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**Volatile profile of white wines fermented with sequential inoculation of *Starmerella bacillaris* and *Saccharomyces cerevisiae***

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1 **Volatile profile of white wines fermented with sequential inoculation of *Starmerella***  
2 ***bacillaris* and *Saccharomyces cerevisiae***

3

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**ABSTRACT**

Mixed fermentations with *Starmerella bacillaris* and *Saccharomyces cerevisiae* affects the chemical composition of wines by modulating various metabolites of enological interest. The current study was carried out to elucidate the effect of sequential inoculation of the above mentioned species on the production of white wines, especially on the chemical and aromatic characteristics of Chardonnay, Muscat, Riesling and Sauvignon blanc wines. Analysis form chemical composition showed that titratable acidity and glycerol content exhibited evident differences among the wines after fermentation. For volatile compounds, mixed fermentations led to a reduction of the total ester, including ethyl acetate, which is a compound responsible for wine deterioration. However, Sauvignon blanc wines fermented by mixed cultures contained significantly higher levels of esters and thiols, both associated with positive sensory attributes. These findings suggest that sequential inoculations posed a great potential in affecting and modulating the chemical and aromatic profile of white wines, especially those produced from Sauvignon blanc grapes.

**Keywords:** non-*Saccharomyces*, *Starmerella bacillaris*, sequential inoculation, white grape varieties, aroma profile

## 62 1. Introduction

63

64 Aroma is an important aspect of grape and wine quality, since it has a substantial  
65 influence on consumer acceptance (Sáenz-Navajas, Ballester, Fernández-Zurbano, Ferreira,  
66 Peyron & Valentin, 2016). Several aroma families construct the volatile composition of  
67 wines, among them alcohols are known to contribute to herbaceous characters, esters and  
68 terpenes to fruity and floral characters, C<sub>13</sub>-norisoprenoids to balsamic and violet aromas  
69 (Dzialo, Park, Steensels, Lievens & Verstrepen, 2017; Swiegers, Bartowsky, Henschke &  
70 Pretorius, 2005). Meanwhile, thiols generally contribute to blackcurrant, passion fruit and  
71 citrus zest descriptors (Francis & Newton, 2005). Many of these metabolic compounds are  
72 produced from non-volatile precursors through complex metabolic reactions, which begin  
73 during grape ripening and continue throughout fermentation, ageing and bottling (Swiegers et  
74 al., 2005).

75 During fermentation the yeasts, through their central glycolytic pathway, transform  
76 the sweet and low aroma must into an alcoholic, high aroma beverage. In this process, each  
77 glucose and fructose molecule is split and converted to ethanol, carbon dioxide and plenty of  
78 volatile metabolites that contribute individually or synergistically to wine composition and  
79 sensory profile, in order to provide energy necessary for cell growth maintenance and  
80 reproduction (Belda et al., 2017; Fleet, 2008; Molina, Guadalupe, Varela, Swiegers, Pretorius  
81 & Agostin, 2009). In addition to this, many volatile metabolites are also released from non-  
82 volatile grape derived precursors by yeast enzymes (Swiegers et al., 2005). Examples are  
83 monoterpenes and C<sub>13</sub>-norisoprenoids, which are released from glycosidic precursors, and  
84 long-chain polyfunctional thiols, which are derived from S-cysteinylated conjugates. The  
85 production of these metabolites is strictly correlated with the fermentation conditions which

86 the yeasts strain(s) is subjected to, that is: strain compatibility, physicochemical and nutrition  
87 parameters (Belda et al., 2017).

88 Grapes and winery equipment contain a large variety of indigenous yeasts, that are  
89 involved in spontaneously fermented wines (Fleet, 2008). Allowing the must to ferment with  
90 indigenous yeasts can potentially increase the complexity of wine aromas due to the diversity  
91 of yeast species and strains, which are present (Belda et al., 2017). However, the lack of  
92 reproducibility and predictability on these fermentations has favoured the use of yeast  
93 starters, generally strains of *Saccharomyces cerevisiae*, with several phenotypes (Fleet,  
94 2008). In addition to the choice of *S. cerevisiae* strain, the use of mixed starter cultures with  
95 selected non-*Saccharomyces* and *S. cerevisiae* yeasts can result in greater complexity and  
96 diversity of volatile metabolites in ways not reachable with pure starter cultures of *S.*  
97 *cerevisiae*, by simulating a spontaneous fermentation (Belda et al., 2017; Englezos et al.,  
98 2016b; Sadoudi et al., 2012).

99 Among non-*Saccharomyces* yeasts, *Starmerella bacillaris* (synonym *Candida*  
100 *zemplanina*) can tolerate relatively high concentrations of ethanol and persist until the middle-  
101 end stages of fermentation, making them more suitable for mixed fermentations (Englezos,  
102 Giacosa, Rantsiou, Rolle & Cocolin, 2017). Recent studies have revealed several potentially  
103 useful winemaking attributes, including high glycerol and low ethanol production, preference  
104 towards fructose rather than glucose, ability to tolerate relative high concentrations of  
105 ethanol, while acetic acid and acetaldehyde production is highly variable among strains  
106 (Englezos et al., 2018, Rantsiou et al., 2017). These phenotypic characteristics make this non-  
107 *Saccharomyces* species an optimum candidate to accompany *S. cerevisiae* in mixed  
108 fermentations (Mestre, Maturano, Combina, Mercado, Toro & Vasquez, 2017). In the last  
109 decade, many studies have focused on mixed fermentations with *Starm. bacillaris* and *S.*  
110 *cerevisiae* to ferment grape must and have made noticeable progress in many aspects,

111 including the importance of strain selection, inoculation density and delay on the chemical  
112 profile of the wines (Englezos et al., 2017). However, several efforts must be performed in  
113 order to establish a link between an inoculation protocol and chemical composition of wines  
114 using the same couple of strains and fermentation conditions.

115 Hence, the present study sought to investigate the effect of mixed fermentations with  
116 *Starm. bacillaris* and *S. cerevisiae* on the aroma profile of some monovarietal white wines.  
117 To this end, four of the world's most planted white wine grape varieties, namely:  
118 Chardonnay, Muscat, Riesling and Sauvignon blanc, were fermented with *Starm. bacillaris*  
119 FC54 and *S. cerevisiae* Uvaferm BC<sup>®</sup> using an inoculation delay of 48 hours. Control  
120 fermentations with *S. cerevisiae* Uvaferm BC<sup>®</sup> were performed in parallel. The aroma profile  
121 of the resultant wines was determined by Head Space-Solid Phase Micro Extraction (HS-  
122 SPME) combined with Gas Chromatography-Mass Spectrometry (GC-MS).

123

## 124 **2. Materials and methods**

125

### 126 *2.1. Strains*

127

128 The yeast strains for this experiment were the commercial *S. cerevisiae* Uvaferm BC<sup>®</sup>  
129 and *Starm. bacillaris* FC54 obtained from Lallemand Inc. (Montreal, Canada) and the yeast  
130 culture collection of DISAFA (Department of Agricultural, Forest and Food Sciences,  
131 University of Turin, Italy), respectively. These strains were selected for their enological  
132 attributes in mixed fermentations in grape must at the laboratory and pilot scale (Englezos et  
133 al., 2016a).

134

### 135 *2.2. Must preparation*

136

137 Four white wine grape varieties (*Vitis vinifera* L.) cultivars, namely Chardonnay,  
138 Muscat, Riesling and Sauvignon blanc were harvested at technological ripening from the  
139 experimental vineyard of the University of Turin at Grinzane Cavour (Cuneo, Piedmont, NW  
140 Italy). After harvesting, the grapes were destemmed, crushed and the juice obtained without  
141 the skins was sterilized by adding 200 mg/L dimethyl dicarbonate from Sigma (Milan, Italy)  
142 as previously described by Delfini, Gaia, Schellino, Strano, Pagliara & Ambrò (2002). The  
143 absence of culturable yeast population in the musts prior to inoculation was checked by  
144 plating an aliquot of the must on Wallerstein laboratory nutrient (WLN) medium  
145 (Biogenetics, Milan, Italy). The sanitization protocol was deemed successful, since no  
146 colonies were formed on the medium after 3-5 days of incubation at 28 °C. Grape musts were  
147 standardized for providing a unified starting point of sugars and YAN (Yeast Assimilable  
148 Nitrogen) for the fermentations. To this end the musts were standardized to  $245 \pm 5$  g/L of  
149 sugar and  $180 \pm 5$  mg/L of YAN using the commercial product Fermaid O<sup>®</sup> from Lallemand  
150 Inc., in order to ensure complete sugar fermentation. The chemical composition of the musts  
151 is reported in Table 1.

152

### 153 2.3. Fermentation trials

154

155 The four musts were each divided into six samples comprising three replicates of each  
156 of two types of inoculation protocols, a. inoculation with *S. cerevisiae* Uvaferm BC<sup>®</sup> (pure  
157 culture fermentation), b. initial inoculation with *Starm. bacillaris* FC54 followed by *S.*  
158 *cerevisiae* Uvaferm BC<sup>®</sup> after 48 hours of fermentation (mixed, sequential inoculation).  
159 Twenty-four fermentations (4 grape varieties x 2 inoculation protocols x 3 replicates = 24) in  
160 total were performed under semi-anaerobic conditions in 1 L sterile glass bottles containing



161 800 mL of must. Each yeast strain was inoculated at  $5.0 \times 10^6$  cells/mL, which corresponds to  
162 a dose of 25 g/hL of ADY (Active Dry Yeast) (Lallemand SAS, Toulouse, France),  
163 previously activated in a sterile glucose solution (5 %), incubated at 37 °C. Fermentors were  
164 fitted with air-lock to ensure semi-anaerobic conditions, after all the oxygen in the headspace  
165 is consumed and kept at 20 °C without shaking. Fermentations were considered finished  
166 when the sum of glucose and fructose was less than 2 g/L. At the end of fermentation,  
167 samples were taken from each fermentor for analysis of the volatile fermentation compounds.

168

#### 169 2.4. Microbiological analysis

170

171 The growth dynamics of the two yeasts during fermentation were monitored by plate  
172 counts. Aliquots of 1 mL were taken from each must at days 0, 2, 4, 7, 9 and 14 (only for the  
173 mixed culture fermentation), diluted in sterile Ringer's solution (Oxoid, Milan, Italy) and  
174 plated on WLN medium. Enumeration of the yeast colonies was performed after 3-5 days of  
175 incubation at 28 °C and the differentiation of the two species was carried out visually as  
176 previously described by Englezos et al. (2018) and subsequently counted. In this medium,  
177 *Starm. bacillaris* forms light to intense green with white border, whereas *S. cerevisiae* forms  
178 creamy white to light green colonies enabling the concurrent enumeration of both species.

179

#### 180 2.5. Chemical analysis

181

182 Extracellular metabolites concentration such as sugars (glucose and fructose),  
183 glycerol, organic acids (citric, tartaric, succinic, malic, lactic and acetic acid) (g/L) and  
184 ethanol (% v/v) were quantified during (0, 2, 4, 7, 9 and 14 days) and at the end of  
185 fermentation were quantified by an Agilent 1260 HPLC system (Agilent Technologies, Santa

186 Clara, CA, USA) using a UV detector (UV100) at 210 nm and a refractive index detector  
187 (RI-150). Analyses were performed isocratically at 0.8 mL min<sup>-1</sup> flow-rate and at 65 °C  
188 column temperature with a 300 mm x 7.8 mm i.d cation exchange column (Aminex HPX-  
189 87H) and a Cation H<sup>+</sup> Microguard cartridge (Bio-Rad Laboratories, Hercules, CA, USA).  
190 The mobile phase was 0.0065 mol L<sup>-1</sup> H<sub>2</sub>SO<sub>4</sub> (Rolle et al., 2018). At the end of fermentation,  
191 total acidity (expressed as g/L of tartaric acid) was determined according to the official  
192 method proposed by the International Organization of Vine and Wine (OIV, 2008), while pH  
193 was registered using an InoLab 730 pH meter (WTW, Weilheim, DE). Total YAN  
194 concentration was determined spectrophotometrically by using two enzymatic kits (catalog  
195 codes: K-Large and K-PANOPA, Megazyme International, Wicklow, Ireland).

196

## 197 *2.6. Volatile profile*

198

199 Volatile compounds formed through yeast metabolism in pure and mixed culture  
200 fermentations were extracted and determined by Head Space – Solid Phase Micro Extraction  
201 (HS-SPME) coupled by Gas Chromatography – Mass Spectroscopy (GC-MS). The  
202 chromatographic and MS conditions were previously described by Sánchez-Palomo, Diaz-  
203 Maroto & Perez-Coello, 2005) and slightly modified by Rolle et al. (2015, 2018). For each  
204 sample, a 5 mL aliquot was transferred to a 20 mL glass headspace vial with a headspace  
205 screw cap, containing 5 mL of water, 2 g of sodium chloride and 1-heptanol solution (200 µL  
206 of 15.52 mg/L solution in 10 % v/v ethanol) as internal standard (IS). The vials were sealed  
207 with 18 mm diameter silicon septa caps (Supelco, Bellefonte, PA, USA) and carefully shaken  
208 to dissolve sodium chloride before the analysis. A 50/30 µm DVB/CAR/PDMS fibre from  
209 Supelco was used to extract the volatile compounds, using a Gerstel MPS2 XL auto sampler  
210 (Gerstel, Baltimore, MD, USA). The fibre was exposed to the headspace of each vial for 20

211 min at 40 °C and inserted into the injection port of the GC apparatus for the thermal  
212 desorption. Injections were carried out in splitless mode at 250 °C for 5 min, during which  
213 the desorption of analytes from the fibre was occurred.

214 Analyses were carried out using an Agilent 7890C gas chromatograph (Little Falls,  
215 DE, USA) associated with an Agilent 5975 mass selective detector and DB-WAXETR  
216 capillary column (30 m x 0.25 mm, 0.25 µm, J&W Scientific Inc., Folsom, CA, USA).  
217 Helium was used as a carrier gas with a flow rate of 1 mL/min. the software used was Agilent  
218 G1701-90057 MSD ChemStation. Chromatographic conditions are as follows: 5 min at 40 °C  
219 and increased at a rate of 2 °C/min to 200 °C for 10 min and 5 °C/min to 220 °C. The oven  
220 was the held at this temperature for 5 min before returning to the initial temperature. The  
221 injection port temperature was 250 °C, the ion source temperature was 150 °C and the  
222 interface temperature was 280 °C. The detection was carried out by electron impact mass  
223 spectroscopy in total ion current (TIC) mode, using an ionisation energy of 70 eV. The mass  
224 acquisition range was between m/z 30-330. Volatile compounds were identified according to  
225 retention indices and mass spectra of pure standards and the NIST database  
226 (<http://webbook.nist.gov/chemistry/>). The VOCs quantification was performed with linear  
227 regression using analytical standards (all from Sigma) where available (Supplementary Table  
228 1) (Englezos et al., 2016b). Quantitative determination was performed using 1-heptanol as  
229 internal standard and calibration with pure standard previously reported and data expressed as  
230 µg/L. The thiols analysis in the wines produced from Sauvignon blanc grapes was performed  
231 using the method reported by Piano et al. (2015) and data expressed as ng/L.

232

233 *2.7. Statistical analyses*

234

235 The data obtained were subjected to statistical analysis using IBM SPSS Statistics  
236 software package (version 19.0, IBM Corp., Armonk, NY, USA). Significant differences  
237 between samples were established using one-way Analysis of Variance (ANOVA). When  
238 statistical differences were found, a Tukey-b post hoc test comparison was performed using  
239  $p < 0.05$  as the threshold significance.

240

### 241 **3. Results and discussion**

242

#### 243 *3.1. Yeast growth during fermentation*

244

245 The yeast growth dynamics during pure and mixed fermentations were followed by  
246 plate counts and the results are illustrated in Fig. 1. In pure culture fermentations, *S.*  
247 *cerevisiae* Uvaferm BC<sup>®</sup> reached the maximum population (about  $5.0\text{-}8.0 \times 10^7$  colony  
248 forming units [cfu]/mL) in two days. The viable population then remained stable until the end  
249 of the fermentation (9 days). In sequential fermentations, *Starm. bacillaris* FC54 reached the  
250 highest cell population on day 4 ( $5.0\text{-}7.0 \times 10^7$  cfu/mL). Its population became undetectable  
251 in sequential inoculations on day 14, while *S. cerevisiae* population remained at levels from  
252  $10^6$  cfu/mL in Sauvignon blanc to  $10^7$  cfu/mL in Muscat wines. *Starm. bacillaris* impacted *S.*  
253 *cerevisiae* population in sequential inoculations. More specifically, *S. cerevisiae* was slightly  
254 lower (range 0.1 to 0.2 Log cfu/mL, data not shown) in comparison to pure culture *S.*  
255 *cerevisiae* fermentations, after similar periods of post-inoculation.

256

#### 257 *3.2. Chemical parameters*

258

259 The extracellular metabolites concentrations, for the fermented wines from each grape  
260 variety and inoculation protocol, are shown in Table 1. While both glucose and fructose were

261 almost consumed (< 2.0 g/L) at the end of fermentation, the strong fructophilic character of  
262 *Starm. bacillaris* compared to *S. cerevisiae* was confirmed on the first 48 hours of  
263 fermentation, in agreement with previous studies (Englezos et al., 2017, 2018; Rantsiou et al.,  
264 2017). As it can be seen in Fig. 2 (right panel) and Supplementary Table 2, *Starm. bacillaris*  
265 consumed on average more fructose and left glucose mostly untouched during this period.  
266 Sequential fermentations started significantly slower as within the first 48 hours only 9.0 g/L  
267 of sugars (mainly fructose) were consumed on average, representing 3% of the total sugars.  
268 At the same time point, pure fermentations with *S. cerevisiae* consumed on average 81.0 g/L  
269 of sugars, representing 34% of total sugars. Sugar consumption rate had a steep increase  
270 when *S. cerevisiae* was inoculated in mixed fermentations, and continued until day 7 after  
271 which rate of sugar consumption slowed and stopped on day 14 in sequential fermentations.  
272 On the other hand, sugar consumption rate decreased on day 4 and stopped on day 9 in pure  
273 fermentations. The length of the sequential fermentations is in line with Englezos et al.  
274 (2016a) who reported a fermentation time three-days longer when sequential fermentations  
275 are compared to pure fermentations with the same *S. cerevisiae* strain Uvaferm BC®.

276 Ethanol production in the sequential fermented wines was slightly lower (0.1 to 0.2 %  
277 v/v) compared to pure fermented wines, independently of the grape variety used as shown in  
278 Table 1. These differences are lower than observed in a previous work (0.5 % v/v) with the  
279 same couple of strains and inoculation delay using red Barbera grape must, compared to pure  
280 fermented wines with *S. cerevisiae* (Englezos et al. 2016a). The lower fermentation  
281 temperature compared to the previous study (20 °C vs. 25 °C), could explain the low sugar  
282 consumption by *Starm. bacillaris* in the first 48 hours of fermentation and as a consequence  
283 the low ethanol reduction in this work.

284 While the ethanol content of the wines was lower in mixed fermentations, the glycerol  
285 content was significant higher for all grape variety used in this study, confirming previous

286 observations (Englezos et al., 2016ab; 2018; Rolle et al., 2018). Glycerol production in the  
287 mixed fermented wines ranged from 9.3 to 10.3 g/L compared to pure fermented wines that  
288 ranged from 7.8 to 8.4 g/L. This increase in glycerol was also reported in previous studies but  
289 in higher levels (more than 4.0 > g/L) (Englezos et al., 2016a). The glycerol yield was  
290 between 0.038 - 0.042 for mixed fermented wines and between 0.032 – 0.035 for the control  
291 wines.

292         Titratable acidity (expressed as g/L of tartaric acid) was in average significantly  
293 higher in sequential fermented wines (7.1 g/L) compared to pure fermented wines (6.3 g/L).  
294 This increase is in line with Sadoudi et al. (2012) and Englezos et al. (2016a) who also  
295 reported that 24 and 48 hours inoculation delay resulted in higher titratable acidity (0.16 –  
296 0.50 g/L) compared to pure fermented wines respectively, resulting in a decrease of pH.  
297 However, the increase of 0.6 – 0.8 g/L observed in this study could not be explained by the  
298 primary organic acids (citric, tartaric, succinic, malic and lactic acid) monitored in study  
299 (Supplementary Table 3), suggesting that other acids (such as  $\alpha$ -ketoglutaric and pyruvic) are  
300 most probably responsible for this increase (van Dijken & Scheffers, 1986). Magyar, Nyitrai-  
301 Sárdy, Leskó, Pomázi & Kállay (2014) reported a significantly higher accumulation of  
302 pyruvic acid by *Starm. bacillaris* compared to *S. cerevisiae* in pure culture fermentation using  
303 synthetic medium. Conversely, pure starter culture fermentations lead to a higher average  
304 decrease of malic acid than mixed starter culture fermentations. *S. cerevisiae* in pure  
305 fermentations consumed on average 0.7 g/L of malic acid, representing a 36% reduction,  
306 while in sequential inoculations the decrease was on average 0.5 g/L representing a 28%  
307 reduction. Rantsiou et al. (2017), reported that pure culture fermentations with *Starm.*  
308 *bacillaris* consumed malic acid on a level of 40% in red Barbera cv. musts with differing  
309 sugar levels (200-330 g/L), which was in line with earlier research by Tofalo et al (2012),  
310 using a red must with 220 g/L of residual sugars.

311

### 312 3.3 Volatile composition

313

314 Identification and quantification of the volatile metabolites was carried out in order to  
315 determine the effect of the inoculation protocol on white wines aroma. As shown in Table 2,  
316 a total of 38 volatile compounds were identified and subsequently divided into 4 volatile  
317 families, including 7 alcohols, 19 esters, 2 fatty acids, 8 terpenes and C<sub>13</sub>-norisoprenoids. The  
318 total aroma volatile composition exhibited significant differences between pure and mixed  
319 culture fermentations, highlighting a metabolic interaction between the two species. In  
320 particular, significant lower levels of volatile compounds were registered for the mixed  
321 compared to pure fermented wines.

322

#### 323 3.3.1 Higher alcohols

324

325 Higher alcohols, known as fusel alcohols, constitute the largest group of volatile  
326 metabolites, synthesized by yeast during alcoholic fermentation (Dzialo et al., 2017). Both  
327 pure and mixed fermentations, independent of the grape variety used, produced the same  
328 levels of alcohols, at concentrations ranging from 9.9 mg/L to 14.8 mg/L, well below the  
329 level of 300 mg/L which enhance the complexity in the wines (Rapp & Versini, 1991). The  
330 only exception was Sauvignon blanc wines, in which the involvement of *Starm. bacillaris* in  
331 the fermentation process increased significantly the levels of this group of metabolites (11.8  
332 µg/L vs 10.7 µg/L). The total concentration of the alcohols in the wines was strongly  
333 associated with the concentration of isoamylic alcohol and 2-phenyl ethanol, which  
334 constituted up to 91% of total alcohols. However, none of them surpassed their perception

335 threshold (Cullere, Escudero, Cacho & Ferrerira, 2004; Ferreira, Lopez & Cacho, 2000;  
336 Guth, 1997; Li, 2006).

337 Isoamylic alcohol (3-methyl-1-butanol), which is produced during fermentation  
338 through deamination and decarboxylation reactions from isoleucine (Molina et al., 2009),  
339 could negatively contribute to wine quality due to the herbaceous aroma. Chardonnay and  
340 Muscat wines produced using pure starter cultures contained significant higher levels of this  
341 metabolite, however in concentrations well below its perception threshold. To the contrary,  
342 no differences were observed for Riesling and Sauvignon blanc wines. 2-phenylethanol,  
343 which is synthesized via Ehrlich pathway through metabolic reactions that involves  
344 transamination of the amino acid L-phenylalanine, could contribute to the wine with a  
345 pleasant rose-like odour (Swiegers et al., 2005). Riesling and Sauvignon blanc wines  
346 produced from mixed starter cultures were distinguished, from the respective wines  
347 fermented exclusively with *S. cerevisiae*, by a significant higher amount of this metabolite.  
348 Therefore, the increased concentration of 2-phenylethanol would potentially increase the  
349 floral aroma in these wines.

350 2-Methyl-1-propanol (also known as isobutanol) is synthesized in the yeast cell  
351 through the valine degradation pathway and contributes to herbaceous notes in the wines  
352 (Dzialo et al., 2017). Chardonnay and Muscat wines produced from pure *S. cerevisiae*  
353 fermentations contained significant higher levels of this metabolite. Conversely, Riesling and  
354 Sauvignon blanc wines fermented with pure cultures, contained significantly lower levels of  
355 this metabolite, suggesting that valine concentration rather than inoculation strategy affects  
356 its production. Moreover, negligible differences were found in wines produced using mixed  
357 cultures independently of the grape variety used. Hexanol, usually has a negative influence  
358 on wine aroma, by imparting a vegetable and herbaceous odour, when the concentration  
359 exceeds 100 mg/L (Satora & Tuszynski, 2010). This metabolite, was present in significant



360 higher levels in mixed starter culture fermented wines, independently of the grape variety  
361 used, but still significantly lower than its olfactory detection threshold.

362

### 363 3.3.2 Esters

364 Fermentation derived esters are responsible for the fruity character of the wines  
365 (Dzialo et al., 2017). In general, mixed fermentations produced Chardonnay and Muscat  
366 wines with significant lower levels of esters, compared to pure fermented wines. To the  
367 contrary, a completely different picture was captured in Sauvignon blanc wines, in fact mixed  
368 starter cultures produced higher levels of this aroma family. No significant differences were  
369 found for Riesling wines, in the amount of total esters produced, between the pure and mixed  
370 fermented wines. Among the identified esters, ethyl esters deriving from medium chain fatty  
371 acids and responsible for the fruity character of the wines were the most representative aroma  
372 family in all the wines produced, accounting for 72 % and 85 % of total esters in the pure and  
373 mixed fermentations, respectively. Ethyl octanoate and ethyl decanoate associated with  
374 pleasant notes “pineapple”, “pear”, and “floral” were the most abundant ethyl esters and  
375 significant differences were registered between pure and mixed fermented wines,  
376 independently of the grape variety used. Significant lower levels were found in mixed  
377 fermented wines. To the contrary, Sauvignon blanc wines fermented by mixed cultures were  
378 characterized by significant higher content of these two compounds. The higher level of ethyl  
379 decanoate in this wine is in line with previous findings (Sadoudi et al., 2012) in sequential  
380 inoculated Sauvignon blanc with 24 h inoculation delay, while ethyl hexanoate was not  
381 affected by the inoculation protocol used in both studies. Concerning the level of ethyl  
382 octanoate in the wines, the results of the present study are in agreement with those of Sadoudi  
383 et al. (2012) who observed a lower level of this compound in pure fermented Sauvignon  
384 blanc wines with *S. cerevisiae*. Conversely, Chardonnay, Muscat and Riesling wines

385 fermented with pure *S. cerevisiae* cultures contained significant higher levels of this  
386 metabolite, indicating that strain selection and grape variety can modulate its production.  
387 Ethyl dodecanoate (pear, fruity, floral) was found in significant higher levels in pure culture  
388 fermented wines compared to mixed culture fermented wines. On the other hand, Sauvignon  
389 blanc wines fermented with mixed cultures contained significant higher levels of this  
390 metabolite compared to the respective control wine suggesting that grape variety rather than  
391 inoculation protocol modulate its production.

392         The second group of esters, called acetate esters, are those formed from acetic acid  
393 and higher alcohols, and are considered to have a greater effect on the perceived aroma than  
394 the ethyl esters (Dzialo et al., 2017). In the current study, the acetate esters identified were  
395 ethyl acetate, hexyl acetate, octyl acetate, 2-phenyl-ethyl-acetate, and 3-methyl-1-butanol  
396 acetate. All wines inoculated with mixed cultures presented significant lower content of this  
397 aroma family. Among the quantified acetate esters two compounds (2-phenyl-ethyl-acetate  
398 and 3-methyl-1-butanol acetate) associated with the positive attributes, “rose”, “honey” and  
399 “banana” presented values above the threshold value in all the wines studied, consequently  
400 they are expected to have an influence on the aroma of the wines. Both compounds were  
401 found to be significantly higher in pure fermented wines independently of the grape variety  
402 used. A significant difference in hexyl acetate, a metabolite with pleasant fruity note was  
403 observed. The amount of this metabolite was above the threshold in the control wine however  
404 below in mixed starter culture fermented wine, the former having 3-14 times the amount  
405 compared to the latter for all the varieties investigated. Similar behaviour was found for 3-  
406 methyl-1-butanol acetate. This reduction was more evident in Chardonnay and Muscat wines,  
407 suggesting that the grape variety may have an influence on the production of these esters.

408         Ethyl acetate and 2-phenyl-ethyl-acetate are the most common esters found in wine.  
409 Contrary to 2-phenyl-ethyl-acetate, ethyl acetate is known to have an unpleasant nail polish,

410 vinegar aroma at concentrations above 150 mg/L (Corison, Ough, Berg & Nelson, 1979). At  
411 concentrations below this limit, this metabolite contributes positively to white wine quality,  
412 with pleasant descriptors such as, pineapple and apple. For both Chardonnay and Muscat  
413 wines fermented by pure cultures, the content of ethyl acetate was above the odour threshold,  
414 while it was lower than the perception threshold in sequential inoculation wines. The  
415 difference between pure and mixed fermentation was statistically significant for these  
416 varieties. Ethyl acetate was not above the threshold in any of the fermentations of Riesling  
417 and Sauvignon blanc. Generally, wines produced with *Starm. bacillaris*, showed a reduction  
418 in ethyl acetate, hexyl acetate and 2-phenyl-ethyl acetate when compared with pure culture  
419 fermented wines.

420

#### 421 3.3.4 Fatty acids

422

423 Two fatty acids, decanoic and octanoic acid were identified across the pure and mixed  
424 fermented wines (Table 2). Both are medium-chain fatty acids (C<sub>6</sub> – C<sub>10</sub>), which can impart a  
425 butter-like, cheesy aroma (Francis & Newton, 2005), however, they impact negatively wine  
426 quality only when their concentration exceeds 20 mg/L (Ribéreau-Gayon, Dubourdieu,  
427 Donèche & Lonvaud, 2006). Wines produced from pure *S. cerevisiae* culture contained  
428 significant higher levels of these metabolites independently of the grape variety used. In these  
429 wines, the decanoic acid ranged from 578 to 616 µg/L and the octanoic acid concentration  
430 from 787 to 1108 µg/L. Even though these volatile fatty acids are well below the  
431 concentration at which become unpleasant, octanoic acid concentration in pure starter  
432 fermentations was present at levels above its perception threshold, which is 500 µg/L. In  
433 small quantities, volatile fatty acids contribute to the aromatic equilibrium of wine, since they  
434 counteract the hydrolysis of their esters (Swiegers et al., 2005).

435

### 436 3.3.3 Terpenes and C<sub>13</sub>-norisoprenoids

437

438 Terpenes are a kind of aroma family responsible for the characteristic floral and fruity  
439 aroma of Muscat and Riesling wines. Generally, they are present in grape berries in free or  
440 bound form and synthesized from glucose via the isoprenoid pathway (Mateo & Jimenez,  
441 2000). The terpenes compounds with high odour activity are linalool, geraniol and nerol.  
442 Geraniol has aromas described as rose-like and linalool aromas described as floral-like  
443 (Swiegers et al., 2005), whereas oxidized geraniol and linalool are described as vegetative  
444 and camphorous respectively. The concentration of monoterpene linalool in mixed fermented  
445 Muscat wines, was almost 21 times above the odour threshold, however significantly lower  
446 (514 µg/L) than in the control wine (647 µg/L). This result suggests that the interaction  
447 between the two yeast species has a negative influence in the expression of the varietal  
448 character of the wines. This result is in line with those reported by Sadoudi and and co-  
449 workers (2012), where a negative interaction was registered between *Starm. bacillaris* and *S.*  
450 *cerevisiae* resulting in a decrease in terpenes content compared to pure fermentations with *S.*  
451 *cerevisiae*. Similarly, linalool and terpenes concentration in Riesling was above the odour  
452 threshold in both inoculation protocols investigated, however no significant differences were  
453 found between the two protocols.

454

### 455 3.3.4 Thiols

456

457 Volatile thiols, such as hydrogen sulphide (H<sub>2</sub>S), ethanethiol and methanethiol are  
458 responsible for wine defects, however, certain volatile thiols are considered important aroma  
459 constituents of Sauvignon blanc wines and other white, rosé and red wines elaborated with

460 different grape varieties (Roland, Schneider, Razungles & Cavelier, 2011). Among these  
461 metabolites, 4-mercapto-4-methylpentan-2-one (4MMP), 3-mercaptohexan-1-ol (3MH) and  
462 its acetate 3-mercaptohexyl acetate (3MHA) contribute positively to the fruity character of  
463 young wines with pleasant notes of box tree, grape fruit and exotic fruit aroma, respectively  
464 (Rolland et al., 2011; Tominaga, Furrer, Henry & Dubourdieu, 1988). These metabolites are  
465 present in grape as non-volatile cysteine or glutathione conjugated precursors and they are  
466 released during the fermentation by yeast through their beta-lyase activity (Murat, Masneuf,  
467 Darriet, Lavigne, Tominaga & Dubourdieu, 2001). The two inoculation protocols used in this  
468 study affected the release of 3MH, while 3MHA was not detected in the samples. Wines  
469 fermented using the sequential inoculation protocol showed a significant higher concentration  
470 (269 ng/L) of 3MH compared to the control wine (198 ng/L), well above the 60 ng/L  
471 perception threshold. This liberation of higher levels of volatile thiols in mixed fermentations  
472 could be explained by the beta-lyase activity that favour the cleavage of the conjugated thiols,  
473 probably due to involvement of *Starm. bacillaris* in the fermentation process (Swiegers &  
474 Pretorius, 2007). Anfang, Brajkovich & Goddard (2009) also reported a significant increase  
475 in 3MH in Sauvignon blanc wines, co-fermented with *Starm. bacillaris* and *S. cerevisiae* in a  
476 ratio of 9:1, compared to pure fermented wines with *S. cerevisiae*. Conversely, co-inoculation  
477 at a ratio 1:9 that favour *S. cerevisiae*, produced wines with similar 3MH content. According  
478 to Sadoudi et al. (2012), inoculation of *S. cerevisiae* 24 hours after *Starm. bacillaris*  
479 inoculation led to the production of wines, with significant lower levels of this metabolite,  
480 compared to the respective control wine. Thus differences in 3MH profile depend on the  
481 initial inoculation ratio and the resulting population dynamics, demonstrating that yeast-  
482 interactions are strain-dependent.

483

## 484 **5. Conclusion**

485

486           The current study examined the effect of mixed fermentations with *Starm. bacillaris*  
487 and *S. cerevisiae* on the production of white wines using four different white grape varieties.  
488 Results, obtained from chemical composition showed that the level of glycerol and titratable  
489 acidity varied significantly among wines after fermentation. For volatile components  
490 determined, inoculation protocol influenced the aroma profile of the wines in a variety-  
491 dependent manner, since only the wines produced from Sauvignon blanc grapes contained  
492 significant higher levels of esters and alcohols compared to pure fermented wines. Since all  
493 the data presented here are obtained from one couple of strains, more investigations are  
494 necessary to access the impact of strain selection on wine composition.

495

#### 496 **Conflict of interest**

497 The authors state no conflict of interest.

498

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601

602 **Table 1**

603 Chemical parameters of musts and wines produced by pure and mixed culture fermentations

Grape variety	Inoculation protocol	Residual sugars (g/L)	Malic acid (g/L)	Acetic acid (g/L)	Succinic acid (g/L)	Glycerol (g/L)	Ethanol (% v/v)	Y(gly/sugar) (g/g)	Y(eth/sugar) (g/g)	pH	TA (g/L)
Chardonnay	Prior inoculation	246.0 ± 2.6	2.55 ± 0.03	< 0.1	0.06 ± 0.01	< 0.1	< 0.1	-	-	3.99 ± 0.01	4.33 ± 0.02
	pure	0.4 ± 0.2	1.55 ± 0.03	0.29 ± 0.10	1.27 ± 0.08	8.4 ± 0.1	14.9 ± 0.1	0.034 ± 0.001	0.061 ± 0.001	3.26 ± 0.27	5.84 ± 0.11
	mixed	0.5 ± 0.1	1.88 ± 0.01	0.28 ± 0.01	1.29 ± 0.02	10.3 ± 0.1	14.7 ± 0.1	0.042 ± 0.001	0.06 ± 0.001	3.35 ± 0.06	6.92 ± 0.06
Sign.	NS	***	NS	NS	***	*	***	*	NS	***	
Muscat	Prior inoculation	244.0 ± 1.2	1.28 ± 0.03	< 0.1	0.05 ± 0.01	< 0.1	< 0.1	-	-	3.81 ± 0.03	3.15 ± 0.04
	pure	0.5 ± 0.1	0.83 ± 0.01	0.31 ± 0.01	0.94 ± 0.01	7.8 ± 0.1	14.8 ± 0.1	0.032 ± 0.001	0.061 ± 0.001	3.22 ± 0.14	6.69 ± 0.04
	mixed	0.7 ± 0.1	0.94 ± 0.01	0.27 ± 0.01	1.14 ± 0.01	9.3 ± 0.1	14.6 ± 0.1	0.038 ± 0.002	0.06 ± 0.02	3.24 ± 0.11	7.16 ± 0.04
Sign.	NS	***	***	***	***	*	***	*	NS	***	
Riesling	Prior inoculation	245.9 ± 1.1	2.26 ± 0.01	< 0.1	0.04 ± 0.01	< 0.1	< 0.1	-	-	3.82 ± 0.01	4.35 ± 0.06
	pure	0.4 ± 0.1	1.44 ± 0.02	0.36 ± 0.03	1.13 ± 0.03	8.6 ± 0.1	14.7 ± 0.1	0.035 ± 0.001	0.06 ± 0.001	3.35 ± 0.08	5.67 ± 0.06
	mixed	0.9 ± 0.1	1.60 ± 0.01	0.32 ± 0.01	1.21 ± 0.01	10.3 ± 0.1	14.6 ± 0.1	0.042 ± 0.001	0.06 ± 0.001	3.34 ± 0.03	6.27 ± 0.05
Sign.	***	***	NS	**	***	*	***	NS	NS	***	
Sauvignon blanc	Prior inoculation	245.7 ± 0.6	1.23 ± 0.01	< 0.1	0.03 ± 0.01	< 0.1	< 0.1	-	-	3.56 ± 0.02	6.51 ± 0.04
	pure	0.7 ± 0.1	0.81 ± 0.01	0.40 ± 0.01	0.92 ± 0.01	8.3 ± 0.1	14.9 ± 0.1	0.034 ± 0.001	0.061 ± 0.001	3.09 ± 0.07	7.08 ± 0.01
	mixed	1.1 ± 0.1	0.86 ± 0.01	0.33 ± 0.01	1.02 ± 0.06	9.8 ± 0.1	14.7 ± 0.1	0.04 ± 0.002	0.06 ± 0.002	3.15 ± 0.03	8.11 ± 0.02
Sign.	***	**	***	*	***	***	***	***	NS	***	

604 The values are mean ± standard deviation of three independent experiments. Sign.: \*, \*\*, \*\*\* and NS indicate significance at  $p < 0.05$ ,  $p < 0.01$ ,  $p < 0.001$  and not significant,

605 respectively. TA: titratable acidity expressed as tartaric acid, Y (gly/sugar consumption): glycerol yield and Y (eth/sugar consumption): ethanol yield.

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Metabolites	Retention index	Perception threshold	Chardonnay		Muscat		Riesling		Sauvignon blanc		Statistical differences						
			Pure	Mixed	Pure	Mixed	Pure	Mixed	Pure	Mixed	Variety	Yeast	Interaction	Chard.	Mus.	Ries.	S.b.
<i>Alcohols</i>																	
2-Methyl-1-propanol	1113	40000a	372 ± 18	271 ± 30	341 ± 58	269 ± 43	153 ± 22	282 ± 66	160 ± 8	264 ± 13	***	NS	***	***	*	**	***
Isoamyl alcohol	1231	30000a	6905 ± 882	4284 ± 436	5163 ± 872	4064 ± 205	4462 ± 882	4196 ± 827	4514 ± 434	4710 ± 494	***	***	***	***	*	NS	NS
Hexanol	1367	8000a	314 ± 51	386 ± 45	72 ± 4	101 ± 9	204 ± 44	287 ± 46	228 ± 21	352 ± 19	***	***	*	*	***	**	***
(R,R)-2,3-Butanediol	1552	120000c	414 ± 102	284 ± 50	619 ± 64	338 ± 51	324 ± 36	240 ± 56	485 ± 202	356 ± 57	***	***	NS	*	***	*	NS
Octanol	1568	900b	7 ± 2	8 ± 4	13 ± 4	12 ± 4	7 ± 2	8 ± 4	7 ± 3	6 ± 5	***	NS	NS	NS	NS	NS	NS
(R,S-meso)-2,3-Butanediol	1587	120000c	96 ± 30	89 ± 19	168 ± 16	108 ± 20	88 ± 20	60 ± 22	122 ± 53	121 ± 14	***	**	NS	NS	***	*	NS
2-phenylethanol	1885	10000a, 14000d	6685 ± 763	8131 ± 1340	7549 ± 1613	8196 ± 1845	4633 ± 955	7369 ± 1999	5176 ± 531	6036 ± 633	***	***	NS	NS	NS	*	*
∑ <i>Alcohols</i>			14793 ± 1002	13454 ± 1689	13925 ± 1697	13088 ± 2002	9871 ± 1527	12442 ± 2877	10693 ± 708	11825 ± 477	***	NS	*	NS	NS	NS	**
<i>Esters</i>																	
Ethyl acetate	nd	7500a	7434 ± 850	3909 ± 397	8488 ± 1330	3650 ± 250	3721 ± 569	3688 ± 737	4530 ± 335	4649 ± 315	***	***	***	***	***	NS	NS
Ethyl butanoate	1040	20d	206 ± 26	119 ± 18	248 ± 51	87 ± 28	108 ± 22	80 ± 25	94 ± 28	152 ± 18	***	***	***	***	***	NS	**
3-Methyl-1-butanol acetate	1131	30c	19718 ± 3338	1653 ± 340	19119 ± 3367	1499 ± 232	5844 ± 1174	1097 ± 141	5857 ± 1074	2327 ± 368	***	***	***	***	***	***	***
Ethyl hexanoate	1249	5d,14a	5755 ± 910	3209 ± 832	5711 ± 1079	2456 ± 421	3079 ± 930	2373 ± 431	2770 ± 417	4168 ± 393	***	***	***	***	***	NS	***
Hexyl acetate	1286	670-1500c	5450 ± 1015	572 ± 141	1493 ± 309	113 ± 26	1830 ± 541	227 ± 36	1902 ± 277	648 ± 85	***	***	***	***	***	***	***
Ethyl 2-hexenoate	1355	-	13 ± 3	20 ± 5	0 ± 0	2 ± 1	5 ± 2	13 ± 5	3 ± 2	17 ± 3	***	***	***	*	*	**	***
Methyl octanoate	1398	200f	92 ± 35	51 ± 18	79 ± 9	25 ± 11	57 ± 32	40 ± 10	36 ± 5	60 ± 7	*	***	***	*	***	NS	***
Ethyl octanoate	1445	2a,5d	42583 ± 12382	18266 ± 6110	39625 ± 6078	12680 ± 1891	21525 ± 7409	12036 ± 3738	15044 ± 1802	23904 ± 2822	***	***	***	**	***	*	***
Octyl acetate	1478	50000e	81 ± 27	3 ± 2	92 ± 26	3 ± 2	13 ± 9	1 ± 1	16 ± 6	13 ± 4	***	***	***	***	***	*	NS
Ethyl nonanoate	1543	1300b	15 ± 7	35 ± 18	17 ± 3	20 ± 19	6 ± 4	8 ± 3	20 ± 22	19 ± 19	NS	NS	NS	*	NS	NS	NS
Methyl decanoate	1599	1200e	70 ± 35	33 ± 17	55 ± 8	12 ± 5	32 ± 23	20 ± 8	17 ± 5	47 ± 6	**	**	***	NS	***	NS	***
Ethyl decanoate	1648	200a	34198 ± 10455	15223 ± 3991	27364 ± 5403	11097 ± 1946	14863 ± 5067	10398 ± 2922	12358 ± 1658	22455 ± 2577	***	***	***	*	***	NS	***
3-Methyl-butyl octanoate	1663	-	169 ± 64	84 ± 35	194 ± 36	65 ± 16	51 ± 50	40 ± 15	50 ± 16	131 ± 30	***	**	***	*	***	NS	***
Ethyl 9-decenoate	1697	-	340 ± 151	75 ± 22	241 ± 51	32 ± 9	74 ± 58	51 ± 16	117 ± 18	103 ± 21	***	***	***	**	***	NS	NS

2-Phenyl-ethyl acetate	1815	250a	2585 ± 602	1350 ± 253	3140 ± 640	1209 ± 177	1404 ± 376	772 ± 198	1950 ± 216	1124 ± 117	***	***	***	**	***	**	***
Ethyl dodecanoate	1834	1500-2000b	3008 ± 802	2869 ± 744	3732 ± 813	1901 ± 432	1963 ± 580	1808 ± 594	1682 ± 358	3887 ± 1585	*	NS	***	NS	***	NS	**
3-Methyl-butyl decanoate	1846	-	136 ± 28	112 ± 35	146 ± 38	76 ± 17	55 ± 39	61 ± 18	75 ± 15	163 ± 132	*	NS	**	NS	**	NS	NS
Ethyl tetradecanoate	1974	800b	104 ± 47	196 ± 27	142 ± 44	109 ± 31	59 ± 32	86 ± 20	133 ± 19	211 ± 176	**	NS	NS	**	NS	NS	NS
Ethyl hexadecanoate	2122	1500b	74 ± 28	146 ± 26	61 ± 25	75 ± 24	65 ± 36	50 ± 14	122 ± 14	100 ± 24	***	NS	***	**	NS	NS	NS
$\Sigma$ Esters			122031 ± 19678	47927 ± 10550	109946 ± 13512	35126 ± 4285	45337 ± 20413	32848 ± 8028	46776 ± 5073	64178 ± 5677	***	***	***	***	***	NS	***
<i>Fatty acids</i>																	
Octanoic acid	1986	500a	898 ± 468	329 ± 140	1108 ± 222	319 ± 140	787 ± 174	313 ± 198	900 ± 104	237 ± 186	NS	***	NS	*	***	**	***
Decanoic acid	2138	1000a	389 ± 320	275 ± 62	578 ± 259	70 ± 17	616 ± 144	76 ± 41	587 ± 97	394 ± 36	NS	***	**	NS	***	***	**
$\Sigma$ Fatty acids			1287 ± 648	604 ± 70	1686 ± 463	389 ± 104	1403 ± 316	389 ± 222	1487 ± 197	631 ± 188	NS	***	NS	*	***	***	***
<i>Terpenes</i>																	
D-Limonene	1205	15g, 200f	0 ± 0	0 ± 0	17 ± 4	9 ± 5	0 ± 0	2 ± 5	0 ± 0	0 ± 0	***	NS	**	NS	*	NS	NS
$\delta$ -3-Carene	1330	-	0 ± 0	0 ± 0	40 ± 19	24 ± 11	13 ± 7	6 ± 5	0 ± 0	0 ± 0	***	*	NS	NS	NS	NS	NS
<i>t</i> -Furan linalool oxyde	1457	-	0 ± 0	0 ± 0	0 ± 0	0 ± 0	5 ± 8	1 ± 2	0 ± 0	0 ± 0	NS	NS	NS	*	NS	NS	*
Linalool	1556	25.2d	2 ± 1	3 ± 2	647 ± 61	514 ± 68	101 ± 26	80 ± 18	4 ± 2	7 ± 2	***	***	***	NS	**	NS	*
Hotrienol	1617	100g	0 ± 0	0 ± 0	42 ± 10	41 ± 8	35 ± 13	13 ± 8	3 ± 1	1 ± 1	***	**	***	*	NS	**	*
$\alpha$ -Terpineol	1707	250c	0 ± 0	0 ± 0	31 ± 5	25 ± 5	13 ± 8	6 ± 2	0 ± 0	0 ± 0	***	**	*	NS	NS	NS	**
Citronellol	1770	100c	2 ± 0	9 ± 4	4 ± 2	11 ± 5	2 ± 0	11 ± 3	2 ± 1	9 ± 4	NS	***	NS	**	**	***	***
Geraniol	1836	30a	0 ± 0	3 ± 3	4 ± 5	13 ± 3	1 ± 2	4 ± 3	1 ± 2	3 ± 3	***	***	*	*	**	NS	NS
$\Sigma$ Terpenes			5 ± 1	15 ± 8	785 ± 85	639 ± 86	169 ± 51	122 ± 30	12 ± 4	21 ± 6	***	**	***	*	*	NS	**
<i>Other metabolites</i>																	
Methionol	1727	1000d	1 ± 2	3 ± 5	2 ± 2	2 ± 4	3 ± 1	9 ± 3	3 ± 1	6 ± 4	**	**	NS	NS	NS	**	NS
$\beta$ -Damascenone	1820	0.055a	15 ± 3	11 ± 5	26 ± 7	19 ± 5	35 ± 11	18 ± 5	15 ± 7	8 ± 3	***	***	NS	NS	NS	*	NS
$\Sigma$ other metabolites			17 ± 4	15 ± 5	28 ± 6	22 ± 5	38 ± 12	27 ± 5	19 ± 8	14 ± 5	***	**	NS	NS	NS	NS	NS
<i>Volatile thiols(ng/L)</i>																	
3-mercaptohexanol	-	60h	-	-	-	-	-	-	198 ± 7	269 ± 41	-	-	-	-	-	-	*
3-mercaptohexyl acetate	-	4h	-	-	-	-	-	-	nd	nd	-	-	-	-	-	-	NS

619 Aroma compounds in wines expressed in  $\mu\text{g/L}$ , as mean  $\pm$  standard deviation of three independent experiments (each replicate was analysed two times (total 6)). Sig: \*, \*\*,  
620 \*\*\* and NS indicate significance at  $p < 0.05$ ,  $p < 0.01$ ,  $p < 0.001$  and not significant, respectively. Chard., Chardonnay; Mus., Muscat, Ries., Riesling, S.b., Sauvignon blanc.  
621 Perception thresholds ( $\mu\text{g/L}$ ) were taken from: (a) Guth (1997), (b) Li (2006), (c) Cullere, Escudero, Cacho & Ferreira (2004), (d) Ferreira, Lopez & Cacho (2000), (e) Li,  
622 Tao, Wang & Zhang (2008), (f) Cheng, Liu, Yue & Zhang (2015) and (g) Zhang, Petersen, Liu & Toldam-Andersen (2015), (h) Tominaga, Furrer, Henry & Dubourdieu  
623 (1998).

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639 **Figure captions**

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641 **Fig.1**

642 Growth dynamics of yeasts during pure (left panel) and mixed culture fermentations (right  
643 panel) using white grape musts: Chardonnay (A, B), Muscat, (C, D), Riesling (E, F) and  
644 Sauvignon blanc (G, H). *Starm. bacillaris* strain FC54 (black circle) and *S. cerevisiae*  
645 Uvaferm BC<sup>®</sup> (white circle). The arrow indicates the *S. cerevisiae* inoculation. Counts are the  
646 mean CFU/mL values  $\pm$  standard deviations of three independent experiments.

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648 **Fig.2**

649 Evolution of metabolites during pure (left panel) and mixed culture fermentations (right  
650 panel) using white grape musts: Chardonnay (A, B), Muscat, (C, D), Riesling (E, F) and  
651 Sauvignon blanc (G, H). Glucose (white circle) fructose (black circle), ethanol (white  
652 diamond) and glycerol (black diamond). Data are the mean  $\pm$  standard deviation of three  
653 independent experiments

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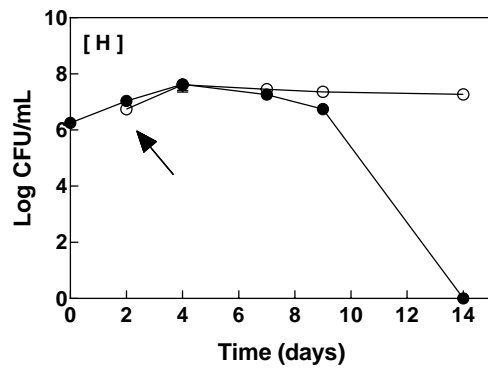
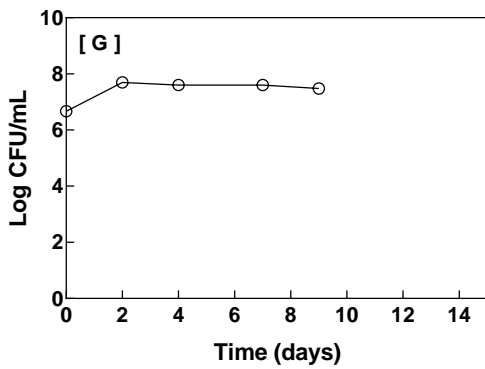
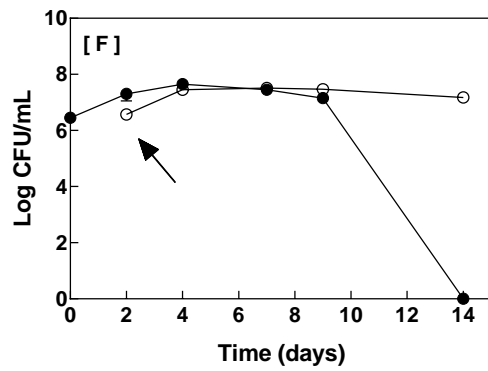
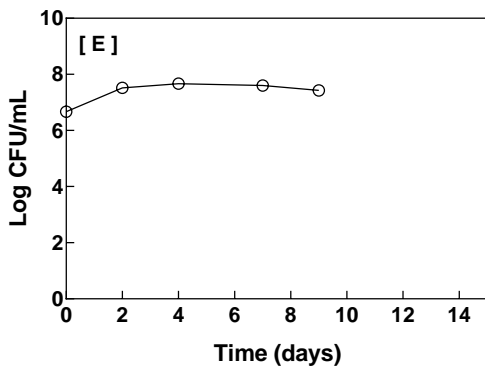
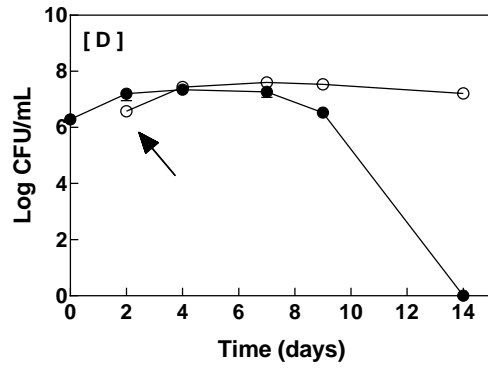
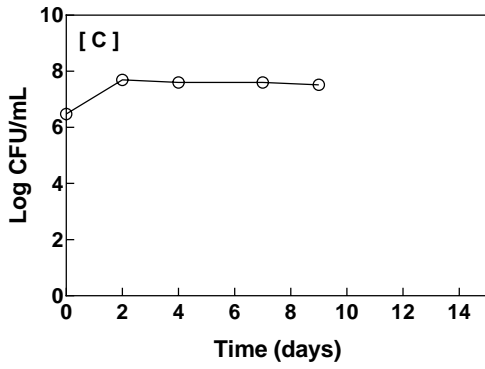
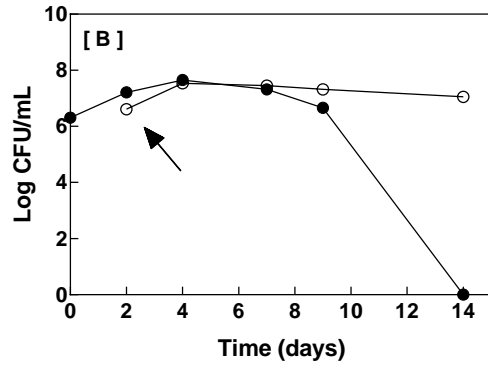
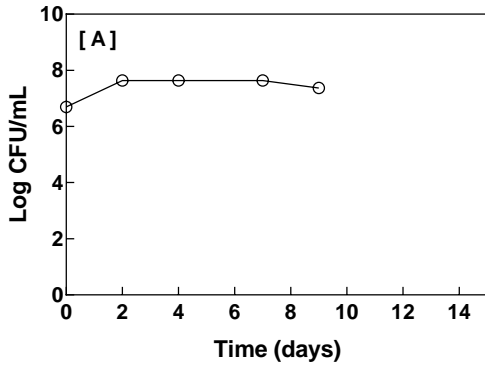
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669 **Figures**

670 **Fig. 1**



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