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Abstract

Decreasing the ethanol content in wine is a current challenge, mainly due to the global climate change and to the consumer preference for wines from grapes with increased maturity. In this study, a central composite design and response surface methodology approach was used to investigate the potential application of *Starmerella bacillaris* (synonym *Candida zemplinina*) in combination with *Saccharomyces cerevisiae*, in mixed (co-inoculated and sequential) cultures, to understand better the mechanism of co-habitation and achieve the objective of reducing the ethanol in wines. Laboratory scale fermentations demonstrated a decrease up to 0.7 % (v/v) of ethanol and an increase of about 4.2 g/L of glycerol when *S. cerevisiae* was inoculated with a delay of 48 h with respect to the inoculation of *Starm. bacillaris.* Pilot scale fermentations, carried out in winemaking conditions, confirmed the laboratory results. This study demonstrates that the combination of strains and inoculation protocol could help to reduce the ethanol content in wines.

Keywords Non-*Saccharomyces* yeast; *Starmerella bacillaris*; Central Composite Design; Response Surface Methodology; Ethanol content reduction.

Introduction

In the last 20 years, there has been an increasing global attention for ethanol content in wines, influenced mainly by the media and the government programs, due to the marketing, social and health associated reasons (Saliba et al. 2013). Wine consumption, in light to moderate amounts (1-2 glass of wine per day), has been well demonstrated to be beneficial for the human health (German and Walzem 2000; Yoo et al. 2010). In opposition, high levels of ethanol consumption and irregular drinking has been shown to be casually correlated with more than sixty different medical conditions (Room et al. 2005). The production of well-structured and full bodied red wines nowadays, is more difficult than previously thought, especially in warm climate wine regions (Jones et al. 2005). Usually, winemakers in order to achieve the optimum phenolic maturation and tannin concentration, necessary for the quality of these wines, postpone the harvest time, which results in a high, to excessive, sugar concentration in the over ripe grapes (Mira de Orduňa 2010). As a consequence, the excessive sugar content could be translated to wines with elevated levels of ethanol, increasing the perception of bitterness, hotness and decreasing the perception of some wine aromas and flavour attributes (Goldner et al., 2009).

Facing the climate change, human health and the constant growing demand for full bodied red wines, it is important to anticipate further increase. In this way, several technological approaches have been proposed, to reduce ethanol content in wine (Pickering 2000), ranging mainly from pre-fermentation (selection of grapevine clones and vineyard management) to post-fermentation approaches (spinning cone column, reverse osmosis etc.), which however, could increase the production costs and affect negatively wine quality (Pickering 2000). One the other hand, in recent years intervening on the yeast ecology during must fermentation is gaining more attention and this is carried out mainly by decreasing the sugar-ethanol yield transformation through the selection of wine yeasts (Contreras et al. 2015; Gobbi et al. 2014; Quirós et al. 2014). For non-*Saccharomyces* yeasts, the quantity of sugar used to produce 1 % (v/v) of ethanol is higher (17.0 – 40.0 g/L) (Englezos et al. 2015; Magyar and Tóth 2011) due to their ability to utilize the carbon to produce biomass and by-products. As a consequence, through their metabolism ethanol concentration does not increase (Contreras et al. 2014; Contreras et al. 2015ab; Gobbi et al. 2015; Gonzalez et al. 2013; Morales et al. 2015; Quiros et al. 2014).

Among, the non-*Saccharomyces* species of oenological interest, *Starmerella bacillaris* (synonym *Candida zemplinina*) (Duarte et al. 2012) is considered as one of the

most promising species to achieve the objective described above. *S. bacillaris* is supposed to be one of the best candidates, due to its ability to produce less ethanol from sugar consumed, tolerate high concentrations of ethanol present in the wine and produce low levels of biogenic amines (Englezos et al. 2015; Magyar and Tóth 2011; Rantsiou et al. 2012; Suzzi et al. 2012; Tristezza et al. 2013). These phenotypic characteristics support the potential use of this wine yeast, in combination with *S. cerevisiae* either in co-inoculated or sequential inoculated fermentations to reduce the potential ethanol content in wine (Giaramida et al. 2013; Gonzalez et al. 2013; Masneuf-Pomarede et al. 2015). However, strain selection and establishment of inoculation protocols are essential in order to moderate yeast growth and produce wines with the aspects described above.

In this context, the aim of this study was to understand the appropriate time of *S. cerevisiae* addition after *S. bacillaris* inoculation in order to achieve a high level of ethanol reduction. A central composite design (CCD) and response surface methodology approach (RSM) was used for this final goal, in order to optimize and find the appropriate inoculation protocol.

Materials and methods

Yeast strains

Two *S. bacillaris* (FC54 and C.z 03) and one *S. cerevisiae* (ScBa49) isolate were obtained from the Yeast Culture Collection of the DISAFA (Dipartimento di Scienze Agrarie, Forestali e Alimentari University of Torino, Italy). *S. bacillaris* MUT 5705 came from the Mycotheca Universitatis Taurinensis - MUT (DBIOS - University of Torino, Italy), while a commercial *S. cerevisiae* wine yeast Uvaferm BC® (Lallemand SA, Montreal, Canada) was used as a reference strain (Table 1). *S. bacillaris* strains were selected for their physiological and enological performance (Englezos et al. 2015) and routinely cultivated on YPD slants (1% yeast extract, 2% bacteriological peptone, 2% glucose and 2% agar, all w/v) or stored at -80 ^{o}C in YPD broth supplemented with 20% glycerol (Sigma, Milano, Italy).

Wine fermentations

Laboratory scale fermentations

132 Grape must of Barbera cultivar (*Vitis vinifera* L.) without the grape skin was obtained from
133 the experimental winery of the University of Torino. Grape must contained 233.2 g/L sugars
134 (116.4 g/L glucose, 116.8 g/L fructose), titratable acidity 8.20 g/L (expressed as tartaric acid),
135 pH 3.20 and absence of ethanol. The initial Yeast Available Nitrogen (YAN) was 197 mg/L
136 composed by the sum of 116 mg/L of AUG (ammonium + urea + L-arginine) and 81 mg/L of
137 PAN (primary amino nitrogen). The must was pasteurized in a water bath at 60˚C for 1 hour
138 and the sterility was checked by plating 100 µL of must on WL Nutrient Agar medium
139 (Biogenetics, Milano, Italy) and incubated them at 28 ^{o}C for 5 days. Under sterile conditions,
140 25 mL of the pasteurized must was distributed onto 50 mL tubes with loose screw cap for all
141 the fermentations performed in this work.
142
143 Pure fermentations
144
145 The oenological performance of the three *S. bacillaris* and two *S. cerevisiae* strains was
146 evaluated by micro-vinification trials in pure culture fermentations. The inoculum of the five
147 yeast strains was prepared by pre-adaptation of the strains in the same must as described
148 above for 48 hours at 25 °C. Afterwards, the yeast cells were stained with methylene blue dye
149 and immediately the viable cell population was counted by using a Thoma hemocytometer
150 chamber (BRAND GMBH + CO KG, Wertheim, Germany). Before inoculation, appropriate
151 amounts of inoculum were calculated and subsequently used to inoculate the musts at an
152 initial cell population of 1.0 x 10^6 cells/mL. All the fermentations were carried out in
153 duplicate under static conditions at 25 °C for 21 days.
154
155 Central Composite Design
156
157 Two factorial CCD were used to understand the appropriate experimental plan to model the
158 delay of *S. cerevisiae* inoculation and the sampling time for the chemical analyses during the
159 fermentation period, as previously described by Torchio et al. (2011). A matrix was
160 generated with two factors, delay of *S. cerevisiae* inoculation (hours) and time of chemical
161 analyses (days of fermentation) at five levels (-α, -1, 0, +1, +α), where α was equal to 1.41
162 factorial units. The corresponding values were calculated in the decoded matrix based on the
163 limit of the design -α and +α. In this study, it was decided that 0 (co-inoculation) and 48h
164 (sequential inoculation) delay would be the extreme values of the *S. cerevisiae* addition and 0
165 and 21 days for the time of chemical analyses (Table S1 in the Supplementary Material).

166

167 After running Response Surface Methodology (RSM), a second-order polynomial regression
168 equation was fitted to the subsequent equation:

169

170 $Y = b_0 + b_1X_1 + b_2X_2 + b_{11}X_1^2 + b_{22}X_2^2 + b_{12}X_1X_2$

171

172 Where, Y is the predicted response of the dependent variable, X_1 (inoculation delay) and X_2
173 (sampling time) are the independent variables that correspond to the response of Y, b_0 is the
174 value in the central point conditions, b_1 and b_2 represent the linear regression coefficient
175 associated with each variable, b_{11} and b_{22} are the quadratic regression coefficient of each
176 independent value, while b_{12} is the regression coefficient of the interaction effect between the
177 two variables. The second-order polynomial equations used to generate the surface curves are
178 presented in Supplementary Material (Table S2 in the Supplementary Material).

179

180 Mixed fermentations

181

182 Mixed fermentations were carried out by inoculating the three *S. bacillaris* and two *S.*
183 *cerevisiae* strains in combination, according to the X_1 of CCD pattern. Five inoculation
184 strategies were carried out: inoculation of the two species simultaneously (co-inoculation)
185 and addition of the *S. cerevisiae* at 7, 24, 41 and 48 hours after *S. bacillaris* inoculation
186 (sequential inoculation). In each case, the musts were inoculated with 48 hours pre-adapted
187 cultures grown in the same must, with the same cell relative density of 1:1 as described above
188 for the pure cultures. Fermentations were carried out in duplicate under static conditions at 25
189 °C for 21 days.

190

191 Pilot-scale fermentations

192

193 Fermentations were performed in 2-hL stainless-steel fermenters with Barbera grape, at the
194 experimental winery of the University of Torino. The must composition was as follows:
195 250.4 g/L of sugars (126.1 g/L glucose, 124.3 g/L fructose), titratable acidity 10.21 g/L
196 (expressed as tartaric acid), pH 3.09, total sulphur dioxide 20 mg/L. The initial YAN was 145
197 mg/L composed by the sum of 55 mg/L of AUG and 90 mg/L of PAN. The best performing
198 couple and inoculation strategy according to the laboratory fermentations were selected for
199 these trials: a pure culture fermentation of *S. cerevisiae* Uvaferm BC® was used as control

200 and a sequential mixed culture which *S. cerevisiae* Uvaferm BC® was inoculated with 48
201 hours delay after *S. bacillaris* MUT 5705 inoculation. Both strains were inoculated with an
202 initial cell population of 1.0 x 10^6 cells/mL as described above for the laboratory scale trials.
203 Fermentations were performed in duplicate at 25 ± 2 °C. Must was pumped up twice a day
204 and racking was carried out when residual sugars were less than 2 g/L. Malolactic
205 fermentation was carried at 20 °C in stainless steel tanks, by inoculating the commercial
206 *Oenococcus oeni* Lalvin VP41® strain (Lallemand SA, Montreal, Canada), according the
207 manufacturer's instructions. At the end of the malolactic fermentation, wines were clarified,
208 supplemented with 50 mg/L of total SO_2 and then bottled and subjected to chemical analysis.
209
210 Microbiological and molecular analysis
211
212 Samples were collected in duplicate at 0, 1, 2, 4, 7, 14 and 21 days from the beginning of
213 fermentation, serially diluted in Ringer's solution (Oxoid, Milan, Italy) and plated on WL
214 Nutrient Agar medium (Biogenetics). Plates were incubated at 28 °C for 5 days and the two
215 types of colonies were differentiated visually as described previously (Rantsiou et al. 2012)
216 and subsequently counted. The enumeration of non-*Saccharomyces* yeasts in the pilot-scale
217 fermentations was carried out using lysine agar medium (Oxoid, Milan, Italy). Concerning
218 pilot-scale trials, 5 putative colonies of *S. bacillaris* and *S. cerevisiae* from each sampling
219 point (30 for each ferment), were isolated and then subjected to molecular characterization by
220 Rep and interdelta-PCR, as suggested by Englezos et al. (2015) and Charpentier et al. (2002)
221 respectively, in order to understand strain dynamics over the fermentation process.
222
223 Chemical analysis
224
225 Ethanol, glycerol, acetic acid production, as well as the glucose and fructose consumption
226 were determined by HPLC using an Agilent 1260 Infinity HPLC apparatus (Milford, MA,
227 USA) as described by Rolle et al. (2012). The concentration of total YAN was determined by
228 using two enzymatic kits (Megazyme International, Wicklow, Ireland) following the kit
229 manufacturer instructions. In particular, total YAN concentration was calculated by the sum
230 of ammonium, urea and L-arginine (AUG) and the concentration of the primary amino
231 nitrogen (PAN).
232
233 Statistical analyses

The data obtained from the different inoculation strategies, were subjected to one-way analysis of variance (ANOVA) by using the statistical software package IBM SPSS Statistics (version 21.0. IBM Corp., Armonk. NY, USA). The ANOVA analysis was coupled by the Duncan test ($p < 0.05$), in order to evaluate the significant differences between the data obtained.

The RSM was performed with the statistical software STATISTICA™, program version 10.0 (StatSoft Inc. Tulsa, USA) to evaluate the results obtained by the CCD pattern applied. The regression models were performed only with R^2 values greater than 0.8 indicating that the variability could be explained by the second-order model equations.

Results

Laboratory scale fermentations

Growth dynamics

The growth dynamics of the *S. bacillaris* and *S. cerevisiae* strains when inoculated in pure cultures in Barbera must are presented in Fig. 1. The five strains grew similarly and reached a cell population of about 10^8 colony forming units (CFU)/mL on the second day of fermentation. On the seventh day, viable cell population started to decrease and no *S. bacillaris* population was observed at the last sampling point (<10 CFU/mL on WLN medium plates), while populations of the *S. cerevisiae* strains (ScBa49 and Uvaferm BC®) ranged from 10^6 to 10^7 CFU/mL, respectively.

In Fig. 2 the growth dynamics of the mixed fermentations with *S. cerevisiae* ScBa49 are illustrated. In the co-inoculated and sequential fermentation (7 hours delay), all *S. bacillaris* and *S. cerevisiae* couples showed comparable growth dynamics, reaching a population of 10^7 to 10^8 CFU/mL in 2 days. Through the rest of the fermentation, *S. cerevisiae* ScBa49 maintained this counts, while *S. bacillaris* populations started to decrease. When the other three inoculation strategies (24, 41 and 48 hours delay) were carried out, all *S. bacillaris* strains used in this study competed with *S. cerevisiae* ScBa49 during the first 7 days of fermentation. After this day, *S. bacillaris* started to decrease and the population became undetectable (<10 CFU/mL on WLN medium plates) after 14 (24 hours delay) and

267 21 days (41 and 48 hours delay), while the viability of the *S. cerevisiae* cells remained stable
268 at 10^6 to 10^7 CFU/mL throughout the whole fermentation process.
269 The population dynamics for the mixed fermentations with *S. cerevisiae* Uvaferm
270 BC® are shown in Fig. 3. Population dynamics within the first four days of fermentation, for
271 the co-inoculated and sequential inoculated trials (7 hours delay), were comparable to those
272 observed for *S. cerevisiae* ScBa49. Afterwards, the population of *S. cerevisiae* Uvaferm BC®
273 started to decrease, with counts that ranged from 10^5 to 10^6 CFU/mL at the end of the
274 monitored period (21 days), in contrast with the *S. cerevisiae* ScBa49 population that
275 remained stable during all the monitored period. Interestingly, sequential inoculations with
276 the highest delay (24, 41 and 48 hours), showed similar population dynamics as for the
277 couples previously tested.
278
279 Chemical composition of the wines
280
281 The mean concentration of sugars, glycerol, organic acids and ethanol in the must and wines
282 obtained from the pure cultures of *S. bacillaris* and *S. cerevisiae*, after 21 days of
283 fermentation, are presented in Table 2. *S. bacillaris* in pure cultures produced partially
284 fermented wines with significant presence of residual sugars (up to 32.6 g/L glucose), while
285 the fructose was totally consumed (<1.0 g/L). Complete fermentation of the sugars was
286 observed only for *S. cerevisiae* strains in pure cultures. The chemical composition of these
287 wines was characterized from the presence of lower levels of glycerol (7.8 – 8.3 g/L) and
288 higher levels of ethanol (13.8 – 14.0) % (v/v) compared to *S. bacillaris* pure cultures.
289 Compared to wines produced with *S. cerevisiae* in pure culture, wines fermented with *S.*
290 *bacillaris* presented significantly higher glycerol yields and lower potential ethanol
291 concentrations.
292 The chemical composition of the wines produced from mixed fermentations carried
293 out with *S. cerevisiae* strains ScBa49 and Uvaferm BC® are presented in Tables 3 and 4,
294 respectively. In mixed fermentations with ScBa49, the five different inoculation protocols
295 resulted in a different consumption of sugars. As it can be seen, inoculation delay up to 7
296 hours, always allowed consumption of all sugars (< 2.9 g/L) from the must at day 21,
297 regardless of the *S. bacillaris* strain used. On the contrary, inoculation delays of 24, 41 and
298 48 hours, always performed poorly, leaving significant higher quantities of sugars (14.1 –
299 27.6 g/L), mainly glucose. A different behaviour was observed for the Uvaferm BC®

300 commercial strain, since all couples fermented all the sugars from the must (< 3.9 g/L) after
301 21 days from the beginning of fermentation, independently of the inoculation delay applied.
302 The glycerol production was also influenced by time of *S. cerevisiae* addition and the
303 sampling time (Table 4). Compared to wines produced by *S. cerevisiae* Uvaferm BC® in pure
304 culture, wines produced by mixed yeast species contained more glycerol. In particular, with a
305 delay of 48 hours the glycerol content of the wines increased up to 4.2 g/L (Table 4). The
306 modeling of glycerol production with RSM reflects that its increase is correlated with the
307 increase of the inoculation delay of *S. cerevisiae*. The production of this metabolite was
308 linearly increased when *S. cerevisiae* yeast strain Uvaferm BC® was inoculated in the first 24
309 hours (R^2=0.985) after *S. bacillaris* addition. Conversely, minor differences were found by
310 increasing the sequential inoculation between 24 and 48 hours. Acetic acid production, was
311 also influenced by the inoculation strategy, however all the couples tested in this study
312 maintained values at levels lower than 0.50 g/L.
313 Regarding ethanol production, in sequentially inoculated fermentations, only the
314 couple *S. bacillaris* MUT 5705 and *S. cerevisiae* Uvaferm BC® produced wine with 0.7 %
315 (v/v) less ethanol compared to *S. cerevisiae* Uvaferm BC® in pure culture (Tab. 4). In this
316 case, the must was initially inoculated with *S. bacillaris* followed by *S. cerevisiae* after 24, 41
317 and 48 hours. Additionally, an interesting observation was the increasing quantity of sugars
318 (g/L) consumed by these couples to produce 1% (v/v) of ethanol, with the increase of
319 inoculation delay. This was particularly evident when the inoculation delay moved to 48
320 hours, highlighting the contribution of *S. bacillaris* to the ethanol reduction. For all the
321 couples tested, the shape of the surface curves (Fig. 4, right panel) also confirmed this trend,
322 indicating a significant linear decrease of the ethanol content when *S. cerevisiae* was
323 inoculated with a delay of minimum 4 hours. However, this decrease was improved with the
324 inoculation delay, with a maximum value at 48 hours (maximum monitored). Taking in
325 consideration these findings and the results from growth dynamics, we hypothesized that the
326 most suitable protocol able to reduce the ethanol at industrial scale could be the sequential
327 inoculation with 48 hours delay. This is also in line with previous studies, in which
328 indigenous *S. cerevisiae* started to grow after 2 days from *S. bacillaris* inoculation
329 (Giaramida et al. 2013). Extended delays were not tested because considered not applicable
330 in real wine-making settings.
331
332 Pilot-scale fermentations
333

334 In order to validate the results obtained at laboratory scale, the best performing couple (MUT
335 5705 and Uvaferm BC®) and the inoculation strategy of 48 h delay were selected to ferment
336 Barbera must at pilot-scale fermentations at 25 ^{o}C. Pure fermentation with Uvaferm BC® was
337 used as control. Both inoculation rates and procedures were as close as possible to laboratory
338 scale fermentations, in order to obtain a better reproducibility. Molecular typing of *S.*
339 *bacillaris* and *S. cerevisiae* isolates by Rep and interdelta-PCR amplification respectively,
340 revealed that these fermentations were guided by the inoculated strains (data not shown). The
341 cell population of Uvaferm BC® in pure culture reached a concentration of about 10^8
342 CFU/mL on the second day, which was maintained to these levels during the whole
343 fermentation period (Fig. S1 in the Supplemental Material, panel a). Indigenous non-
344 *Saccharomyces* yeasts were detected at concentration of 10^5 - 10^6 CFU/mL during the first
345 two days, after which they rapidly decreased to undetectable levels (< 10 colonies on lysine
346 medium) on the fourth day. In addition, wild *S. bacillaris* strains, which were determined to
347 be different from the inoculated MUT 5706 by Rep-PCR profiling, were found in the must at
348 concentrations up to 10^6 CFU/mL during the first four days, after this point a remarkable
349 decrease of cell population was observed. When *S. cerevisiae* Uvaferm BC® was inoculated
350 with 48 hours delay after *S. bacillaris* MUT 5705 inoculation (Fig. S1 in the Supplemental
351 Material, panel b), Uvaferm BC® cell population was affected by MUT 5705 and it was not
352 able to reach counts of 10^8 CFU/mL. This allowed MUT 5705 to maintain relative high cell
353 population (about 10^6 – 10^7 CFU/mL) until the seventh day of fermentation. The same pattern
354 was seen for the non-*Saccharomyces* during the first four days, afterwards rapidly decreased
355 to undetectable levels.
356 The chemical composition of the wines produced from the pilot-scale fermentation is
357 given in Table 5. Sequential fermentations consumed sugars slower than Uvaferm BC® in
358 pure culture (10 days vs 7 days). The wine produced from sequential inoculation contained
359 significantly more glycerol (13.4 g/L vs 12.0 g/L) than Uvaferm BC®, while the ethanol
360 content was reduced by 0.5 % (v/v). On the contrary, acetic acid production after malolactic
361 fermentation for the sequential inoculation was reduced compared to that observed for
362 Uvaferm BC® (0.34 g/L vs 0.47 g/L). A significant increase of 0.5 in total acidity was seen
363 for the sequentially inoculated wine, with a parallel decrease of pH.
364
365 **Discussion**
366

367 One possible approach to reduce the ethanol content of wines is by fine-tuning yeast ecology
368 during must fermentation. The selection and use of non-*Saccharomyces* wine yeasts can
369 potentially lead to a reduction of the overall sugar-ethanol yield during alcoholic
370 fermentation. In the study presented here, the possibility of using *S. bacillaris* in combination
371 with *S. cerevisiae* was investigated in order to regulate the ethanol production in a must with
372 a high initial sugar concentration (233.2 g/L). In order to find the appropriate time of *S.*
373 *cerevisiae* addition after *S. bacillaris* inoculation, a CCD approach was selected to model the
374 chemical composition of the wine produced with particular attention on the ethanol and
375 glycerol concentration. With RSM, several combinations of *S. bacillaris*/*S. cerevisiae* at
376 different inoculation times were tested simultaneously with a small number of experiments
377 able to generate large amounts of information, according to the CCD experiment plan, which
378 permits to uncover interactions between variables (Bezerra et al. 2008).
379 In pure fermentations, *S. bacillaris* strains produced wines with residual sugars,
380 composed exclusively by glucose, confirming the preference of this species for fructose
381 (Englezos et al. 2015; Magyar and Tóth 2011). The impact of this non-*Saccharomyces* yeast
382 on the chemical composition of the wine was evident with a higher production of glycerol, in
383 agreement with previous studies (Englezos et al. 2015; Magyar and Tóth 2011; Magyar et al.
384 2014), higher amounts of sugars used to produce 1% of alcohol and slightly higher
385 production of acetic acid compared to the *S. cerevisiae* strains, in accordance with previous
386 studies (Sadoudi et al. 2012; Soden et al. 2000).
387 When mixed fermentation trials were performed, the co-inoculation of the two species
388 did not show a significant reduction of ethanol content and the chemical composition of these
389 wines was very similar to the control wines produced by the *S. cerevisiae* strains in pure
390 cultures. This behavior is confirming the high competitive ability of *S. cerevisiae* over non-
391 *Saccharomyces* yeast cells, probably due to the depletion of nutrients present in the must,
392 cell-to-cell contact-mediated mechanisms or due to the production of toxic metabolites
393 (Andorrà et al. 2010; Nissen et al. 2003; Pérez-Nevado et al. 2006) and underlines the need to
394 understand better the mechanism of this co-habitation.
395 In this context, the early growth of *S. bacillaris* in the sequential inoculations with the
396 highest delays (24, 41 and 48 hours) limited the subsequent growth of the two *S. cerevisiae*
397 strains. One possible explanation for this behaviour is that *S. bacillaris* decreased the nutrient
398 concentration by subtracting large quantities of organic nitrogen from the must (data not
399 shown) (Andorrà et al. 2010; Medina et al. 2012). Indeed, since only the Uvaferm BC®
400 commercial strain totally consumed the sugars in these fermentations, it can be hypothesized

401 that this strain has probably lower demands in nutrients (e.g. nitrogen) compared to ScBa49
402 wild strain. These results suggest that nutrient concentration and strain selection have a
403 fundamental role on the fermentation rate of the mixed fermentations with *S. bacillaris* and *S.*
404 *cerevisiae*.

405 Sequential fermentations performed with the strain Uvaferm BC®, changed positively
406 the chemical composition of the wines produced, especially in terms of glycerol. Glycerol
407 production was influenced by the time of *S. cerevisiae* addition and the sampling time (Table
408 4). The higher concentration of glycerol is in agreement with previous studies (Giaramida et
409 al. 2013; Suzzi et al. 2012),.

410 Interestingly, the inoculation delay changed dramatically the sugar to ethanol
411 conversion rate of alcoholic fermentation. More specifically, in the sequentially inoculated
412 fermentations, yeasts consumed more sugars to produce 1.0 % (v/v) of ethanol, compared to
413 *S. cerevisiae* Uvaferm BC® in pure culture highlighting the impact of *S. bacillaris* for ethanol
414 reduction (Bely et al. 2013; Englezos et al. 2015; Giaramida et al. 2013). However, the
415 results revealed that only the couple MUT 5705 and Uvaferm BC® sequentially inoculated
416 with a minimum of 24 hours delay was able to consume up to 17.5 g/L of sugars to produce
417 1.0% of ethanol, while the official European Economic Community (EEC) ethanol
418 conversion factor is 16.83 g/L (Ribéreau Gayon et al. 2006). The coefficient of determination
419 (R^2) was 0.88 indicating a good correlation between the inoculation delay and fermentation
420 efficiency (g/L of sugar used for 1 % v/v ethanol production).

421 These results let us to hypothesize that *S. bacillaris* diverts carbon derived from the
422 glycolytic pathway away from ethanol production to the synthesis of biomass and production
423 of by-products, in order to maintain intracellular NADH/NAD+ redox balance ensuring
424 continuous operation of the metabolic processes. These products include glycerol,
425 monocarboxylic (acetic acid and pyruvic acid), dicarboxylic (succinic acid and α-ketoglutaric
426 acid) and tricarboxylic acids (citric acid and isocitric acid), and aroma volatile compounds
427 (van Dijken and Scheffers 1986). The overproduction of titratable acidity observed in the
428 pilot scale sequential fermentation could result from the swift of carbon flux towards organic
429 acid production, since *S. bacillaris* is considered high producer of α-ketoglutaric acid and
430 pyruvic acid (Magyar et al. 2014; Mangani et al. 2011).

431 In order to confirm laboratory scale fermentations, the best performing couple (MUT
432 5705 and Uvaferm BC®) and inoculation strategy (48 hours delay) were used to ferment
433 Barbera must in pilot scale fermentations. The presence and dominance of the inoculated
434 yeast strains was confirmed using Rep-PCR and interdelta PCR amplification for the *S.*

bacillaris and *S. cerevisiae*, in order to exclude contributions of indigenous strains. Pilot-scale results confirmed the findings observed in laboratory settings, with the only exception of acetic acid production, which was registered to be higher in pure culture fermentation. The effect of MUT 5705 on wine composition was apparent. As in the laboratory scale fermentation, production of glycerol was higher in the sequential trial than in pure culture fermentation, while ethanol production showed a significant reduction. The pH was also lower and the titratable acidity higher, for wine produced from sequential than this produced from pure culture fermentation.

In summary, this study presents a fermentation protocol tested under both laboratory and pilot scale conditions to reduce ethanol levels in wines. This protocol is based on the inoculation of the grape must with *S. bacillaris* MUT 5705 and *S. cerevisiae* Uvaferm BC® after 48 hour from the beginning of the fermentation. Furthermore, the exploitation of this inoculation protocol could be further investigated using other varieties of grape musts, in order to understand the impact of the co-habitation of these species to wine composition in terms of aroma and flavour. In the future, the decrease of ethanol as described here, could help winemakers to decide the appropriate time to harvest their grapes, without the risk of excessive sugar content, which can be converted in high levels of ethanol in wine.

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Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest.

Ethical approval This paper does not contain any studies with human participants or animals.

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590 **Table 1** Strains used in this study

Strain	Species	Geographical region of isolation	Collection
FC54	*S. bacillaris*	Friuli Venezia Giulia (ITALY)	DISAFA
MUT 5705	*S. bacillaris*	Friuli Venezia Giulia (ITALY)	MUT[a]
C.z 03	*S. bacillaris*	Piedmont (ITALY)	DISAFA
ScBa49	*S. cerevisiae*	Piedmont (ITALY)	DISAFA
Uvaferm BC®	*S. cerevisiae*	France	LALLEMAND

591 [a]MUT= Mycotheca Universitatis Taurinensis (DBIOS - University of Torino, Italy)

592 **Table 2** Concentration of sugars, glycerol, organic acids and ethanol in the must and wines obtained from pure fermentations of *S.*
593 *bacillaris* and *S. cerevisiae* strains

Treatment	Residual sugars (g/L)	Glucose (g/L)	Fructose (g/L)	Acetic acid (g/L)	Glycerol (g/L)	Ethanol (% v/v)	Fermentation efficiency[1]	Potential ethanol[2] (% v/v)	Glycerol yield (g/L)[3]
Must	233.2 ± 0.1	116.4 ± 0.1	116.8 ± 0.1	< 0.1	< 0.1	< 0.1	/	/	/
S. bacillaris									
FC54	21.7 ± 10.4[b]	21.5 ± 10.6	0.2 ± 0.2	0.40 ± 0.02	11.8 ± 0.8	12.1 ± 0.7	18.1 ± 0.7[c]	13.4 ± 0.0[a]	0.06 ± 0.00[b]
MUT 5705	29.7 ± 9.9[b]	29.7 ± 10.0	0.1 ± 0.1	0.41 ± 0.02	12.9 ± 0.3	12.0 ± 0.6	17.0 ± 0.1[ab]	13.7 ± 0.0[b]	0.07 ± 0.01[b]
C.z 03	32.6 ± 11.2[b]	32.4 ± 11.4	0.2 ± 0.2	0.37 ± 0.03	12.6 ± 0.4	11.7 ± 0.7	17.1 ± 0.1[b]	13.7 ± 0.0[b]	0.07 ± 0.01[b]
S. cerevisiae									
ScBa49	1.0 ± 0.2[a]	0.3 ± 0.1	0.7 ± 0.0	0.26 ± 0.04	7.8 ± 0.1	13.8 ± 0.0	16.8 ± 0.0[ab]	13.9 ± 0.0[c]	0.03 ± 0.00[a]
Uvaferm BC®	0.5 ± 0.1[a]	0.1 ± 0.1	0.4 ± 0.0	0.14 ± 0.01	8.3 ± 0.3	14.0 ± 0.2	16.6 ± 0.2[a]	14.1 ± 0.2[d]	0.04 ± 0.01[a]
Sig[4]	***	/	/	/	/	/	***	***	***

594 [1]Fermentation efficiency (sugars used to produce 1.0 % of ethanol (v/v)): initial and residual sugar concentrations were used to calculate
595 the fermentation efficiency
596 [2]Potential ethanol (% v/v) = ethanol produced + ((residual glucose + residual fructose) * 0.06)
597 [3]Glycerol yield = glycerol produced / (initial sugar concentration – final sugar concentration)
598 All data are expressed as average value ± standard deviation (n = 2). Different superscript letters within the same column indicate
599 significant differences among the strains according to the Duncan test ($p < 0.05$)
600 [4]Sig: *** indicate significance at $p < 0.001$

601 **Table 3** Concentration of sugars, glycerol, organic acids and ethanol in the wines obtained from mixed fermentations using the *S.*
602 *cerevisiae* strain ScBa49

Strains and inoculation strategy	Residual sugars (g/L)	Glucose (g/L)	Fructose (g/L)	Acetic acid (g/L)	Glycerol (g/L)	Ethanol (% v/v)	Fermentation efficiency[1]
Pure fermentation ScBa49	$1.0 \pm 0.2^{aA\alpha}$	$0.3 \pm 0.1^{aA\alpha}$	0.7 ± 0.1	$0.26 \pm 0.05^{aA\alpha}$	$7.8 \pm 0.1^{aA\alpha}$	$13.8 \pm 0.1^{bC\gamma}$	16.6 ± 0.3^{A}
Couple: FC54 and ScBa49							
Co-inoculation	2.0 ± 0.1^{a}	0.2 ± 0.1^{a}	1.7 ± 0.1	0.34 ± 0.02^{b}	8.2 ± 0.3^{a}	13.8 ± 0.1^{b}	16.6 ± 0.1
Inoculation delay: 7 h.	2.4 ± 1.4^{a}	0.4 ± 0.1^{a}	2.0 ± 1.3	0.39 ± 0.01^{b}	9.6 ± 0.3^{b}	13.7 ± 0.2^{b}	16.7 ± 0.2
Inoculation delay: 24 h.	23.1 ± 0.1^{b}	22.6 ± 0.9^{b}	0.4 ± 0.1	0.51 ± 0.01^{c}	11.8 ± 0.2^{c}	12.4 ± 0.2^{a}	16.8 ± 0.3
Inoculation delay: 41 h.	26.3 ± 7.9^{b}	26.2 ± 7.9^{b}	0.4 ± 0.1	0.49 ± 0.01^{c}	12.0 ± 0.5^{c}	12.3 ± 0.5^{a}	17.1 ± 0.1
Inoculation delay: 48 h.	23.2 ± 8.5^{b}	23.2 ± 8.5^{b}	0.4 ± 0.1	0.49 ± 0.01^{c}	12.0 ± 0.3^{c}	12.1 ± 0.5^{a}	17.1 ± 0.5
Sig[2]	**	**	NS	***	***	**	NS
Couple: MUT 5705 and ScBa49							
Co-inoculation	1.8 ± 0.2^{A}	0.3 ± 0.1^{A}	1.5 ± 0.3	0.32 ± 0.06^{AB}	8.5 ± 0.2^{B}	14.0 ± 0.1^{D}	16.4 ± 0.1^{A}
Inoculation delay: 7 h.	2.9 ± 0.1^{A}	0.3 ± 0.1^{A}	2.6 ± 0.1	0.40 ± 0.02^{B}	9.5 ± 0.1^{C}	13.8 ± 0.1^{C}	16.7 ± 0.3^{AB}
Inoculation delay: 24 h.	14.1 ± 2.7^{B}	13.5 ± 2.9^{B}	0.5 ± 0.2	0.50 ± 0.01^{C}	11.7 ± 0.3^{D}	12.8 ± 0.1^{B}	17.3 ± 0.1^{BC}
Inoculation delay: 41 h.	24.5 ± 0.3^{C}	24.1 ± 0.3^{C}	0.4 ± 0.1	0.51 ± 0.01^{C}	12.2 ± 0.1^{E}	12.4 ± 0.1^{A}	17.3 ± 0.4^{BC}
Inoculation delay: 48 h.	27.6 ± 0.7^{D}	27.2 ± 0.1^{D}	0.6 ± 0.3	0.51 ± 0.05^{C}	12.8 ± 0.1^{F}	12.3 ± 0.1^{A}	17.5 ± 0.2^{C}
Sig[2]	***	***	NS	**	***	***	*
Couple: C.z 03 and ScBa49							
Co-inoculation	$0.9 \pm 0.1^{\alpha}$	$0.3 \pm 0.1^{\alpha}$	0.7 ± 0.1	$0.34 \pm 0.02^{\beta}$	$8.5 \pm 0.1^{\beta}$	$13.9 \pm 0.2^{\gamma}$	16.8 ± 0.4
Inoculation delay: 7 h.	$1.3 \pm 0.1^{\alpha}$	$0.3 \pm 0.1^{\alpha}$	1.0 ± 0.1	$0.40 \pm 0.02^{\beta}$	$9.7 \pm 0.3^{\gamma}$	$13.8 \pm 0.1^{\gamma}$	16.7 ± 0.1
Inoculation delay: 24 h.	$20.0 \pm 1.4^{\beta}$	$19.6 \pm 1.4^{\beta}$	0.4 ± 0.1	$0.47 \pm 0.01^{\gamma}$	$12.5 \pm 0.2^{\delta}$	$12.6 \pm 0.1^{\beta}$	16.9 ± 0.3
Inoculation delay: 41 h.	$23.6 \pm 1.2^{\gamma}$	$23.6 \pm 1.2^{\gamma}$	0.4 ± 0.1	$0.49 \pm 0.01^{\gamma}$	$12.6 \pm 0.2^{\delta}$	$12.1 \pm 0.1^{\alpha}$	17.2 ± 0.1
Inoculation delay: 48 h.	$25.8 \pm 0.1^{\delta}$	$25.8 \pm 0.1^{\delta}$	0.4 ± 0.1	$0.47 \pm 0.02^{\gamma}$	$12.5 \pm 0.4^{\delta}$	$12.3 \pm 0.1^{\alpha}$	17.2 ± 0.1
Sig[2]	***	***	NS	***	***	***	NS

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604 [1]Fermentation efficiency (sugars used to produce 1.0 % of ethanol (v/v)): initial and residual sugar concentrations were used to calculate
605 fermentation efficiency
606 All data are expressed as average value ± standard deviation (n = 2). Different superscript Latin, UPPER Latin and Greek letters within the
607 same column indicate significant differences among the couples FC54 and ScBa49, MUT 5705 and ScBa49 and C.z 03 and ScBa49
608 according to the Duncan test ($p < 0.05$) respectively
609 [2]Sig: *, **, *** and NS indicate significance at $p < 0.05$, $p < 0.01$, $p < 0.001$ and not significant respectively
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621 **Table 4** Mean concentration of sugars, glycerol, organic acids and ethanol in the wines obtained from mixed fermentations with the *S.*
622 *cerevisiae* strain Uvaferm BC®

Strains and inoculation strategy	Residual sugars (g/L)	Glucose (g/L)	Fructose (g/L)	Acetic acid (g/L)	Glycerol (g/L)	Ethanol (% v/v)	Fermentation efficiency[1]
Pure fermentation Uvaferm BC®	$0.5 \pm 0.1^{A\alpha}$	$0.1 \pm 0.1^{A\alpha}$	0.4 ± 0.1	$0.14 \pm 0.01^{aA\alpha}$	$8.3 \pm 0.3^{aA\alpha}$	14.0 ± 0.2^{B}	16.6 ± 0.2^{A}
Couple: FC54 and Uvaferm BC®							
Co-inoculation	0.8 ± 0.1	0.4 ± 0.2	0.4 ± 0.1	0.19 ± 0.05^{a}	8.8 ± 0.1^{ab}	14.0 ± 0.1	16.6 ± 0.1
Inoculation delay: 7 h.	0.6 ± 0.1	0.2 ± 0.1	0.4 ± 0.1	0.19 ± 0.05^{a}	9.5 ± 0.1^{b}	14.0 ± 0.2	16.7 ± 0.2
Inoculation delay: 24 h.	1.3 ± 0.6	1.0 ± 0.5	0.3 ± 0.1	0.37 ± 0.01^{b}	12.5 ± 0.2^{c}	13.8 ± 0.3	16.8 ± 0.3
Inoculation delay: 41 h.	3.9 ± 2.5	3.6 ± 2.4	0.3 ± 0.1	0.46 ± 0.09^{b}	12.5 ± 0.3^{c}	13.4 ± 0.2	17.1 ± 0.1
Inoculation delay: 48 h.	3.1 ± 1.9	2.8 ± 1.9	0.3 ± 0.1	0.41 ± 0.05^{b}	12.6 ± 0.6^{c}	13.5 ± 0.3	17.0 ± 0.5
Sig[2]	NS	NS	NS	**	***	NS	NS
Couple: MUT 5705 and Uvaferm BC®							
Co-inoculation	0.7 ± 0.1^{AB}	0.2 ± 0.1^{A}	0.5 ± 0.1	0.19 ± 0.05^{A}	9.1 ± 0.1^{B}	14.1 ± 0.1^{B}	16.4 ± 0.1^{A}
Inoculation delay: 7 h.	0.6 ± 0.1^{A}	0.2 ± 0.1^{A}	0.3 ± 0.1	0.19 ± 0.05^{A}	9.9 ± 0.1^{C}	13.9 ± 0.2^{B}	16.7 ± 0.3^{AB}
Inoculation delay: 24 h.	0.8 ± 0.2^{AB}	0.5 ± 0.2^{AB}	0.3 ± 0.1	0.34 ± 0.01^{B}	12.5 ± 0.2^{D}	13.4 ± 0.1^{A}	17.3 ± 0.1^{BC}
Inoculation delay: 41 h.	1.7 ± 0.4^{C}	1.3 ± 0.4^{C}	0.4 ± 0.1	0.42 ± 0.03^{B}	12.9 ± 0.1^{D}	13.4 ± 0.3^{A}	17.3 ± 0.4^{BC}
Inoculation delay: 48 h.	1.1 ± 0.1^{B}	0.9 ± 0.0^{B}	0.3 ± 0.1	0.42 ± 0.04^{B}	12.5 ± 0.2^{D}	13.3 ± 0.1^{A}	17.5 ± 0.2^{C}
Sig[2]	**	**	NS	***	***	*	*
Couple: C.z 03 and Uvaferm BC®							
Co-inoculation	$0.7 \pm 0.1^{\beta}$	$0.2 \pm 0.1^{\alpha}$	0.5 ± 0.1	$0.17 \pm 0.05^{\alpha}$	$9.1 \pm 0.4^{\beta}$	13.9 ± 0.4	16.8 ± 0.4
Inoculation delay: 7 h.	$0.5 \pm 0.1^{\alpha}$	$0.2 \pm 0.1^{\alpha}$	0.4 ± 0.1	$0.19 \pm 0.04^{\alpha}$	$9.5 \pm 0.1^{\beta}$	13.9 ± 0.1	16.7 ± 0.1
Inoculation delay: 24 h.	$1.1 \pm 0.1^{\gamma}$	$0.7 \pm 0.1^{\beta}$	0.4 ± 0.1	$0.34 \pm 0.03^{\beta}$	$12.8 \pm 0.1^{\delta}$	13.7 ± 0.2	16.9 ± 0.3
Inoculation delay: 41 h.	$1.9 \pm 0.1^{\delta}$	$1.6 \pm 0.1^{\gamma}$	0.3 ± 0.1	$0.41 \pm 0.03^{\beta}$	$12.2 \pm 0.3^{\gamma\delta}$	13.5 ± 0.1	17.2 ± 0.1
Inoculation delay: 48 h.	$2.5 \pm 0.1^{\varepsilon}$	$2.2 \pm 0.1^{\delta}$	0.3 ± 0.1	$0.40 \pm 0.01^{\beta}$	$12.0 \pm 0.2^{\gamma}$	13.4 ± 0.1	17.2 ± 0.1
Sig[2]	***	***	NS	***	***	NS	NS

[1]Fermentation efficiency: (sugars used to produce 1.0 % of ethanol(v/v)): initial and residual sugar concentrations were used to calculate fermentation efficiency

All data are expressed as average value ± standard deviation (n = 2). Different superscript Latin, UPPER Latin and Greek letters within the same column indicate significant differences among the couples FC54 and Uvaferm BC®, MUT 5705 and Uvaferm BC® and C.z 03 and Uvaferm BC® according to the Duncan test ($p < 0.05$) respectively

[2]Sig: *, **, *** and NS indicate significance at $p < 0.05$, $p < 0.01$, $p < 0.001$ and not significant respectively

642 **Table 5** Mean concentration of sugars, glycerol, organic acids and ethanol in the wines produced from pure (Uvaferm BC®, control) and

643 sequential (MUT 5705 and Uvaferm BC®) culture fermentations at pilot scale (2hL)

Parameter	Must	Control	Sequential	Sign[1]
Residual sugars (g/L)	250.4 ± 2.5	< 2.0	< 2.0	NS
Acetic acid (g/L)	< 0.1	0.47 ± 0.07	0.34 ± 0.04	**
Glycerol (g/L)	< 0.1	12.0 ± 0.4	13.4 ± 0.1	***
Ethanol (% v/v)	< 0.1	15.4 ± 0.0	14.9 ± 0.1	***
pH	3.09 ± 0.01	3.38 ± 0.00	3.35 ± 0.00	***
Titratable acidity (g/L)	10.21 ± 0.14	6.71 ± 0.04	7.18 ± 0.08	***

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645 All data are expressed as average value ± standard deviation (n = 2)

646 [1]Sig: **, *** and NS indicate significance at $p < 0.01$, $p < 0.001$ and not significant respectively, between control and sequential fermented

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650 **Figures legends**

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652 **Fig. 1** Growth dynamics of *S. bacillaris* (A) and *S. cerevisiae* (B) strains in pure
653 cultures. *S. bacillaris* strains: FC54 (-○-), MUT 5705 (-◊-), C.z 03 (-□-) and *S.*
654 *cerevisiae* strains: ScBa49 (-♦-) and Uvaferm BC® (-◊-). Counts are the mean CFU/mL
655 values ± standard deviations of two independent experiments

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657 **Fig. 2** Growth dynamics of mixed fermentations performed with the three *S. bacillaris*
658 strains and the *S. cerevisiae* strain ScBa49. *S. bacillaris*/*S. cerevisiae* couples: FC54 (-
659 ○-), ScBa49 (-•-), MUT 5705 (-◊-) and ScBa49 (-◆-), C.z 03 (-□-) and ScBa49 (-■-).
660 Counts are the mean CFU/mL values ± standard deviations of two independent
661 experiments. The arrow indicates the *S. cerevisiae* inoculation

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663 **Fig. 3** Growth dynamics of mixed fermentations performed with the three *S. bacillaris*
664 strains and the *S. cerevisiae* strain Uvaferm BC®. *S. bacillaris*/*S. cerevisiae* couples :
665 FC54 (-○-) and Uvaferm BC® (-•-), MUT 5705 (-◊-) and Uvaferm BC® (-◆-), C.z 03 (-
666 □-) and Uvaferm BC® (-■-). Counts are the mean CFU/mL values ± standard deviations
667 of two independent experiments. The arrow indicates the *S. cerevisiae* inoculation

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669 **Fig. 4** Response surface curves fitted to experimental data points corresponding to the
670 glycerol (g/L) (left panel) and ethanol (% v/v) (right panel) production as a function of
671 *S. cerevisiae* inoculation and time of the chemical analyses. *S. bacillaris*/*S. cerevisiae*
672 couples: FC54 and Uvaferm BC® (panel A), MUT 5705 and Uvaferm BC® (panel B),
673 C.z 03 and Uvaferm BC® (panel C).

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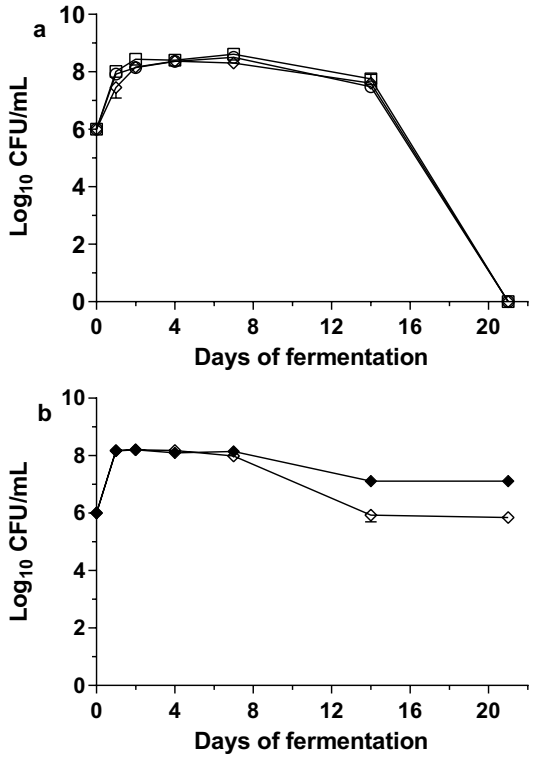
682 **Fig.1**



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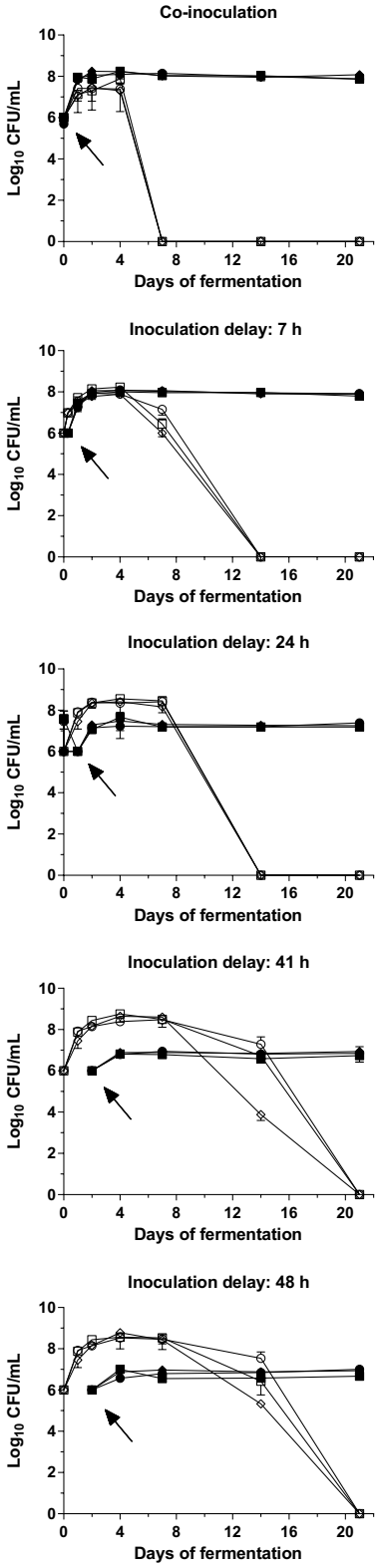
699 **Fig. 2**



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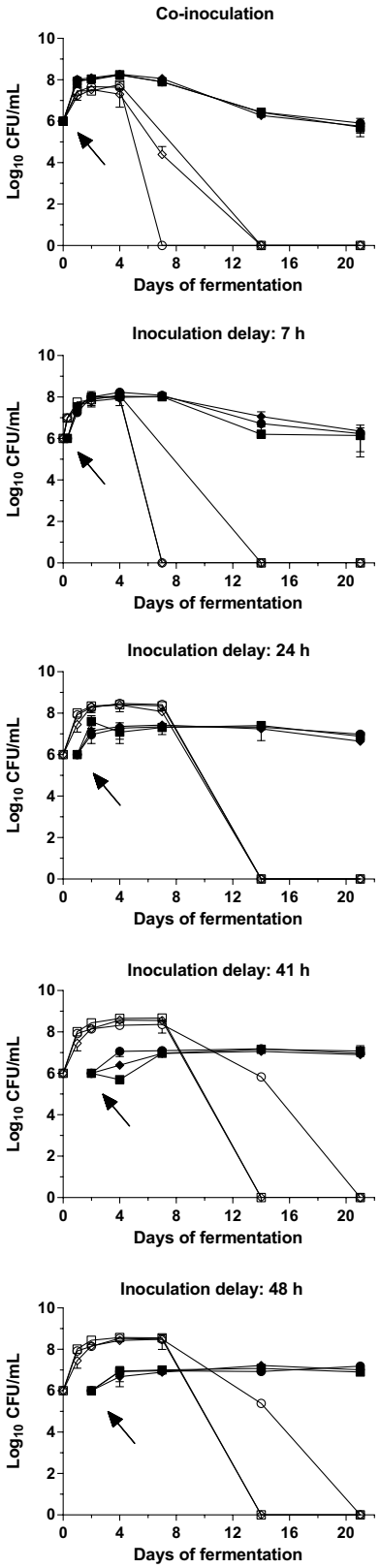
702 **Fig.3**


Co-inoculation
Inoculation delay: 7 h
Inoculation delay: 24 h
Inoculation delay: 41 h
Inoculation delay: 48 h
Log10 CFU/mL
Days of fermentation
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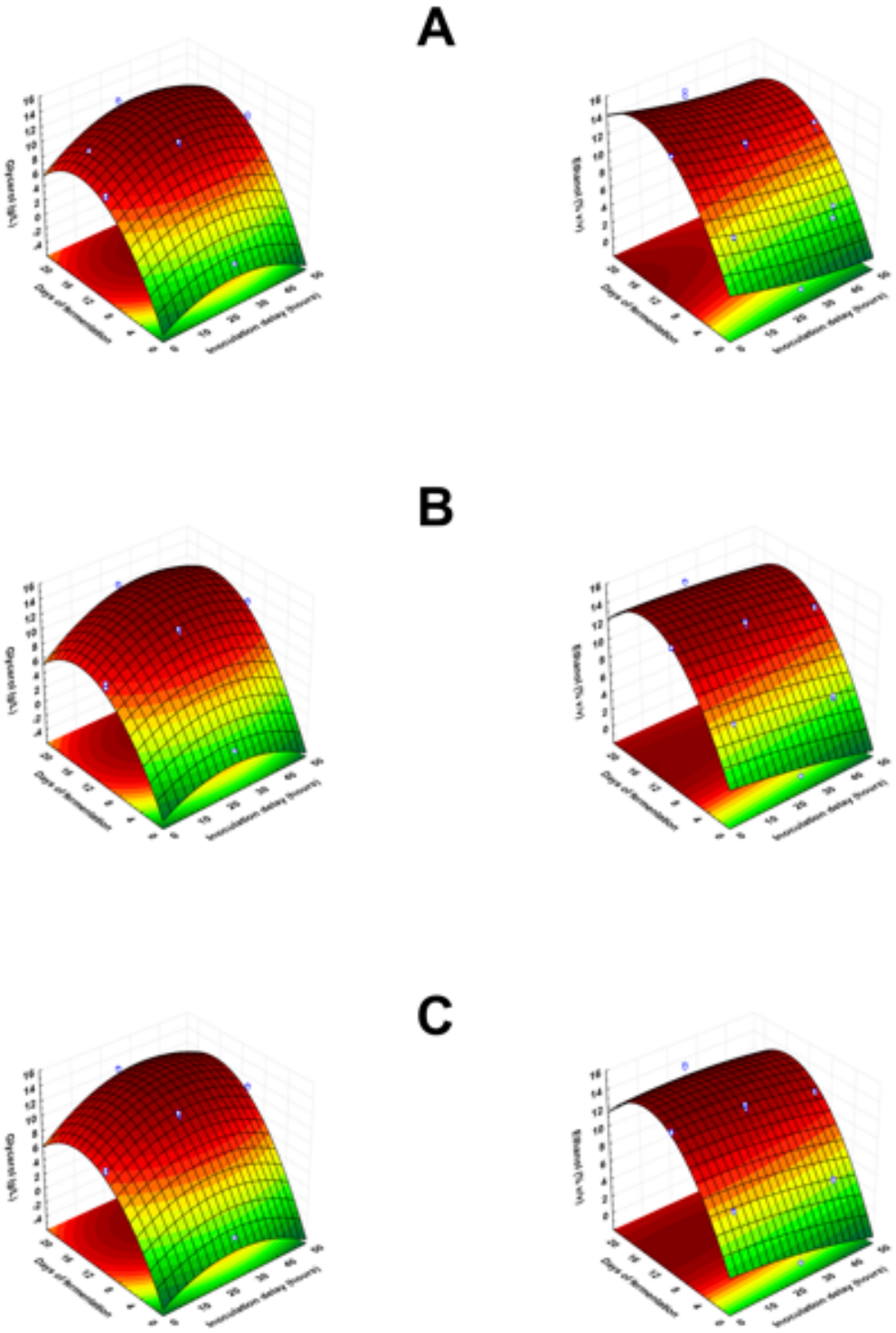
704 **Fig. 4**



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709 **Table S1** Matrix used for the CCD analysis

Experiments	Inoculation delay (hours)	Chemical analyses (days)	Inoculation delay (hours)	Chemical analyses (days)
1	-1	-1	7	3.0
2	1	-1	41	3.0
3	-1	1	7	18.0
4	1	1	41	18.0
5	-1.41	0	0	10.5
6	1.41	0	48	10.5
7	0	-1.41	24	0.0
8	0	1.41	24	21.0
9-13	0	0	24	10.5

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733 **Table S2.** Second-order polynomial regression equations used to generate the surface

734 curves for the three couples of *S. bacillaris* and *S. cerevisiae*.

Chemical compounds	Couples	b_0	b_1	b_2	b_{11}	b_{22}	b_{12}
Glycerol	FC54 and Uvaferm BC®	-4.941	0.2513	1.849	-5.217E-03	-6.280E-02	3.900E-03
	MUT 5705 and Uvaferm BC®	-4.767	0.2443	1.781	-5.167E-03	-6.036E-02	5.390E-03
	C.z03 and Uvaferm BC®	-4.941	0.2513	1.849	-5.217E-03	-6.281E-02	3.929E-03
Ethanol	FC54 and Uvaferm BC®	3.238	-0.1163	1.563	7.870E-04	-4.892E-02	1.111E-03
	MUT 5705 and Uvaferm BC®	2.398	-0.0506	1.701	-4.563E-04	-5.708E-02	2.460E-03
	C.z03 and Uvaferm BC®	2.256	-0.0301	1.721	-7.940E-04	-5.922E-02	2.939E-03

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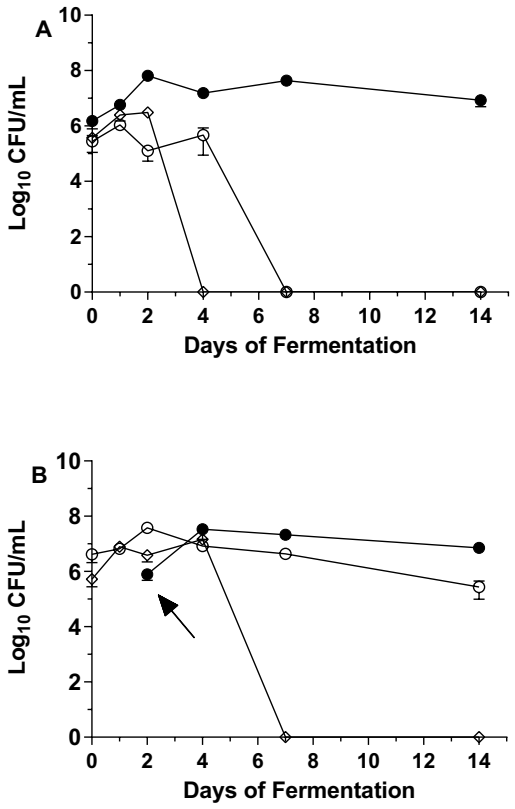
759 **FigS1.**



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761 **Fig. S1** Growth dynamics of yeast during pilot scale fermentations. (a) Control culture
762 fermentation, (b) Sequential fermentation. *S. bacillaris* (-○-), *S. cerevisiae* (-•-) and
763 indigenous non-*Saccharomyces* yeasts (-◊-). Counts are the mean CFU/mL values ±
764 standard deviations of two independent experiments. The arrow indicates the *S.*
765 *cerevisiae* inoculation

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