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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 Orduña 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

Grape must of Barbera cultivar (*Vitis vinifera* L.) without the grape skin was obtained from the experimental winery of the University of Torino. Grape must contained 233.2 g/L sugars (116.4 g/L glucose, 116.8 g/L fructose), titratable acidity 8.20 g/L (expressed as tartaric acid), pH 3.20 and absence of ethanol. The initial Yeast Available Nitrogen (YAN) was 197 mg/L composed by the sum of 116 mg/L of AUG (ammonium + urea + L-arginine) and 81 mg/L of PAN (primary amino nitrogen). The must was pasteurized in a water bath at 60˚C for 1 hour and the sterility was checked by plating 100 µL of must on WL Nutrient Agar medium (Biogenetics, Milano, Italy) and incubated them at 28 ^{o}C for 5 days. Under sterile conditions, 25 mL of the pasteurized must was distributed onto 50 mL tubes with loose screw cap for all the fermentations performed in this work.

Pure fermentations

The oenological performance of the three *S. bacillaris* and two *S. cerevisiae* strains was evaluated by micro-vinification trials in pure culture fermentations. The inoculum of the five yeast strains was prepared by pre-adaptation of the strains in the same must as described above for 48 hours at 25 °C. Afterwards, the yeast cells were stained with methylene blue dye and immediately the viable cell population was counted by using a Thoma hemocytometer chamber (BRAND GMBH + CO KG, Wertheim, Germany). Before inoculation, appropriate amounts of inoculum were calculated and subsequently used to inoculate the musts at an initial cell population of 1.0 x 10^6 cells/mL. All the fermentations were carried out in duplicate under static conditions at 25 °C for 21 days.

Central Composite Design

Two factorial CCD were used to understand the appropriate experimental plan to model the delay of *S. cerevisiae* inoculation and the sampling time for the chemical analyses during the fermentation period, as previously described by Torchio et al. (2011). A matrix was generated with two factors, delay of *S. cerevisiae* inoculation (hours) and time of chemical analyses (days of fermentation) at five levels (-α, -1, 0, +1, +α), where α was equal to 1.41 factorial units. The corresponding values were calculated in the decoded matrix based on the limit of the design -α and +α. In this study, it was decided that 0 (co-inoculation) and 48h (sequential inoculation) delay would be the extreme values of the *S. cerevisiae* addition and 0 and 21 days for the time of chemical analyses (Table S1 in the Supplementary Material).

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

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

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

Mixed fermentations

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

Pilot-scale fermentations

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

and a sequential mixed culture which *S. cerevisiae* Uvaferm BC® was inoculated with 48 hours delay after *S. bacillaris* MUT 5705 inoculation. Both strains were inoculated with an initial cell population of 1.0 x 10^6 cells/mL as described above for the laboratory scale trials. Fermentations were performed in duplicate at 25 ± 2 °C. Must was pumped up twice a day and racking was carried out when residual sugars were less than 2 g/L. Malolactic fermentation was carried at 20 °C in stainless steel tanks, by inoculating the commercial *Oenococcus oeni* Lalvin VP41® strain (Lallemand SA, Montreal, Canada), according the manufacturer's instructions. At the end of the malolactic fermentation, wines were clarified, supplemented with 50 mg/L of total SO_2 and then bottled and subjected to chemical analysis.

Microbiological and molecular analysis

Samples were collected in duplicate at 0, 1, 2, 4, 7, 14 and 21 days from the beginning of fermentation, serially diluted in Ringer's solution (Oxoid, Milan, Italy) and plated on WL Nutrient Agar medium (Biogenetics). Plates were incubated at 28 °C for 5 days and the two types of colonies were differentiated visually as described previously (Rantsiou et al. 2012) and subsequently counted. The enumeration of non-*Saccharomyces* yeasts in the pilot-scale fermentations was carried out using lysine agar medium (Oxoid, Milan, Italy). Concerning pilot-scale trials, 5 putative colonies of *S. bacillaris* and *S. cerevisiae* from each sampling point (30 for each ferment), were isolated and then subjected to molecular characterization by Rep and interdelta-PCR, as suggested by Englezos et al. (2015) and Charpentier et al. (2002) respectively, in order to understand strain dynamics over the fermentation process.

Chemical analysis

Ethanol, glycerol, acetic acid production, as well as the glucose and fructose consumption were determined by HPLC using an Agilent 1260 Infinity HPLC apparatus (Milford, MA, USA) as described by Rolle et al. (2012). The concentration of total YAN was determined by using two enzymatic kits (Megazyme International, Wicklow, Ireland) following the kit manufacturer instructions. In particular, total YAN concentration was calculated by the sum of ammonium, urea and L-arginine (AUG) and the concentration of the primary amino nitrogen (PAN).

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

21 days (41 and 48 hours delay), while the viability of the *S. cerevisiae* cells remained stable at 10^6 to 10^7 CFU/mL throughout the whole fermentation process.

The population dynamics for the mixed fermentations with *S. cerevisiae* Uvaferm BC® are shown in Fig. 3. Population dynamics within the first four days of fermentation, for the co-inoculated and sequential inoculated trials (7 hours delay), were comparable to those observed for *S. cerevisiae* ScBa49. Afterwards, the population of *S. cerevisiae* Uvaferm BC® started to decrease, with counts that ranged from 10^5 to 10^6 CFU/mL at the end of the monitored period (21 days), in contrast with the *S. cerevisiae* ScBa49 population that remained stable during all the monitored period. Interestingly, sequential inoculations with the highest delay (24, 41 and 48 hours), showed similar population dynamics as for the couples previously tested.

Chemical composition of the wines

The mean concentration of sugars, glycerol, organic acids and ethanol in the must and wines obtained from the pure cultures of *S. bacillaris* and *S. cerevisiae*, after 21 days of fermentation, are presented in Table 2. *S. bacillaris* in pure cultures produced partially fermented wines with significant presence of residual sugars (up to 32.6 g/L glucose), while the fructose was totally consumed (<1.0 g/L). Complete fermentation of the sugars was observed only for *S. cerevisiae* strains in pure cultures. The chemical composition of these wines was characterized from the presence of lower levels of glycerol (7.8 – 8.3 g/L) and higher levels of ethanol (13.8 – 14.0) % (v/v) compared to *S. bacillaris* pure cultures. Compared to wines produced with *S. cerevisiae* in pure culture, wines fermented with *S. bacillaris* presented significantly higher glycerol yields and lower potential ethanol concentrations.

The chemical composition of the wines produced from mixed fermentations carried out with *S. cerevisiae* strains ScBa49 and Uvaferm BC® are presented in Tables 3 and 4, respectively. In mixed fermentations with ScBa49, the five different inoculation protocols resulted in a different consumption of sugars. As it can be seen, inoculation delay up to 7 hours, always allowed consumption of all sugars (< 2.9 g/L) from the must at day 21, regardless of the *S. bacillaris* strain used. On the contrary, inoculation delays of 24, 41 and 48 hours, always performed poorly, leaving significant higher quantities of sugars (14.1 – 27.6 g/L), mainly glucose. A different behaviour was observed for the Uvaferm BC®

commercial strain, since all couples fermented all the sugars from the must (< 3.9 g/L) after 21 days from the beginning of fermentation, independently of the inoculation delay applied.

The glycerol production was also influenced by time of *S. cerevisiae* addition and the sampling time (Table 4). Compared to wines produced by *S. cerevisiae* Uvaferm BC® in pure culture, wines produced by mixed yeast species contained more glycerol. In particular, with a delay of 48 hours the glycerol content of the wines increased up to 4.2 g/L (Table 4). The modeling of glycerol production with RSM reflects that its increase is correlated with the increase of the inoculation delay of *S. cerevisiae*. The production of this metabolite was linearly increased when *S. cerevisiae* yeast strain Uvaferm BC® was inoculated in the first 24 hours (R^2=0.985) after *S. bacillaris* addition. Conversely, minor differences were found by increasing the sequential inoculation between 24 and 48 hours. Acetic acid production, was also influenced by the inoculation strategy, however all the couples tested in this study maintained values at levels lower than 0.50 g/L.

Regarding ethanol production, in sequentially inoculated fermentations, only the couple *S. bacillaris* MUT 5705 and *S. cerevisiae* Uvaferm BC® produced wine with 0.7 % (v/v) less ethanol compared to *S. cerevisiae* Uvaferm BC® in pure culture (Tab. 4). In this case, the must was initially inoculated with *S. bacillaris* followed by *S. cerevisiae* after 24, 41 and 48 hours. Additionally, an interesting observation was the increasing quantity of sugars (g/L) consumed by these couples to produce 1% (v/v) of ethanol, with the increase of inoculation delay. This was particularly evident when the inoculation delay moved to 48 hours, highlighting the contribution of *S. bacillaris* to the ethanol reduction. For all the couples tested, the shape of the surface curves (Fig. 4, right panel) also confirmed this trend, indicating a significant linear decrease of the ethanol content when *S. cerevisiae* was inoculated with a delay of minimum 4 hours. However, this decrease was improved with the inoculation delay, with a maximum value at 48 hours (maximum monitored). Taking in consideration these findings and the results from growth dynamics, we hypothesized that the most suitable protocol able to reduce the ethanol at industrial scale could be the sequential inoculation with 48 hours delay. This is also in line with previous studies, in which indigenous *S. cerevisiae* started to grow after 2 days from *S. bacillaris* inoculation (Giaramida et al. 2013). Extended delays were not tested because considered not applicable in real wine-making settings.

Pilot-scale fermentations

In order to validate the results obtained at laboratory scale, the best performing couple (MUT 5705 and Uvaferm BC®) and the inoculation strategy of 48 h delay were selected to ferment Barbera must at pilot-scale fermentations at 25 °C. Pure fermentation with Uvaferm BC® was used as control. Both inoculation rates and procedures were as close as possible to laboratory scale fermentations, in order to obtain a better reproducibility. Molecular typing of *S. bacillaris* and *S. cerevisiae* isolates by Rep and interdelta-PCR amplification respectively, revealed that these fermentations were guided by the inoculated strains (data not shown). The cell population of Uvaferm BC® in pure culture reached a concentration of about 10^8 CFU/mL on the second day, which was maintained to these levels during the whole fermentation period (Fig. S1 in the Supplemental Material, panel a). Indigenous non-*Saccharomyces* yeasts were detected at concentration of 10^5 - 10^6 CFU/mL during the first two days, after which they rapidly decreased to undetectable levels (< 10 colonies on lysine medium) on the fourth day. In addition, wild *S. bacillaris* strains, which were determined to be different from the inoculated MUT 5706 by Rep-PCR profiling, were found in the must at concentrations up to 10^6 CFU/mL during the first four days, after this point a remarkable decrease of cell population was observed. When *S. cerevisiae* Uvaferm BC® was inoculated with 48 hours delay after *S. bacillaris* MUT 5705 inoculation (Fig. S1 in the Supplemental Material, panel b), Uvaferm BC® cell population was affected by MUT 5705 and it was not able to reach counts of 10^8 CFU/mL. This allowed MUT 5705 to maintain relative high cell population (about 10^6 – 10^7 CFU/mL) until the seventh day of fermentation. The same pattern was seen for the non-*Saccharomyces* during the first four days, afterwards rapidly decreased to undetectable levels.

The chemical composition of the wines produced from the pilot-scale fermentation is given in Table 5. Sequential fermentations consumed sugars slower than Uvaferm BC® in pure culture (10 days vs 7 days). The wine produced from sequential inoculation contained significantly more glycerol (13.4 g/L vs 12.0 g/L) than Uvaferm BC®, while the ethanol content was reduced by 0.5 % (v/v). On the contrary, acetic acid production after malolactic fermentation for the sequential inoculation was reduced compared to that observed for Uvaferm BC® (0.34 g/L vs 0.47 g/L). A significant increase of 0.5 in total acidity was seen for the sequentially inoculated wine, with a parallel decrease of pH.

Discussion

One possible approach to reduce the ethanol content of wines is by fine-tuning yeast ecology during must fermentation. The selection and use of non-*Saccharomyces* wine yeasts can potentially lead to a reduction of the overall sugar-ethanol yield during alcoholic fermentation. In the study presented here, the possibility of using *S. bacillaris* in combination with *S. cerevisiae* was investigated in order to regulate the ethanol production in a must with a high initial sugar concentration (233.2 g/L). In order to find the appropriate time of *S. cerevisiae* addition after *S. bacillaris* inoculation, a CCD approach was selected to model the chemical composition of the wine produced with particular attention on the ethanol and glycerol concentration. With RSM, several combinations of *S. bacillaris*/*S. cerevisiae* at different inoculation times were tested simultaneously with a small number of experiments able to generate large amounts of information, according to the CCD experiment plan, which permits to uncover interactions between variables (Bezerra et al. 2008).

In pure fermentations, *S. bacillaris* strains produced wines with residual sugars, composed exclusively by glucose, confirming the preference of this species for fructose (Englezos et al. 2015; Magyar and Tóth 2011). The impact of this non-*Saccharomyces* yeast on the chemical composition of the wine was evident with a higher production of glycerol, in agreement with previous studies (Englezos et al. 2015; Magyar and Tóth 2011; Magyar et al. 2014), higher amounts of sugars used to produce 1% of alcohol and slightly higher production of acetic acid compared to the *S. cerevisiae* strains, in accordance with previous studies (Sadoudi et al. 2012; Soden et al. 2000).

When mixed fermentation trials were performed, the co-inoculation of the two species did not show a significant reduction of ethanol content and the chemical composition of these wines was very similar to the control wines produced by the *S. cerevisiae* strains in pure cultures. This behavior is confirming the high competitive ability of *S. cerevisiae* over non-*Saccharomyces* yeast cells, probably due to the depletion of nutrients present in the must, cell-to-cell contact-mediated mechanisms or due to the production of toxic metabolites (Andorrà et al. 2010; Nissen et al. 2003; Pérez-Nevado et al. 2006) and underlines the need to understand better the mechanism of this co-habitation.

In this context, the early growth of *S. bacillaris* in the sequential inoculations with the highest delays (24, 41 and 48 hours) limited the subsequent growth of the two *S. cerevisiae* strains. One possible explanation for this behaviour is that *S. bacillaris* decreased the nutrient concentration by subtracting large quantities of organic nitrogen from the must (data not shown) (Andorrà et al. 2010; Medina et al. 2012). Indeed, since only the Uvaferm BC® 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.*

435 *bacillaris* and *S. cerevisiae*, in order to exclude contributions of indigenous strains. Pilot-
436 scale results confirmed the findings observed in laboratory settings, with the only exception
437 of acetic acid production, which was registered to be higher in pure culture fermentation. The
438 effect of MUT 5705 on wine composition was apparent. As in the laboratory scale
439 fermentation, production of glycerol was higher in the sequential trial than in pure culture
440 fermentation, while ethanol production showed a significant reduction. The pH was also
441 lower and the titratable acidity higher, for wine produced from sequential than this produced
442 from pure culture fermentation.
443 In summary, this study presents a fermentation protocol tested under both laboratory
444 and pilot scale conditions to reduce ethanol levels in wines. This protocol is based on the
445 inoculation of the grape must with *S. bacillaris* MUT 5705 and *S. cerevisiae* Uvaferm BC®
446 after 48 hour from the beginning of the fermentation. Furthermore, the exploitation of this
447 inoculation protocol could be further investigated using other varieties of grape musts, in
448 order to understand the impact of the co-habitation of these species to wine composition in
449 terms of aroma and flavour. In the future, the decrease of ethanol as described here, could
450 help winemakers to decide the appropriate time to harvest their grapes, without the risk of
451 excessive sugar content, which can be converted in high levels of ethanol in wine.
452
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457
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459
460 **Conflict of interest** The authors declare that there is no conflict of interest.
461
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464
465 **References**
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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)

Table 2 Concentration of sugars, glycerol, organic acids and ethanol in the must and wines obtained from pure fermentations of *S. 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]	***	/	/	/	/	/	***	***	***

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

[2]Potential ethanol (% v/v) = ethanol produced + ((residual glucose + residual fructose) * 0.06)

[3]Glycerol yield = glycerol produced / (initial sugar concentration – final sugar concentration)

All data are expressed as average value ± standard deviation (n = 2). Different superscript letters within the same column indicate significant differences among the strains according to the Duncan test ($p < 0.05$)

[4]Sig: *** indicate significance at $p < 0.001$

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

[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 ScBa49, MUT 5705 and ScBa49 and C.z 03 and ScBa49 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

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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Figures legends

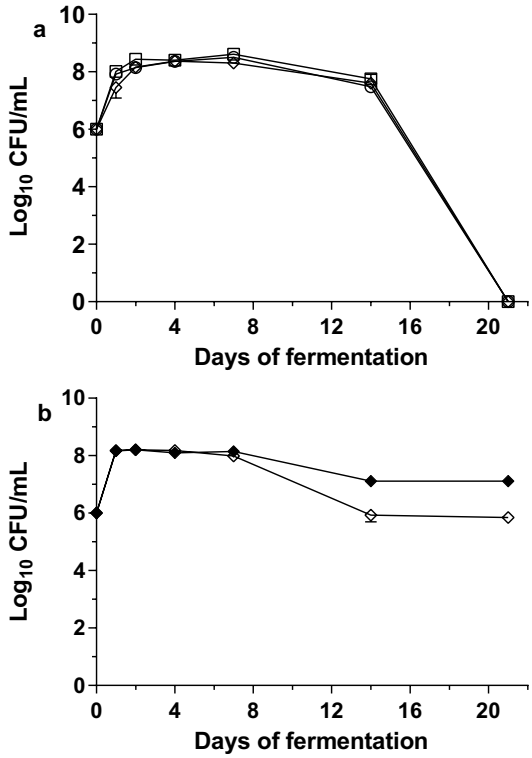
Fig. 1 Growth dynamics of *S. bacillaris* (A) and *S. cerevisiae* (B) strains in pure cultures. *S. bacillaris* strains: FC54 (-○-), MUT 5705 (-◊-), C.z 03 (-□-) and *S. cerevisiae* strains: ScBa49 (-♦-) and Uvaferm BC® (-◊-). Counts are the mean CFU/mL values ± standard deviations of two independent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

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

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

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

682 **Fig.1**



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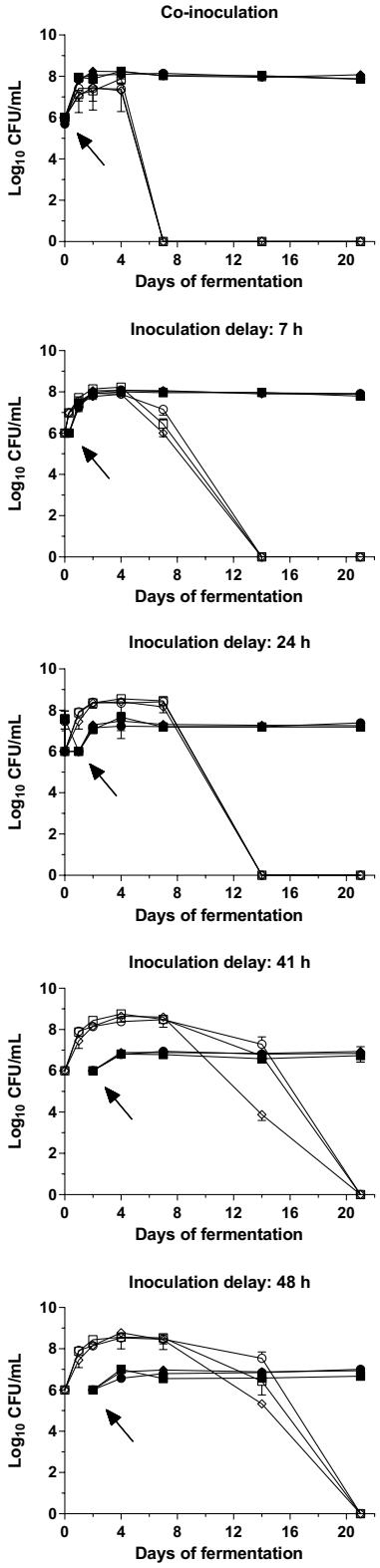
699 **Fig. 2**



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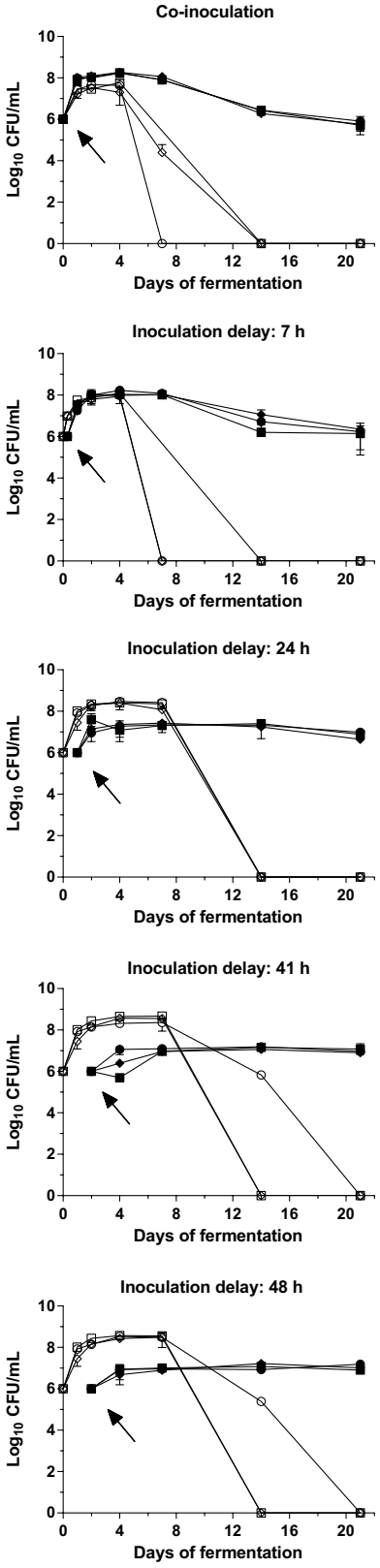
701

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
0
2
4
6
8
10
0
4
8
12
16
20

703

704 **Fig. 4**

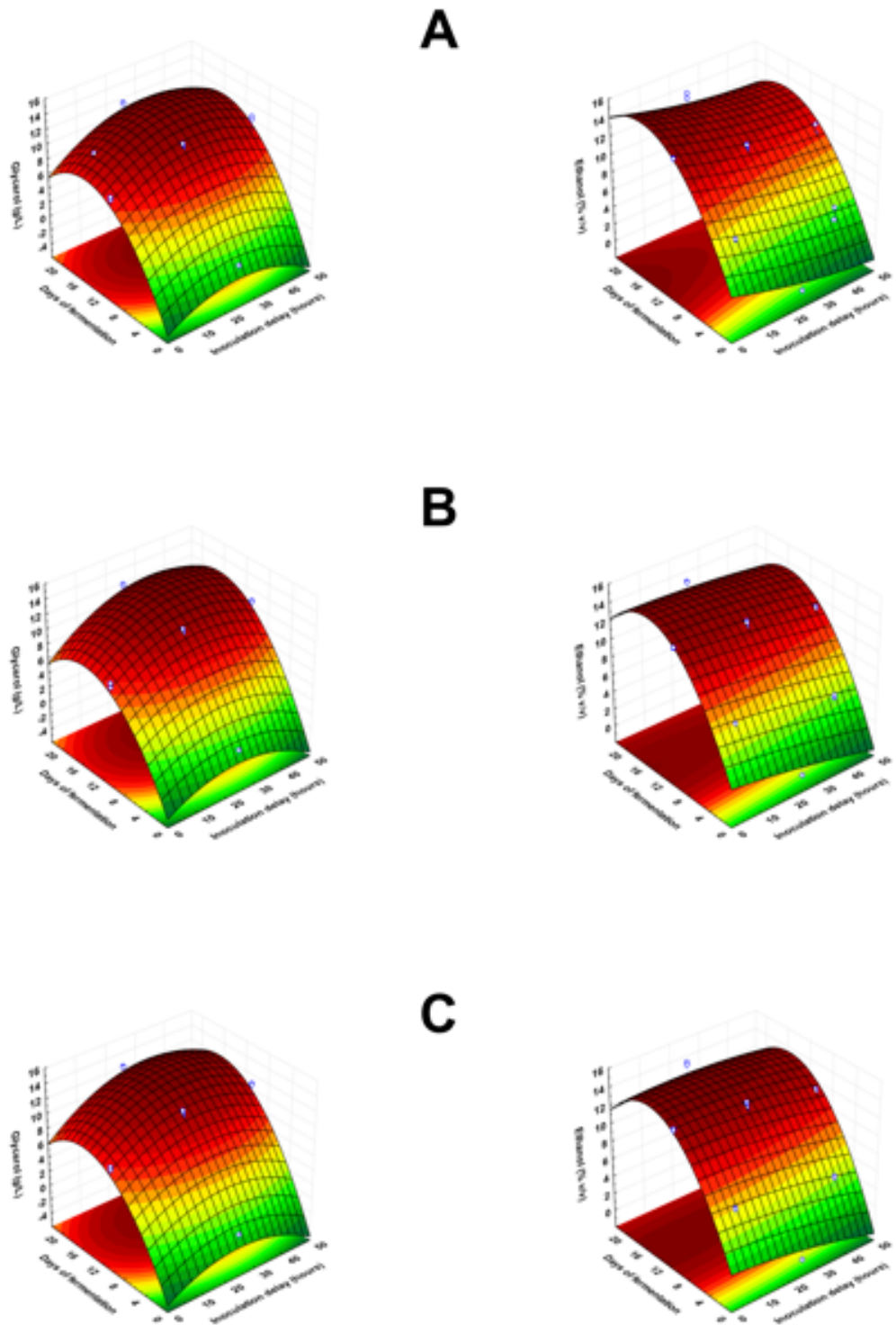


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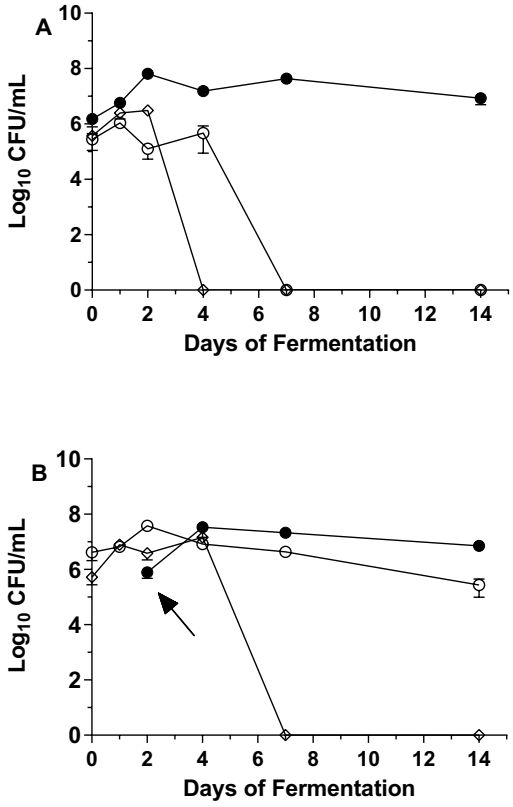
Table S1 Matrix used for the CCD analysis

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

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

759 **FigS1.**



760

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

766