Fourteenth Australian Wine Industry Technical Conference
- 584 36. **Magyar, I., and T. Toth.** 2011. Comparative evaluation of some oenological
585 properties in wine strains of *Candida stellata*, *Candida zemplinina*, *Saccharomyces*
586 *uvarum* and *Saccharomyces cerevisiae*. *Food Microbiology* **28**:94-100.
- 587 37. **Medina, K., E. Boido, E. Dellacassa, and F. Carrau.** 2012. Growth of non-
588 *Saccharomyces* yeasts affects nutrient availability for *Saccharomyces cerevisiae*
589 during wine fermentation. *International Journal of Food Microbiology* **157**:245-250.
- 590 38. **Nissen, T. L., U. Schulze, J. Nielsen, and J. Villadsen.** 1997. Flux distributions in
591 anaerobic, glucose-limited continuous cultures of *Saccharomyces cerevisiae*.
592 *Microbiology* **143**:203-218.
- 593 39. **Palacios, A., F. Raginel, and A. Ortiz-Julien.** 2007. Can the selection of
594 *Saccharomyces cerevisiae* yeast lead to variations in the final alcohol degree of
595 wines? . *Australian and New Zealand Grapegrower and Winemaker* **527**:71-75.
- 596 40. **Rantsiou, K., S. Campolongo, V. Alessandria, L. Rolle, F. Torchio, and L.**
597 **Cocolin.** 2013. Yeast populations associated with grapes during withering and
598 their fate during alcoholic fermentation of high-sugar must. *Australian Journal of*
599 *Grape and Wine Research* **19**:40-46.
- 600 41. **Rapp, A., and H. Mandery.** 1986. Wine Aroma. *Experientia* **42**:873-884.
- 601 42. **Renouf, V., O. Claisse, and A. Lonvaud-Funel.** 2005. Understanding the
602 microbial ecosystem on the grape berry surface through numeration and

- 603 identification of yeast and bacteria. Australian Journal of Grape and Wine
604 Research **11**:316-327.
- 605 43. **Renouf, V., P. Strehaiano, and A. Lonvaud-Funel.** 2007. Yeast and bacteria
606 analysis of grape, wine and cellar equipments by PCR-DGGE. Journal International
607 des Sciences de la Vigne et du Vin **41**:51-61.
- 608 44. **Ribereau-Gayon, P., D. Dubourdieu, B. Doneche, and A. Lonvaud-Funel.** 2000.
609 The microbiology of wine and vinification, 2nd ed. John Wiley and Sons, New York,
610 USA.
- 611 45. **Rodriguez, M. E., C. A. Lopes, R. J. Barbagelata, N. B. Barda, and A. C.**
612 **Caballero.** 2010. Influence of *Candida pulcherrima* Patagonian strain on alcoholic
613 fermentation behaviour and wine aroma. International Journal of Food Microbiology
614 **138**:19-25.
- 615 46. **Rojas, V., J. V. Gil, F. Pinaga, and P. Manzanares.** 2001. Studies on acetate
616 ester production by non-*Saccharomyces* wine yeasts. International Journal of Food
617 Microbiology **70**:283-289.
- 618 47. **Sadoudi, M., R. Tourdot-Marechal, S. Rousseaux, D. Steyer, J. J. Gallardo-**
619 **Chacon, J. Ballester, S. Vichi, R. Guerin-Schneider, J. Caixach, and H.**
620 **Alexandre.** 2012. Yeast-yeast interactions revealed by aromatic profile analysis of
621 Sauvignon Blanc wine fermented by single or co-culture of non-*Saccharomyces*
622 and *Saccharomyces* yeasts. Food Microbiology **32**:243-253.
- 623 48. **Schmidt, S. A., S. Dillon, R. Kolouchova, P. A. Henschke, and P. J. Chambers.**
624 2011. Impacts of variations in elemental nutrient concentration of Chardonnay
625 musts on *Saccharomyces cerevisiae* fermentation kinetics and wine composition.
626 Applied microbiology and biotechnology **91**:365-75.

- 627 49. **Siebert, T. E., H. E. Smyth, D. L. Capone, C. Neuwohner, K. H. Pardon, G. K.**
628 **Skouroumounis, M. Herderich, M. A. Sefton, and A. P. Pollnitz.** 2005. Stable
629 isotope dilution analysis of wine fermentation products by HS-SPME-GC-MS.
630 Analytical and Bioanalytical Chemistry **381**:937-947.
- 631 50. **Soden, A., I. L. Francis, H. Oakey, and P. A. Henschke.** 2000. Effects of co-
632 fermentation with *Candida stellata* and *Saccharomyces cerevisiae* on the aroma
633 and composition of Chardonnay wine. Australian Journal of Grape and Wine
634 Research **6**:21-30.
- 635 51. **Swiegers, J. H., and I. S. Pretorius.** 2005. Yeast modulation of wine flavor.
636 Advances in Applied Microbiology **57**:131-175.
- 637 52. **Thornton, R. J., and S. B. Rodriguez.** 1996. Deacidification of red and white
638 wines by a mutant of *Schizosaccharomyces malidevorans* under commercial
639 winemaking conditions. Food Microbiology **13**:475-482.
- 640 53. **Tofalo, R., C. Chaves-Lopez, F. Di Fabio, M. Schirone, G. E. Felis, S. Torriani,**
641 **A. Paparella, and G. Suzzi.** 2009. Molecular identification and osmotolerant profile
642 of wine yeasts that ferment a high sugar grape must. International Journal of Food
643 Microbiology **130**:179-187.
- 644 54. **Toro, M. E., and F. Vazquez.** 2002. Fermentation behaviour of controlled mixed
645 and sequential cultures of *Candida cantarellii* and *Saccharomyces cerevisiae* wine
646 yeasts. World Journal of Microbiology and Biotechnology **18**:347-354.
- 647 55. **Ugliano, M., and P. A. Henschke.** 2009. Yeast and wine flavour, p. 313-392. *In* M.
648 V. Moreno-Arribas and C. Polo (ed.), Wine Chemistry and Biochemistry. Springer,
649 New York.

- 650 56. **Varela, C., D. Kutyna, P. A. Henschke, P. J. Chambers, M. J. Herderich, and I.**
651 **S. Pretorius.** 2008. Taking control of alcohol. The Australian and New Zealand
652 Wine Industry Journal **23**:41-43.
- 653 57. **Varela, C., T. Siebert, D. Cozzolino, L. Rose, H. McLean, and P. A. Henschke.**
654 2009. Discovering a chemical basis for differentiating wines made by fermentation
655 with 'wild' indigenous and inoculated yeasts: role of yeast volatile compounds.
656 Australian Journal of Grape and Wine Research **15**:238-248.
- 657 58. **Viana, F., J. V. Gil, S. Genoves, S. Valles, and P. Manzanares.** 2008. Rational
658 selection of non-*Saccharomyces* wine yeasts for mixed starters based on ester
659 formation and enological traits. Food Microbiology **25**:778-785.
- 660 59. **Zohre, D. E., and H. Erten.** 2002. The influence of *Kloeckera apiculata* and
661 *Candida pulcherrima* yeasts on wine fermentation. Process of Biochemistry
662 **38**:319-324.

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	<i>Saccharomyces cerevisiae</i>	0.44	98.5
AWRI258*	<i>Hanseniaspora valbyensis</i>	0.06	19.6
AWRI1499	<i>Dekkera bruxellensis</i>	0.09	8.5
AWRI1199	<i>Pichia fermentans</i>	0.11	2.5
AWRI1159*	<i>Candida stellata</i>	0.18	63.5
AWRI1101	<i>Dekkera anomala</i>	0.19	6.3
AWRI1128	<i>Dekkera anomala</i>	0.24	7.3
AWRI1032	<i>Schwanniomyces occidentalis</i>	0.29	14.4
AWRI1656*	<i>Metschnikowia pulcherrima</i>	0.31	22.7
AWRI1052*	<i>Kluyveromyces marxianus</i>	0.31	37.7
AWRI1045	<i>Williopsis mrakii</i>	0.32	16.2
AWRI1047*	<i>Wickerhamomyces subpelliculosus</i>	0.33	47.3
AWRI1005*	<i>Kluyveromyces marxianus</i>	0.33	33.8
AWRI863*	<i>Hanseniaspora uvarum</i>	0.33	20.5
AWRI1103*	<i>Dekkera bruxellensis</i>	0.34	32.2
AWRI1043*	<i>Pichia ciferrii</i>	0.34	46.0
AWRI1149*	<i>Metschnikowia pulcherrima</i>	0.35	42.7
AWRI747*	<i>Torulaspora pretoriensis</i>	0.35	28.9
AWRI141*	<i>Schizosaccharomyces pombe</i>	0.35	25.1
AWRI1165	<i>Candida diversa</i>	0.35	14.4
AWRI442*	<i>Schizosaccharomyces malidevorans</i>	0.36	32.4
AWRI1157	<i>Debaryomyces hansenii</i>	0.38	11.2
AWRI60	<i>Zygopichia spp</i>	0.39	6.3
AWRI1181	<i>Kluyveromyces lactis</i>	0.40	12.8
AWRI1220*	<i>Issatchenkia orientalis</i>	0.40	22.0
AWRI1152*	<i>Torulaspora delbrueckii</i>	0.41	41.9
AWRI1046	<i>Cyberlindnera saturnus</i>	0.42	18.9
AWRI1053	<i>Debaryomyces vanriijiae</i> var. <i>vanriijiae</i>	0.42	5.9
AWRI1578	<i>Zygosaccharomyces bailii</i>	0.45	44.3
AWRI58	<i>Kluyveromyces marxianus</i>	0.45	58.5
AWRI56	<i>Zygosaccharomyces rouxii</i>	0.47	15.5
AWRI861	<i>Lachancea thermotolerans</i>	0.48	31.7
AWRI1665	<i>Torulaspora castellii</i>	0.48	68.1
AWRI1425	<i>Hanseniaspora uvarum</i>	0.48	14.9
AWRI1164	<i>Trigonopsis cantarellii</i>	0.48	9.7

AWRI1821	<i>Pichia kluyveri</i>	0.50	11.8
AWRI1124	<i>Issatchenkia terricola</i>	0.50	8.6
AWRI1158	<i>Hanseniaspora uvarum</i>	0.50	10.4
AWRI1161	<i>Candida sake</i>	0.50	5.9
AWRI1552	<i>Meyerozyma guilliermondii</i>	a	2.4
AWRI1127	<i>Dekkera bruxellensis</i>	a	1.0
AWRI1095	<i>Pichia membranifaciens</i>	a	0.7
AWRI1051	<i>Pichia anomala</i>	a	0.0
AWRI1098	<i>Hasegawaea japonica</i>	a	0.0
AWRI1044	<i>Pichia holstii</i>	a	0.0
AWRI1094	<i>Pachysolen tannophilus</i>	a	0.0
AWRI2053	<i>Pichia spp</i>	a	0.0
AWRI1006	<i>Yamadazyma mexicanum</i>	a	0.0
AWRI743	<i>Rhodotorula glutinis</i>	a	0.0
AWRI69	<i>Sporobolomyces roseus</i>	a	0.0
AWRI950	<i>Dekkera custersiana</i>	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 ± 0.02	99.7^a ± 0.2	9.0^a ± 0.3	1.63^a ± 0.09
AWRI1170	0.33 ^c ± 0.01	80.4 ^b ± 1.4	4.3 ^c ± 0.2	0.65 ^d ± 0.02
AWRI1149	0.35 ^b ± 0.00	99.7 ^a ± 0.1	6.9 ^b ± 0.0	0.78 ^c ± 0.04
AWRI874	0.37 ^b ± 0.02	68.0 ^c ± 7.4	5.1 ^c ± 0.5	0.73 ^c ± 0.05
AWRI1191	0.41 ^{ab} ± 0.07	82.5 ^b ± 4.8	5.7 ^{bc} ± 1.5	0.14 ^e ± 0.03
AWRI448	0.48 ^a ± 0.02	76.6 ^{bc} ± 10.3	7.3 ^b ± 0.8	0.29 ^b ± 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
<i>Esters</i>				
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
<i>Higher alcohols</i>				
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
<i>Volatile Acids</i>				
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/L]	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

707	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
708	Values are means ± standard deviation of three independent replicates.				
709	Shared superscript letters (a, b, c) in the same column indicate no significant difference,				
710	different superscript letters in the same column indicate differences are significant (LSD				
711	test, p< 0.05).				
712	LOD indicates below Limit of Detection (0.5 µg/L).				
713	§ total excludes ethyl acetate				
714	† total excludes acetic acid.				



