1 **Aroma profiling of an aerated fermentation of natural grape must with selected yeast strains**
2 **at pilot scale**

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26 **Abstract**

27 The use of non-*Saccharomyces* strains in aerated conditions has proven effective for alcohol content
28 reduction in wine during lab-scale fermentation. The process has been scaled up to 20 L batches, in
29 order to produce lower alcohol wines amenable to sensory analysis. Sequential instead of
30 simultaneous inoculation was chosen to prevent oxygen exposure of *Saccharomyces cerevisiae*
31 during fermentation, since previous results indicated that this would result in increased acetic acid
32 production. In addition, an adaptation step was included to facilitate non-*Saccharomyces*
33 implantation in natural must. Wines elaborated with *Torulaspora delbrueckii* or *Metschnikowia*
34 in aerated conditions contained less alcohol than control wine (*S. cerevisiae*, non-
35 aerated). Sensory and aroma analysis revealed that the quality of mixed fermentations was affected
36 by the high levels of some yeast amino acid related byproducts, which suggests that further progress
37 requires a careful selection of non-*Saccharomyces* and the use of specific N-nutrients.

38

39

40 **Keywords**

41 reduced alcohol wine, aerobic fermentation, non-*Saccharomyces*, sensory analysis

42

43 **Highlights**

44 Aerated fermentation with non-*Saccharomyces* strains for reduced alcohol wine was scaled up.

45 Sensory analysis of wines in comparison with standard (*S. cerevisiae*, non aerated) was performed.

46 Wines elaborated with different strains had different aroma profiles.

47 Volatile compound analysis identifies the compounds responsible for differences in aroma nuances.

48 **1. Introduction**

49 *Saccharomyces cerevisiae*, the yeast carrying alcoholic fermentation of grape must, constitutes a
50 minor fraction of the microbiota found on sound ripe grapes (Wang et al., 2015). Other yeast
51 species, collectively known as non-*Saccharomyces* in this field, are much more abundant and
52 considered to play an important role during the first hours of grape must fermentation (Fleet and
53 Heard, 1993). Cell counts of the yeast genera *Hanseniaspora*, *Pichia*, *Metschnikowia* or
54 *Torulaspora* can be moderately high during a short time when alcohol levels are still low, before *S.*
55 *cerevisiae* takes over the fermentation process. There are many evidences that some non-
56 *Saccharomyces* yeast species can positively contribute to the aroma profile, sensory complexity,
57 and color stability of wines (Andorr et al., 2012; Comitini et al., 2011; Gobbi et al., 2013; Viana et
58 al., 2008; Sadoudi et al., 2012). Many authors have suggested the controlled use of these strains in
59 combination with *S. cerevisiae* in order to improve aromatic complexity of wine (Ciani et al., 2010;
60 Fleet, 2008; Padilla et al., 2016).

61 Nowadays, most yeast-producing companies have non-*Saccharomyces* yeast starters in their
62 catalogs, and among them, *Torulaspora delbrueckii* is the most represented in the market. Mixed
63 cultures of *T. delbrueckii*/ *S. cerevisiae* have been proposed to reduce the acetic acid content and to
64 enhance organoleptic profiles of wines (Moreno et al., 2001; Jolly et al., 2003; Bely et al., 2008).

65 The competitive advantage of *S. cerevisiae* over all the other yeast species during grape must
66 fermentation translates into a small variability in alcohol yield between different isolates of this
67 species. For that reason, the alcohol yield variability of non-*Saccharomyces* wine yeasts has been
68 explored by several authors (Ciani et al., 2016; Ciani and Maccarelli 1998; Comitini et al., 2011;
69 Domizio et al., 2011).

70 A recent proposal to reduce the ethanol content of wine considers the use of aerobic conditions
71 in order to allow for respiro-fermentative metabolism of grape juice sugars. Non-*Saccharomyces*
72 yeast strains are used in order to overcome the limitations due to the Crabtree positive character of

S. cerevisiae (Gonzalez et al., 2013). Relevant parameters to assess the potential usefulness of yeast strains for this purpose were not only their respiratory capacity under high sugar conditions, but the production of acetic acid and the amount of sugars consumed during the aerobic stage (Quirós et al., 2014). The feasibility of the process was proven at the laboratory scale by co-inoculation of *Metschnikowia pulcherrima* and *S. cerevisiae*, and controlled aeration during the first 48 h (Morales et al., 2015). A maximal reduction of 3.7% ABV (alcohol by volume) was achieved for the fermentation of a natural grape must (260 g/L sugars), as compared to anaerobic fermentation with *S. cerevisiae*. Considering additional parameters, like keeping dissolved oxygen levels as low as possible, and avoiding excess volatile acidity, a 2.2% ABV reduction was achieved under optimized conditions. The aim of this work was to scale-up this process to pilot scale in order to identify potential bottlenecks outside the controlled conditions of the laboratory, and to produce wines amenable to sensory analysis. A strain of *M. pulcherrima* and a commercial strain of *Torulaspora delbrueckii* were used.

The effect of the commercial strain *T. delbrueckii* Viniferm NSTD on wine quality had been previously analyzed under standard fermentation conditions (Belda et al., 2015). The mouthfeel properties of wine produced at semi-pilot scale in a sequential inoculation with *S. cerevisiae* were preferred by a sensory panel, and correlated with an increase in the mannoprotein content.

2. Materials and methods

2.1. Strains and laboratory media

Strain *M. pulcherrima* Mp591, used in preliminary winemaking experiments, was provided by Agrovin S.A. (Alcázar de San Juan, Spain). *M. pulcherrima* strains used in the screening were

98 isolated from grapes in La Rioja, Spain, and belong to the Microwine group strain collection
99 (Instituto de Ciencias de la Vid y del Vino, Logroño, Spain). *M. pulcherrima* CECT 12841
100 (Morales et al., 2015) was used as a reference for the screening. *M. pulcherrima* Mp395, used in the
101 final fermentation trial, was selected in the screening among other isolates of this species, based on
102 the amount of sugars consumed, ethanol yield, and low aroma impact in a synthetic must. *S.*
103 *cerevisiae* Viniferm Carácter and *T. delbrueckii* Viniferm NSTD are commercial strains from
104 Agrovin S.A. (Alcázar de San Juan, Spain).

105 Synthetic grape must contained: 100 g/L glucose, 100 g/L fructose, 6 g/L citric acid, 6 g/L malic
106 acid, 0.764 g/L ammonium chloride, 1.7 g/L Yeast Nitrogen Base without ammonium sulphate and
107 amino acids, and 18 mg/L myo-inositol, pH adjusted to 3.5 with NaOH.

108

109 2.2. Screening of *M. pulcherrima* strains

110

111 *M. pulcherrima* strains were grown on YPD (2% glucose, 1% yeast extract, 2% peptone) for 48
112 hours at 25°C and 200 rpm. Cells were washed 2 times and resuspended in water to OD₆₀₀=10.
113 Then, 250 ml Erlenmeyer flasks containing 50 ml synthetic grape must were inoculated with 1 ml
114 preculture, covered with an aluminium foil, and incubated for 4 days at 200 rpm at 18°C. After this
115 time, consumed sugars and metabolites produced were determined by HPLC as described in section
116 2.5. Experiments were carried out in duplicate.

117

118 2.3. Non-Saccharomyces inoculum preparation for winemaking

119

120 Non-*Saccharomyces* strains were grown in YPD for 48 hours at 25°C and 200 rpm. After
121 centrifugation, aliquots of 8000 units OD₆₀₀ were suspended in 1 L pasteurized natural white must,
122 and incubated for 3 days at 150 rpm and 22°C to adapt them to grape must. Natural must was

123 pasteurized in the autoclave by heating up to reach 105°C and leaving to cool down inside. The
124 whole culture was then used to inoculate 20 L of fresh natural non-sterile grape must (see below).

125

126 2.4. Scaled-up aerated winemaking procedure

127

128 Natural Viura-Malvasía white must was racked overnight at 4°C. It contained 21% sugars, 237
129 mg/L total assimilable nitrogen, and 35 mg/L total SO₂, pH 3.43. Batches of 20 L in 30 L vats (36
130 cm diameter, resulting in a column of liquid about 20 cm high) were inoculated with 1 L
131 conditioned inoculum of *M. pulcherrima* or *T. delbrueckii*. Batches of 21 L were inoculated with *S.*
132 *cerevisiae* following the instructions of manufacturer (30 g/HL). In this way, the input volume of
133 grape must in the whole process was the same for all conditions (21 L). Each tank was
134 supplemented with 1.4 g/L tartaric acid, and 0.3 g/L Actimax Natura (Agrovin S.A., Spain). Three
135 vats were fermented for each condition, using independent inocula. Vats inoculated with non-
136 *Saccharomyces* were sparged with compressed air at 200 mL/h through submerged ceramic
137 spargers. Gas flow was controlled with MFC17 mass flow controllers (Aalborg Instruments and
138 Controls, Inc.; Orangeburg, NY), previously calibrated with an electronic precision flowmeter
139 (Agilent Technologies, Santa Clara, CA). Room temperature was maintained at 18°C.

140 Temperature and density were monitored daily. Density was measured with a portable digital
141 densitometer (Densito 30PX, Mettler Toledo GmbH, Analytical, Schwerzenbach, CH). At day 4, air
142 flow was stopped, 50 mg/L potassium bisulfite was added and, one hour later, vats were inoculated
143 with *S. cerevisiae*, following the instructions of manufacturer (30 g/HL). At day 5, 0.3 g/L Actimax
144 Plus (Agrovin S.A., Spain) was added in all vats, control vats included. After sugar depletion, on
145 day 9, 90 mg/L potassium bisulfite was added in each vat, headspace filled with nitrogen and vats
146 closed and kept 10 days at 10°C. Finally, wine was transferred into colored glass bottles and kept at
147 4°C.

148 Implantation of yeast starter cultures was monitored along the fermentation. Samples of days 0, 4
149 and 8 were plated on YPD, and DNA of 5 isolated colonies extracted (Looke et al., 2011). The
150 presence of *M. pulcherrima* or *T. delbrueckii* was confirmed by PCR amplification of d1/d2 LSU
151 26S DNA and sequencing (Kurtzman and Robnett 1998). Amplification of interdelta elements
152 (Legras and Karst, 2003) was used to verify implantation at the *S. cerevisiae* strain level.

153 Production and consumption of the main fermentation-related metabolites in daily samples was
154 determined by HPLC.

155

156 2.5. HPLC analysis of main fermentation metabolites

157

158 Production and consumption of the main fermentation-related metabolites in daily samples,
159 (glucose, fructose, glycerol, acetic acid and ethanol) were determined in duplicate using a Surveyor
160 Plus chromatograph (Thermo Fisher Scientific, Waltham, MA) equipped with a refractive index and
161 a photodiode array detector (Surveyor RI Plus and Surveyor PDA Plus, respectively). Hyper REZ
162 XP carbohydrate H+8 μm column and guard (Thermo Fisher Scientific) were used and maintained
163 at 50°C. Elution was performed with 1.5 mM H₂SO₄ as mobile phase, at a flow rate of 0.6 mL/min.
164 Prior to injection, samples were filtered through 0.22- μm -pore-size nylon filters and diluted 10-fold.

165 One way analysis of variance was carried out on the main fermentation metabolites. Means of
166 biological replicates were compared using Tukey's test, with significance level set at 5%. All
167 analyses were performed using SPSS Statistics v. 23 program (IBM, Armonk, NY).

168

169 2.6. Sensory analysis of wines

170

171 Sensory analysis was performed one month after bottling. The starting point was a sorting task to
172 select exemplars representative for sensory differences observed in the sensory space. These

173 samples were further characterized (flash profile) by a panel of semi-trained panelists and their
174 aroma quality was finally evaluated by a panel of wine experts. In the three tasks, samples were
175 presented simultaneously attending to a random order different for each assessor. Twenty-mL
176 samples were poured in dark wine glasses (ISO 3591, 1977) labelled with 3-digit random codes and
177 covered by plastic Petri dishes. All samples were served at room temperature and evaluated in
178 individual booths. Panelists were not informed about the nature of the samples to be evaluated.

179

180 2.6.1. *Sorting task*

181 The sorting task consisted in grouping wines by similarity and generating descriptors to
182 differentiate the wines. A total of eleven wines (9 vats + 2 duplicates) were evaluated. Vats Sc1,
183 Sc2 and Sc3, were elaborated with *S. cerevisiae*; Mp4, Mp5 and Mp6, elaborated with *M.*
184 *pulcherrima*; Td7, Td8 and Td9, elaborated with *T. delbrueckii*. The sorting task was carried out by
185 a panel of eighteen wine experts (11 women and 7 men, ranging from 23 to 63 years of age, average
186 = 35) in two independent sessions. In a first session, the panel was asked to group samples by
187 orthonasal aroma; and in a second session, according to in-mouth sensations (aroma, mouthfeel and
188 taste). No limits to number of groups were given. Panelists were asked to write a maximum of 3
189 words describing each group of wines.

190

191 2.6.1.1. *Sorting task data analysis*

192 An individual similarity binary matrix (11 wines x 11wines) was built with data of each panelist,
193 where 1 means similar and 0 means different. A co-occurrence matrix, obtained by sum of all
194 panelists, was submitted to a non-parametric Multidimensional Scaling (MDS) analysis (absolute
195 model) in order to obtain a spatial representation of wines. The quality and the reliability of
196 representations were evaluated by Shepard diagrams and Kruskal's stress value. Finally,
197 Hierarchical cluster analysis (HCA) with the Ward criterion was performed on the matrix consisting

198 of wines x coordinates of the retained MDS dimensions. All analyses were carried out with
199 XLSTAT (2015 version).

200 A list of 12 descriptors for these wines was made with terms generated by panel members,
201 avoiding hedonic and quantity adjectives, and grouping words belonging to the same category
202 (Franco-Luesma et al., 2016). Descriptors are listed in Table 1.

203

204 2.6.2. *Aroma characterization: flash profile*

205 A flash profile was carried for wine aroma characterization. The panel was formed by 13 semi-
206 trained assessors (8 women and 5 men, ranging from 25 to 39 years old, average = 31) with
207 experience in sensory description of wine. The task was similar to classical flash profile, with some
208 modifications carried out with the aim of facilitating the interpretation of attributes, which deems
209 difficult in this methodology given the absence of consensus and training of participants. Therefore,
210 references for the 12 terms obtained in sorting task (Table 1) were built and presented to
211 participants. This familiarization task finished when panelists could correctly match terms with
212 reference standards. Afterwards, they were presented with the six samples, four representing each
213 group formed in previous task, and 2 duplicates. In a first session, assessors were asked to provide
214 the descriptors differentiating each wine. In a second session, they were asked to rank the six wines
215 attending to each one the terms chosen to differentiate among samples. A non-structured 10 cm
216 continuous length scale anchored with the words “absence” and “high intensity” on the left and
217 right ends was provided for each descriptor.

218

219 2.6.2.1. *Flash profile data analysis*

220 Principal Component Analysis (PCA) was performed with the mean intensity scores of
221 descriptors that were individually discriminant in a two-way ANOVA (participants as random and

222 wines as fix factors) and that were used by more than half of panelists. Analyses were carried out
223 with XLSTAT software (version 2015).

224

225 2.6.3. *Aroma quality evaluation*

226 Evaluation of aroma quality was carried out by a panel of 12 wine experts (7 women, ranging
227 from 27 to 62 years old, average = 38). They were all oenologists, who had attended wine-tasting
228 classes and had relevant professional experience in winemaking (Parr et al., 2002). Assessors were
229 presented with seven wines: the four representing each group formed in sorting task and three
230 control samples. The control wines comprised one young white wine (elaborated with Viura) of
231 high quality (C_hq) and two white wines of low quality representing reduction (C_Red) and
232 oxidation (C_ox) defects. Reduction defect was generated by spiking wines with hydrogen sulfide
233 ($60 \mu\text{g L}^{-1}$) and methanethiol ($20 \mu\text{g L}^{-1}$) and oxidation with methional ($90 \mu\text{g L}^{-1}$) and
234 phenylacetaldehyde ($180 \mu\text{g L}^{-1}$). Participants were asked to smell each sample from left to right
235 and to score their aroma quality on a nine-point scale (1=very poor; 3=poor; 5=average; 7=good and
236 9=very good) based on orthonasal olfaction.

237 2.6.3.1. *Aroma quality data analysis*

238 A two-way ANOVA was carried on quality scores with assessors as random factor and wines as
239 fixed factor, followed by Fischer post-hoc pairwise comparison (95%) test.

240

241 2.7. *Volatile compounds analysis*

242

243 Major volatile compounds were isolated by liquid-liquid extraction and analyzed in a gas
244 chromatograph with flame ionization detector (GC-FID) as described (Ortega et al., 2001). Minor
245 and trace volatile compounds were isolated through solid-phase extraction (SPE) and analyzed by

246 gas chromatography coupled to a mass spectrometry detection system (GC-MS), as described
247 (Lopez et al., 2002).

248 Polyfunctional mercaptans were analyzed and quantified by GC-MS with negative chemical
249 ionization (NCI) after SPE derivatization with 2,3,4,5,6-pentafluorobenzylbromide (PFBBBr)
250 (Mateo-Vivaracho et al., 2008).

251 Free Volatile Sulfur Compounds (VSCs) were determined by direct static headspace analysis
252 using a GC coupled with a pulsed flame photometric detection system (GC-PFPD) (Franco-Luesma
253 and Ferreira, 2014).

254 Free forms of aldehydes (methional, isobutyraldehyde, isovaleraldehyde, and
255 phenylacetaldehyde) were quantified by SPME followed by GC-MS as described (Bueno et al.,
256 2014).

257

258 *2.7.1. Volatile compound data analysis*

259 Quantitative data of volatile compounds were transformed into Odor Activity Values (OAV) by
260 dividing them by their corresponding sensory thresholds (ST). The OAV of the limits of detection
261 and quantification was also calculated and used as minimal value when that of compound was lower
262 (San Juan et al., 2011). Odorants with similar chemical and sensory properties were grouped in
263 aroma vectors (Loscos et al., 2007, Saenz_Navajas et al., 2015). Table 2 shows the composition of
264 the fourteen aroma vectors constructed. To rank compounds or families of compounds in
265 accordance to the differentiation ability, the quotient between the maximum OAV and minimum
266 OAV was worked out for each compound or family. Value max/min = 1.5 was established as
267 threshold.

268

269 *2.8. Multivariate analysis*

270

Principal Component Analysis (PCA) was calculated with sensory descriptors as active variables and chemical compounds (expressed as OAVs) as supplementary variables. Only chemical compounds presenting OAV>1.5 in at least one wine were considered. The statistical analyses were carried out with XLSTAT software (Version 2014.2.02).

3. Results and discussion

3.1. Selection of a *Metschnikowia pulcherrima* strain

Some strains of *M. pulcherrima* had shown good properties to be used in aerobic fermentation for alcohol level reduction (Quirós et al., 2014) and one of them was successfully used at laboratory scale in co-inoculation with *S. cerevisiae* (Morales et al., 2015). For that reason, we decided to make a screening among different grape isolates of *M. pulcherrima* to select a good candidate for further development.

The screening involved 11 *M. pulcherrima* recent isolates, in addition to *M. pulcherrima* CECT 12841, from the previous work (Morales et al., 2015), as a reference. Strains were grown in a synthetic must with vigorous agitation for 4 days at 18°C and parameters considered important for the correct behavior of strains in aerated fermentation were measured. Results are presented in Figure 1. The strain with the lowest ethanol yield was Mp274, but it also showed the highest acetic acid yield (see plot) and ethyl acetate production (data not shown). Strain Mp440 had the lowest acetic acid yield and very low ethanol yield, but the amount of sugars consumed was lower than other strains. There was a group of 5 strains with a low acetic acid yield and similar ethanol yield: Mp374, Mp395, Mp411, Mp416 and Mp711. Among them, Mp395 and Mp711 showed the highest amounts of consumed sugars and glycerol production. All strains could ferment a synthetic must

295 with 400 g/L sugars, and consumed between 86 g/L (Mp594) and 138 g/L (Mp395) in 4 days at
296 25°C (data not shown).

297

298 3.2. Preliminary pilot-scale tests

299

300 Two fermentation assays were run during the 2015 harvest season (prior to *M. pulcherrima* strain
301 selection). Non-*Saccharomyces* strains were grown in YPD for 48 h, centrifuged and then
302 inoculated in must at initial OD600 of 0.4. An aeration regime of 60 L/h (3 VVH) was maintained
303 for 48 h in vats inoculated with non-*Saccharomyces*. After this time, aeration was stopped, and *S.*
304 *cerevisiae* was added as dry yeast at 30 g/HL. Nitrogen supplementation was performed at the
305 beginning and after inoculation of *S. cerevisiae*, as described in Materials and Methods. Room
306 temperature was set at 20°C. The wines produced in aerated conditions contained less alcohol than
307 the control (see Table 4), but acetic acid was over the limits of acceptability (data not shown). In
308 addition, microbiological analyses showed that in these conditions, native must microbiota
309 prevailed over the inoculated non-*Saccharomyces* strains 24 h after inoculation.

310 Considering these results, a second trial including reduced airflow (12 L/h or 0.6 VVH) and a
311 step of adaptation of strains to must conditions was run. For the latter, strains were grown for 48 h
312 in YPD, cells were then collected and suspended in 1 L of pasteurized must at OD 8. Cells were
313 incubated for 3 days with vigorous agitation and then used to inoculate 20 L fresh grape must.
314 Room temperature was set at 20°C. In these conditions, inoculated non-*Saccharomyces* strains
315 prevailed over wild microbiota at least until *S. cerevisiae* inoculation. Reduction in alcohol levels
316 was moderate (see Table 4), but still significant, and acetic acid produced was very low in all
317 conditions. This fermentation was performed at the very end of the 2015 harvest season, and counts
318 of *S. cerevisiae* in must were high. Must contained 0.5 % ethanol (v/v) just before inoculation. For
319 that reason, we decided to repeat this assay under more suitable conditions.

320

321 3.3. Optimized pilot-scale aerobic fermentation

322

323 This experiment was carried during the 2016 harvest season with a white must containing 21%
324 sugars, pH 3.43, and 237 mg/L total assimilable nitrogen. *T. delbrueckii* NSTD and *M. pulcherrima*
325 Mp395 were conditioned as previously described. Room temperature was set at 18°C and aeration
326 in non-*Saccharomyces* vats at 12 L/h. Must was racked overnight at 4°C, just before inoculation,
327 and was still cold at inoculation time. After 2 days, the temperature increase in vats indicated
328 microbial activity in non-*Saccharomyces* vats (see Figure 2). For that reason, aeration was kept till
329 day 4, longer than in previous assays. Potassium bisulfite was added just after aeration stopping,
330 and 1 hour later *S. cerevisiae* added in non-*Saccharomyces* vats, as active dry yeast at 30 g/HL. On
331 day 5, total nitrogen was below 15 mg/L and an extra addition of nitrogen supplements was done in
332 all vats to help *S. cerevisiae* activity. On day 9, density indicated that sugars were depleted in all
333 vats so 90 mg/L potassium bisulfite was added in each vat, head space filled with nitrogen and vats
334 closed and kept for 10 days at 10°C. Then, wine was transferred into colored glass bottles and kept
335 at 4°C.

336 Microbiological analysis showed that must contained 2.6×10^3 cells/ml just before inoculation.
337 Maximal counts in non-*Saccharomyces* vats, higher than 10^8 cells/ml, were found on day 2. The
338 color of colonies in plates indicated that *M. pulcherrima* was dominant in the vats where it had been
339 inoculated. Maximal counts in *S. cerevisiae* vats were reached on day 4, lower than 10^7 cells/ml,
340 and maintained constant till the end of fermentation.

341 On day 4, before inoculation with *S. cerevisiae*, counts in *M. pulcherrima* vats were 1 log unit
342 lower than on day 2, and the color indicated that a third of colonies were other microorganisms. All
343 five sequenced non-*Metschnikowia* colonies were *S. cerevisiae*. Counts in *T. delbrueckii* vats for

344 this time point were about 2 log units lower than on day 2. All five sequenced colonies were *T.*
345 *delbrueckii*.

346 The must density curve followed the same pattern than residual sugars, plotted in Figure 2. Sugar
347 consumption in non-*Saccharomyces* vats was appreciated earlier than in *S. cerevisiae* vats. On day
348 2, there were 203, 176 and 153 g/L residual sugars for *Saccharomyces*, *Metschnikowia* and
349 *Torulaspora* vats respectively. On day 4, before addition of *S. cerevisiae*, residual sugars in *S.*
350 *cerevisiae* and in *M. pulcherrima* vats were similar, around 50% of initial sugars, while in *T.*
351 *delbrueckii* vats the 75% of initial sugars had been consumed. Sugars had been exhausted on day 7
352 in *S. cerevisiae* vats and on day 8 in non-*Saccharomyces* vats. The aerated process had taken only
353 one day more than the traditional one.

354 Table 5 shows metabolites found at the end of fermentation. A moderate reduction in ethanol
355 content, but still significant, was achieved by the end of fermentation. The levels of acetic acid were
356 low in all samples. Moreover, levels were significantly lower in non-*Saccharomyces*, aerated
357 fermentations than in *S. cerevisiae* fermentations. *M. pulcherrima* produced the highest levels of
358 glycerol.

359

360 3.4. Sensory analysis

361 Results of the sorting task based exclusively on orthonasal aroma perception are summarized in
362 the dendrogram shown in Figure 3. Samples group in three stable clusters perfectly matching the
363 yeast used. Wines belonging to the same cluster were grouped together at least 10 times (56% of
364 participants), except the Mp4 wine which was grouped with Mp5 and Mp6 six (33%) and four
365 (22%) times, respectively, which suggests that is the least similar to the other two replicates. For
366 that reason, this cluster containing *M. pulcherrima* wines was split into two for wine
367 characterization. Results of the sorting task based on the overall flavor (aroma, taste and mouth-feel

368 properties) produced similar results (Supplementary Figure S1) which suggests that most sensory
369 differences are mainly driven by aroma properties.

370 Wines Sc1, Td7, Mp5 and Mp4 (with replicates of Sc1* and Td7* as controls) were chosen as
371 group representative for wine aroma characterization and were subjected to orthonasal descriptive
372 analysis by means of flash profile with a panel of semi-trained assessors. Training consisted in
373 familiarization with terms and references obtained from the sorting task and given in Table 1.
374 Sixteen different terms were generated including the 12 attributes in Table 1, together with *meat*,
375 *grain*, lemon (cited by just one participant) and *red fruit* (cited by two participants). The more cited
376 terms (at least 7 out of 13 panelists) were: *oxidation*, *spirit-like*, *dried fruit*, *nuts-walnut*, *reduction*,
377 *white fruit-pear* and *tropical fruit-banana*. As the pairs *dried fruits/nuts-walnuts* and
378 *oxidation/spirit-like* were strongly correlated ($r>0.90$) they were further considered as single terms
379 under the labels *dried fruit/nuts* and *oxidation/spirit-like*.

380 Figure 4 shows the projection of wines on the graph obtained with the first and second principal
381 components of PCA analysis, representing respectively 58% and 38% of variance. Duplicate
382 samples group together in the plot, indicating the reliability of panel. Three groups of wines can be
383 observed in the graph, coinciding with yeasts used. This result suggests that even if wine Mp4
384 seems to be relatively different from Mp5, they present aroma commonalities that make them to be
385 more similar to each other than to *S. cerevisiae* or *T. delbrueckii* wines. The first PC confronts the
386 terms *white fruit-pear* and *tropical fruit-banana*, mainly attributed to wines elaborated with *S.*
387 *cerevisiae* yeasts, to *dried fruit/nuts* and *reduction*, which characterize *T. delbrueckii* wines. The
388 second PC is basically driven by the term *oxidation/spirit-like*, which seems to be predominant in
389 *M. pulcherrima*, especially in Mp4 and to a lesser extent in Mp5. This can be clearly seen in the
390 spider plot shown in Figure 5, which confirms that *S. cerevisiae* wines have maxima scores for
391 *white fruit-pear* and *tropical fruit-banana*, *T. delbrueckii* wines for *dried fruit/nuts* and *reduction*
392 and, *M. pulcherrima* wines for *oxidation/spirit-like*.

Aroma quality was also assessed and results are summarized in Figure 6. As seen in the Figure, scores for experimental wines ranged from 3.9 (poor-average quality) for both *M. pulcherrima* wines to 6.7 (good quality) for *S. cerevisiae* wine. *T. delbrueckii* wine was classified as average quality.

3.5. Volatile compound analysis

Table 6 shows the quantitative data of more than 80 volatile compounds found in the 4 exemplars analyzed used in sensory analyses. Concentrations are within the normal range of occurrence in wines (San Juan et al., 2012; Swiegers et al., 2005) with some exceptions, since levels of ethyl dihydroxycinnamate, methionol and β -phenylethanol are unusually high in *T. delbrueckii* sample, and those of 2-methyl-1-propanol (isobutanol) in *M. pulcherrima* samples.

Data of aroma compound concentration were converted into OAVs and further grouped with other aroma molecules with similar odors into aroma vectors, as shown in Table 3. The biplot with the two first components of the PCA made on sensory data and aroma vectors is given in Figure 7. The plot makes it possible to identify the aroma vectors potentially responsible for the sensory differences observed between samples. The fruity character of wines elaborated with *S. cerevisiae* is consistent with the higher levels of acetates, especially 3-mercaptopentyl acetate (MHA), and ethyl esters. The lowest aroma quality of *Metschnikowia* wines is no doubt related to their oxidation and spirit/like character and to their negligible fruity character. These sensory notes can be attributed to the highest levels of aliphatic fusel alcohols, which have been found to impair the perception of fruitiness and give a spirit note (de-la-Fuente-Blanco et al., 2017), and to the highest levels of Strecker aldehydes and of acetaldehyde, which are responsible for the oxidative notes. Finally, the reductive odor note found in *Torulaspora* wines should be related to their highest levels in VSCs (Franco-Luesma et al., 2016), while the dry fruit/nut character may be related to the highest levels of methionol (San-Juan et al., 2011) and of cinnamates. The fact that the oxidation notes were found

only in *Metschnikowia* wines and not in *Torulaspora* wines indicates that this defect is related to the strain used, rather than to the process of aeration on its own. Strain selection for commercial purposes would require the analysis of volatile compounds produced under aerated conditions.

It is noteworthy that many compounds explaining aroma differences are related to the amino acid metabolism of the different yeast strains. This is the case of fusel alcohols and their acetates, of Strecker aldehydes, and of the most important VSCs: H₂S and methanethiol. Attending to present data, it seems that some of these compounds are most likely responsible for some of the aromatic problems detected in *Metschnikowia* wines (oxidation, lack of fruitness) and *Torulaspora* wines (reduction). Thus, it can be hypothesized that a specific reengineering of the nitrogen supplementations provided to the yeast may produce wines with much improved sensory characters and yet reduced levels of ethanol.

4. Conclusions

In summary, we have shown the feasibility of scaling up aerated fermentation conditions, and the use of non-*Saccharomyces* yeast strains, for reducing ethanol content of wines. One key point in the optimization process has been the improvement of the inoculum preparation step, to warrant pre-adaptation of non-*Saccharomyces* cells to grape must, as well as an active metabolism ever since the inoculation time. Aeration conditions could not be extrapolated directly from the relative air flows (vvh) under laboratory conditions, and probably increasing the depth of the tanks would require further reduction in air flows. Since we have previously shown the increased production of acetic acid by *S. cerevisiae* under aerated conditions, sequential inoculation, with *S. cerevisiae* being inoculated after aeration is stopped, seems to be a better choice than co-inoculation with non-*Saccharomyces* strains. The secondary problem of nutrient depletion by the non-*Saccharomyces* starter, before inoculation of standard wine yeasts, has been easily addressed by a rational use of yeast nutrients in key moments of the process. However, results of sensory and aroma analysis

443 suggest that those nutrients should be specifically formulated to limit the formation of problematic
444 compounds such as VSCs, Strecker aldehydes or fusel alcohols. While the current protocol allowed
445 circumventing the problem of acetic acid production, further optimization will be required to
446 develop an industrially feasible protocol for aerated fermentation with non-*Saccharomyces* yeast
447 strains. Topics to be further addressed are the problem of adjusting oxygenation levels to improve
448 alcohol reduction, the non-*Saccharomyces* strain selection, and the formulation of specific nutrients
449 to limit the formation of aroma compounds of demonstrated negative character.

450

451 **Abbreviation**

452 **HCA**, Hierarchical Cluster Analysis; **MDS**, Multidimensional Scaling; **OAV**, Odor Activity Value;
453 **PCA**, Principal Component Analysis; **YAN**, Yeast Assimilable Nitrogen.

454

455 **Acknowledgements**

456

457 Agrovin S.A. is acknowledged for providing yeast strains and enological products.

458 This work was supported by grants MINECO/FEDER RTC-2014-2186-2 (SOUNDWINE)
459 developed in collaboration with Agrovin S.A., and AGL2015-63629-R, and EU project YeSVitE.
460 (7FP-IRSES-GA n° 612441). JAC and PSN are the recipients of a MINECO Formación
461 Postdoctoral contract from the Spanish Government.

462

463 **Supplementary data**

464

465 Figure S1. Tree diagram obtained from Hierarchical Cluster Analysis (HCA) with the Ward
466 criterion performed on data from sorting task based on aroma taste and mouth-feel properties.

467 List S1. References for Odor threshold in Table 6.

468

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Figure Captions

Figure 1. Sugars consumed and metabolites produced by *Metschnikowia pulcherrima* strains in
synthetic must (200 g/L sugars) at 18°C.

587 **Figure 2.** Monitoring of fermentation parameters.

588 **Figure 3.** Tree diagram obtained from Hierarchical Cluster Analysis (HCA) with the Ward criterion
589 of wines performed with data from MDS of orthonasal aroma descriptors as variables.

590 **Figure 4.** Projection of wines used in flash profile (4 wines + 2 replicates) and discriminant
591 attributes on the two first dimensions (PC1 and PC2) of the PCA performed with selected aroma
592 descriptors.

593 **Figure 5.** Sensory description of wine samples (average for duplicate samples Sc1 and Td7).

594 **Figure 6.** Mean aroma quality ratings of studied wines (including controls: C_ox, C_red, C_hq.
595 Different letters indicate the existence of a significant difference between samples ($\alpha < 0.05$) (Fischer
596 post-hoc test). Error bars are calculated as $s/(n)^{1/2}$; s, standard deviation; n, number of assessors.

597 **Figure 7.** Projection of sensory descriptors (blue color), chemical vectors (red color), and wines on
598 the two first dimensions (PC1 and PC2) of the PCA performed with sensory descriptors as active
599 variables and chemical variables (expressed as OAVs) as supplementary variables.

600

601