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# Impact of *Starmerella bacillaris* and *Zygosaccharomyces bailii* on ethanol reduction and *Saccharomyces cerevisiae* metabolism during mixed wine fermentations

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**Abstract**

The bulk of grape juice fermentation is carried out by the yeast *Saccharomyces cerevisiae*, but non-*Saccharomyces* yeasts can modulate many sensorial aspects of the final products in ways not well understood. In this study, some of such non-conventional yeasts were screened as mixed starter cultures in a fermentation defined medium in both simultaneous and sequential inoculations. One strain of *Starmerella bacillaris* and another of *Zygosaccharomyces bailii* were chosen by their distinct phenotypic footprint and their ability to reduce ethanol levels at the end of fermentation, particularly during simultaneous vinification. *S. bacillaris* losses viability strongly at the end of mixed fermentation, while *Z. bailii* remains viable until the end of vinification. Interestingly, for most non-*Saccharomyces* yeasts, simultaneous inoculation helps for survival at the end of fermentation compared to sequential inoculation. *S. cerevisiae* viability was unchanged by the presence of the either yeast. Characterization of both strains indicates that *S. bacillaris* behavior is overall more different from *S. cerevisiae* than *Z. bailii*. *S. bacillaris* has a less strict glucose repression mechanism and molecular markers like catabolite repression kinase Snf1 is quite different in size. Besides, *S. cerevisiae* transcriptome changes to a bigger degree in the presence of *S. bacillaris* than when inoculated with *Z. bailii*. *S. bacillaris* induces the translation machinery and repress vesicular transport. Both non-*Saccharomyces* yeast induce *S. cerevisiae* glycolytic genes, and that may be related to ethanol lowering, but there are specific aspects of carbon-related mechanisms between strains: *Z. bailii* presence increases the stress-related polysaccharides trehalose and glycogen while *S. bacillaris* induces gluconeogenesis genes.

**Keywords:** wine; non-*Saccharomyces* yeasts; mixed fermentations; ethanol; transcriptomics; *Starmerella bacillaris*; *Zygosaccharomyces bailii.*

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## 1. Introduction

Producing wine from grape juice in a traditional way relies on spontaneous fermentation carried out by the microorganisms present on the grape surface and the winery equipment (Ribéreau-Gayon et al., 2006). That implies a complex ecological environment where first yeast, filamentous fungi and bacteria coexist, next only some species of yeasts adapted to the acidic and low oxygen environment proliferate and at the end species of *Saccharomyces* take over due to their high fermentative power and tolerance to ethanol to complete fermentation. The non-*Saccharomyces* (NS), so-called non-conventional yeasts, despite their low fermentative power, contribute greatly to the final product (Jolly et al., 2014)(Benito et al., 2019), as these yeasts produce specific metabolites affecting the wine aroma, or produce enzymes that help liberating volatile compound from the grape (Capece and Romano, 2019). The use of non-*Saccharomyces* yeasts as starters might satisfy an additional request of winemakers, as it could be a potential tool for the reduction of alcohol content in wine. Nowadays, the increase of alcohol levels in wine is one of the main challenges affecting the winemaking sector, due to global climate change which determined an increase of grape maturity (De Orduna, 2010). In this context, the interest for reduction of ethanol content in wine was increased and among the available tools addressed to this aim, the microbiological approach appears a promising way. In particular, researchers' interest was addressed to investigate the wide variability in ethanol yield among non-*Saccharomyces* yeasts, that could be a potential tool for the reduction of alcohol content in wine (Gonzalez et al., 2013) (Contreras et al., 2015). Low ethanol yield for instance was found in mixed fermentations with some strains belonging to *Hanseniaspora*, and *Zygosaccharomyces* (Gobbi et al., 2014) and *Starmerella* (Milanovic et al., 2012) genera .

Modern enology has developed pure yeast starters to obtain a more reliable fermentation. The usual way to delivered them is in the form of active dry yeast (Pérez-Torrado et al., 2015). Inoculation with strains of *S. cerevisiae* make the whole fermentation process more robust and reliable, but it lacks some of the richness that more microbiologically complex fermentations have. This aspect contributed to an increased interest on the use of non-*Saccharomyces* yeasts in winemaking (Benito et al.,

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2019)(Padilla et al., 2016) and in the latest years NS yeasts have been added to the portfolio of yeast manufacturers, so dry starters for *Torulaspora*, *Metschnikowia*, *Kluyveromyces*, *Wicheranomyces*, *Lanchacea* and *Schizosaccharomyces* are marketed [7]. Furthermore, some strains that are not so widespread in the market have interesting winemaking properties, but they need more physiological characterization.

Apiculate yeasts *Hanseniaspora* are the main species present on mature grapes and they produce enzymes and aroma compounds that expand the diversity of wine color and flavor (Martin et al., 2018). A strain of *Hanseniaspora uvarum* is able to reduce ethanol in mixed fermentations (Gobbi et al., 2014). *Starmerella bacillaris* (former *Candida zemplinina*) is an interesting species for the enological point of view. It is a fructophilic yeast and a high glycerol producer, but it produces low ethanol after fermentation, both alone and in mixed fermentation (Englezos et al., 2016)(Lemos Junior et al., 2021). It produces high alcohols, such as benzyl alcohol that inhibit fungal growth. However, it is only produced as cream yeast (Roudil et al., 2019). *Zygosaccharomyces* have been regarded as spoilage yeasts due to their high tolerance to osmotic and acidic stress (Escott et al., 2018). *Zygosaccharomyces* found in grapes and musts are able to increase the production of higher alcohol and reduce acetoin. *Z. bailii* produced wine with reduced ethanol concentration (Contreras et al., 2015)(Zhu et al., 2020).

Since most non-*Saccharomyces* yeasts are unable of completing alcoholic fermentation, *S. cerevisiae* strains should be added in simultaneous or sequentially inoculum modality. When planning mixed fermentations, simultaneous inoculation is the simplest way to proceed, but sequential inoculation (first NS and then *Saccharomyces*) give time to the non-conventional yeast to contribute to the final product with no competitions from *Saccharomyces*. Grape must fermentation, even with just one yeast strains inoculated, is a complex ecological system. There are many kind of interactions, both biotic and abiotic, that link the performance of wine yeasts (Ciani et al., 2016). The most obvious of these interaction is the competition for the nutrients of the grape juice, usually the less abundant ones, like amino acids and vitamins (Medina et al., 2012). More direct interactions rely in the production of toxic molecules, like killer factors, antimicrobial peptides and medium-chain fatty acids. Furthermore, direct

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cell-to-cell interactions have been linked to the early death of *T. delbrueckii*, *K. thermotolerans* and S*. bacillaris* by *S. cerevisiae* (Renault et al., 2013)(Nissen et al., 2003)(Englezos et al., 2019b). All those interactions are crossed, so we need more information on the molecular causes behind the metabolic relationships between yeasts that coexist during fermentation.

In this work, a series of potential interesting wine species, were screened in a defined medium for the use as mixed starters with *S. cerevisiae*, by exploring different inoculation regimes in order to establish the inoculum conditions and mixed starter culture that enable the production of wine with reduced ethanol concentration. Two good ethanol-reducing strains (a *S. bacillaris* and a *Z. bailii*) that have an overall impact in metabolites production during fermentations were chosen for a deep analysis of strain physiology. *S. bacillaris* dies at a highest rate than *Z. bailii* in such fermentations. The physiology of those yeasts in selective media happened to be quite different, so the impact of them in the *S. cerevisiae* transcriptomic during mixed fermentations in synthetic grape juice was evaluated to see common and differential impacts. *S. bacillaris* behavior is less similar to *S. cerevisiae* than *Z. bailii*, and cause a greater change in *S. cerevisiae* transcription. Both yeast induce glycolysis, but S. *bacillaris* up-regulates also gluconeogenesis and *Z. bailii* the synthesis of stress polysaccharides like trehalose and glycogen.

## 2. Materials and methods

### 2. 1. Yeast strains

Eight non-*Saccharomyces* yeast strains, belonging to UNIBAS Yeast Collection (UBYC), University of Basilicata (Potenza, Italy), were tested in the first step of this study. The tested strains were the following: one *Debaryomyces polymorphus* (Db2), two *Hanseniaspora uvarum* strains (H3 and H9), four *Starmerella bacillaris* strains (St1, St2, St5 and St8) and one *Zygosaccharomyces bailii* (Zb1). All the strains were previously isolated during spontaneous lab-scale fermentations of grape of different varieties, directly collected in the vineyard, or fruit

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(prickly pear). These strains were identified by restriction analysis of the amplified ITS region (Granchi et al., 1999); the results of restriction analysis were confirmed by analysis of ITS sequences, which were then blasted with NCBI database. In addition, one commercial *S. cerevisiae* strain, EC1118 (Lallemand Inc.), was used.

### 2.2. Stress and growth tests

Strain tolerance to ethanol and $SO_2$, expressed as the ratio between the growth in microplates in broth with (14% v/v ethanol and 150 mg/L $SO_2$ respectively) and without the stress factor. β-glucosidase and β-xylosidase were measured as previously described (Manzanares et al., 2000)(Manzanares et al., 1999). For the spot analysis, stationary cultures grown in YPD medium (2% glucose, 2% peptone, 1% yeast extract) overnight were serially diluted and 5 µl drops were spotted in YPD, YPS (changing glucose by 2% sucrose), YPG (2% glycerol), SD (25 glucose, 0.17% Yeast Nitrogen Base, 0.5% ammonium sulfate) or SPro (SD with 0.5% proline as nitrogen source). Glucose analog 2-deoxyglucose (2DG) was added to the YPS plates at 200 ng/ml for glucose repression tests. For the $H_2O_2$ oxidative stress test, the equivalent of 2 $OD_{600}$ of overnight YPD cultures were spread on YPD plates and paper circles containing 5 circles containing 5 µl of 30% $H_2O_2$ was placed in the middle and diameter of inhibition halos were measured the following day. To test growth in molasses, the biomass propagation experiments were performed in molasses medium (Torrellas et al., 2020) diluted to 60 g/L sucrose. Cells were cultivated at 30 C with shaking (180 rpm) and growth was followed by $OD_{600}$ and ethanol determined enzymatically with a kit (Megazyme International Ireland Ltd).

### 2.3. Mixed fermentations

The selected strains were tested in laboratory scale fermentations using synthetic grape juice medium (Fleet, 1993), indicated in the OIV-OENO 370-2012 resolution. The final

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concentration of sugars was 230 g/L (115 g/L of glucose and 115 g/L of fructose), pH adjusted to 3.5. The fermentations were carried out in sterile 130 mL flasks filled with 100 mL of synthetic must, equipped with stoppers and kept under static conditions at 26°C. The flasks were inoculated with 48-h pre-cultures grown in YPD broth at 26°C with shaking. Each non-*Saccharomyces* strain was inoculated in combination with the *S. cerevisiae* strain in two different modalities, sequential (SeF) and simultaneous (SiF) inoculum. In the SeF trials, the inoculum ratio was 1:1, with an inoculation ratio of $1x10^7$ cells/mL for both the strains, but the non-*Saccharomyces* strain was inoculated at time 0, whereas the *S. cerevisiae* strain was added when the alcohol content reach about 5% (v/v). In the SiF trials, the two strains were simultaneously inoculated, but at different inoculum levels ($1x10^3$ cells/mL for *S. cerevisiae* strain and $1x10^7$ cells/mL for the non-*Saccharomyces* strain). *S. cerevisiae* EC1118 at concentration of $1x10^7$ cells/mL was used as control.

The fermentation kinetics were monitored daily by measuring the weight loss of the flasks (due to the carbon dioxide release) and sugar consumption. The kinetic growth of yeast strains was checked by plate counting of fermenting must samples on two different agar media, Wallerstein Laboratory (WL) Nutrient Agar medium (Sigma-Aldrich) (Pallmann et al., 2001) and Lysine Agar medium (Oxoid Unipath Ltd, Hampshire, UK) with addition of bromocresol green. Dilution plates containing a statistically representative number of colonies were counted.

### 2.4. Analytical Determinations

Experimental wines obtained from the inoculated fermentation were analyzed for conventional chemical parameters, such as ethanol, total acidity, malic and lactic acid, volatile acidity, residual sugars, glucose, fructose, pH, by a Fourier Transfer Infrared WineScan instrument (OenoFoss™, Hillerød, Denmark). The content of the main secondary influencing wine aroma, such as acetaldehyde, n-propanol, isobutanol, amyl alcohols, ethyl acetate and

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172 acetoin, were determined by direct injection gas chromatography of 1 µl sample into a 180 cm
173 × 2 mm glass column packed with 80/120 Carbopack B/5% Carbowax 20 M (Supelco, Bellefonte,
174 PA). The column was run from 70 to 140 °C, the temperature being ramped up at a rate of 7
175 °C/min. The carrier gas was helium at a flow rate of 20 ml/min. Levels of the secondary
176 compounds were determined by calibration lines, as described by Capece et al. (Capece et al.,
177 2013). Analysis of variance (ANOVA) was used to evaluate differences in chemical compounds
178 of the experimental wines obtained by different inoculation modalities, by using Tukey's test to
179 compare the mean values. Principal component analysis (PCA) was carried out on the data of
180 wines produced from mixed starters at laboratory scale. The PAST3 software ver. 3.20
181 (Hammer, Harper, & Ryan, 2018) was used for the statistical analyses.
182
183 **2.5. Western blot**
184 To analyze Snf1 activation and peroxiredoxin status, proteins were extracted by fast cell
185 lysis with trichloroacetic acid (TCA) (Orlova et al., 2008). 5.5% TCA was added to 5 $OD_{600}$ units
186 of cells, the mix was incubated on ice for 15 min and centrifuged. The pellet was washed with
187 acetone twice and resuspended in 150 µL of 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, and broken
188 with 150 µL of 0.2 M NaOH. SDS-PAGE was carried out in an Invitrogen Novex mini-gel device,
189 gel was blotted onto PVDF membranes a Novex semy dry blotter (Invitrogen). The membrane
190 was probed with either anti-AMPKα (Thr172, Cell Signalling Technologies), anti 2-Cys-Prx
191 (Abcam) or anti-Prx-$SO_3$ (abcam) antibodies. Anti-actin (Sigma) was used as loading control. The
192 ECL Western blotting detection system (GE) was used following the manufacturer's instructions
193 in a ImageQuant LAS500 (GE).
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196 **2.6. Transcriptomic analysis**

197 Fermentation were carried out in synthetic must, inoculating simultaneously *S. cerevisiae*
198 always at $1x10^6$ cells/mL and either non-*Saccharomyces* at $1x10^7$ cells/mL. Cells were taken
199 after 24 h of growth. Total RNA was isolated from yeast pellet using the RNeasy mini kit (Qiagen)
200 following the manufacturer's instructions for yeast. RNA quality was assessed by using
201 TapeStation System 4200 (Agilent Technologies, Santa Clara, CA, USA). All samples presented
202 an RNA Integrity Number (RIN) value ≥ 8.00. Libraries were prepared using 1 µg of RNA with
203 TruSeq® stranded mRNA Preparation Kit (Illumina, San Diego, CA, USA) followed by sequencing
204 with a Novaseq 6000 sequencer using 150 base read lengths in paired-end mode (Illumina, San
205 Diego, CA, USA) according to manufacturer's protocol in the facilities of ADM LifeSequencing
206 (Paterna, Spain). Bcl format files were processed to transform them to FASTQ format files using
207 the BCL2FASTQ (version 2.20) software. The reads were trimmed based on their quality,
208 (threshold < Q20) with bbmap software. Adapters and duplicated reads produced by Illumina
209 sequencing were furthermore trimmed with cutadapt v3.0. Clean sequences were mapped
210 against EC1118 Saccharomyces cerevisiae reference genome with SALMON software (Patro et
211 al., 2017). This software maps against the reference genomes and creates a count matrix that
212 will be used in further steps. Differential expression was measured using DESeq2 software (Love
213 et al., 2014) . Enrichment functional analysis of the DEG was carried out using gene ontology
214 (GO) databases implemented in the GENEONTOLOGY api (Mi et al., 2019) which performs
215 enrichment by Fisher's Exact Test and corrects by FDR.
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## 3. Results

### 3.1. Characterization of eight non-*Saccharomyces* strains by mixed fermentations: sequential and co-inoculations.

Eight wild non-*Saccharomyces* strains, belonging to different species and coded with Db2 (*Debaryomyces polymorphus)*, Ha3, Ha9 (*Hanseniaspora uvarum*), St1, St2, St5, St8 (*Starmerella bacillaris*) and Zb1 (*Zygosaccharomyces bailli*), were tested in the first step of the research activity. Table 1 shows the characteristics of enological interest of the selected yeast strains, like enzyme activities and stress tolerance. *H. uvarum* (Ha3, Ha9) and *D. polymorphus* (Db2) have high β-glycosidase and β-xylosidase activities, *Z. bailii* (Zb1) has medium levels, while *S. bacillaris* strains (St1, St2, St5, St8) showed low levels of enzymatic activities, only a low β-glucosidase activity was exhibited by St1 and St2 strains and none β-xylosidase whatsoever. Regarding the other technological parameters, high ethanol tolerance was found in *D. polymorphus* and *Z. bailii*, while *H. uvarum* and *S. bacillaris* strains were more sensitive to this compound. Only the *D. polymorphus* strain showed a low level of sulphur dioxide tolerance, while the rest, as expected for non-*Saccharomyces* yeasts, are very sensitive to this chemical.

Strains were then tested as mixed starters with a commercial *S. cerevisiae* strain in a defined, standardized synthetic grape juice that will make easier transcriptomic analysis (see below). In this step, the fermentative behavior of mixed starter cultures was tested by using two modality of inoculation, simultaneous and sequential inoculum. The fermentation kinetics, represented by $CO_2$ release, of mixed cultures in micro-vinification trials are showed in Fig 1. Regarding simultaneous fermentation (Fig. 1A), *S. cerevisiae* alone is the fastest one, as expected. Next, all three *S. bacillaris* strains behave in a similar way, starting fermentation at day 1. The rest of strains have a slower pace, being *H. uvarum* and *D. polymorphus* quite similar. All the simultaneous fermentations were completed in 14 days, without significant differences among them, except for the mixed fermentation with Zb1+Sc starter, which was completed in 16 days. At the end of the process, the maximum $CO_2$ production was found in the fermentation inoculated with St1+Sc starter (about 13.01 g/100 mL), whereas the lowest amount (about 11.99 g/100 mL) was detected in the fermentation inoculated with Ha9+Sc starter. A similar trend for

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246 $CO_2$ production was observed in sequential fermentations (Fig. 1B), with constant increase in $CO_2$
247 production from the first fermentation day for all non-*Saccharomyces* strains, although clearly slower
248 than *S. cerevisiae* alone. After the fourth fermentation day, it was observed a high increase of $CO_2$
249 production, as a consequence of the addition of *S. cerevisiae*. The amount of $CO_2$ produced at the end
250 of fermentation was similar in all the trials, ranging between 13.91 and 15.17 g/100 ml, with highest
251 production found for fermentation inoculated with St8+Sc starter. The duration of fermentation was
252 higher for mixed cultures than for single starter culture; in fact, all the mixed starters completed the
253 process in 15 days, with similar trend all the trials, whereas fermentation inoculated with pure culture
254 of *S. cerevisiae* (control fermentation) ends the process at day 11. The evolution of sugar consumption
255 during the mixed fermentations (Supplementary Fig. S1), reflects the same trend observed for $CO_2$
256 production, as expected.

257 The studies on yeast population dynamics during inoculated fermentation with mixed starter
258 cultures will help in understanding the interactions between yeast strains (Fig. 2). The persistence level
259 of non-*Saccharomyces* strains during the mixed fermentations was variable in function of yeast
260 strain/species and inoculation modality. In fact, in all the fermentations the presence of non-
261 *Saccharomyces* strains at the end of the process was higher in simultaneous inoculum than sequential
262 inoculum, except for fermentations inoculated with *H. uvarum* strains, in which no differences between
263 two modalities of inoculum were found. In mixed fermentations inoculated with *D. polymorphus* strain
264 (Fig. 2A), in simultaneous modality cells decrease steadily, while in the sequential inoculum, Db2 strain
265 reached a maximum of yeast cells ($1.6 \times 10^8$ UFC/mL) in 3 days, after that the viable count decreased
266 and at the 10th day of the fermentation no *D. polymorphus* cells were found. The evolution of yeast cells
267 of *H. uvarum* strains (Ha3 in Fig. 2B, Ha9 in Supplementary Figure 2) during the process followed the
268 same trend in both the inoculum modalities, with a decrease of yeast cells after 4 days of fermentation.
269 Furthermore, after 10 days of fermentation, no *H. uvarum* colonies were found on plates. In mixed
270 fermentations with *S. bacillaris* strains (St8 in Fig. 2C, rest in Supplementary Fig. 2), for simultaneous
271 inoculum a slight increase of cell count was observed in the first two days of fermentations, after that

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cell count slightly was reduced and at the end of the process a number of viable cells ranging between 2 x $10^2$ and 3.4 x $10^3$ UFC/mL was found. Regarding fermentations inoculated in sequential modality, for St8 (Fig. 2C) and St5 (Supplementary Figure 2) strains, in the first days of fermentation a trend similar to simultaneous inoculum was found, with an increase of viable cells, whereas after the third fermentation day the number of viable cells decreased and at the end of the process no *S. bacillaris* cells were found. Regarding mixed fermentation with *Z. bailii* (Figure 2D), in simultaneous inoculum, the number of Zb1 cells remains constant in the first two days of the process, after that a reduction in number of viable cells was observed, although a number quite high of viable cells was found at the end of the fermentation (6 x $10^6$ cells/mL). For sequential inoculum, during the first four days, *Z. bailii* cells increased, after that a high reduction of number of viable cells is observed and in the final wine the number of *Z. bailii* cells was about 1 x $10^5$ cells/mL. As regards the evolution of *S. cerevisiae* EC1118 population in mixed fermentations, similar cell count was observed in both the inoculum modalities and the evolution of *S. cerevisiae* cells in pure culture, used as control, reflects the typical growth kinetic, with the presence of high cell number until the end of the fermentations.

### 3.2. Analysis of experimental wines obtained from mixed starter cultures

The experimental wines were analysed for oenological parameters and main volatile compounds and the data are shown in Tables 2 (simultaneous trials) and 3 (sequential trials). As already reported, all the starter cultures completed the fermentations. Regarding co-inoculum, all the samples from mixed fermentations contained an ethanol concentration lower than control sample (Figure 3A), with values ranging from 11.58 to 12.19 % (v/v), whereas the ethanol content of wine from single fermentation was 12.38 % (v/v). As regards volatile acidity, the activity of non-*Saccharomyces* strains did not increase it; in fact, the volatile acidity of samples inoculated with mixed starters was lower or very similar to level detected in control wine, except for the mixed fermentations inoculated with Db2+Sc and Ha3+Sc starters. The experimental wines were analysed also for the content of volatile compounds usually present in high quantity in wines, and involved in the wine flavour. The content of acetaldehyde ranged between 38.72 mg/L (Zb1+Sc) and 65.95 mg/L (Ha9+Sc), being the latter the only case where more

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298 acetaldehyde is produced compared with the control. The ethyl acetate was found in concentrations
299 ranging from 7.71 mg/L (St8+Sc) to 28.95 mg/L (Db2+Sc), with higher levels than the control when *D.*
300 *polymorphus* and *H. uvarum* were used. Generally, mixed starters produced experimental wines
301 characterized by lower amount of alcohols (n-propanol, isobutanol, amyl alcohols) than the wine from
302 *S. cerevisiae* EC1118 strain, used as control, except for n-propanol. The highest difference between
303 single and mixed starter wines was found for fermentation performed by mixed starter including *S.*
304 *bacillaris* strains, which contained lower amounts of both D-amyl and isoamyl alcohols than
305 experimental wine fermented with EC1118 strain.

306 The samples obtained by mixed starter sequentially inoculated with EC1118 strain contained
307 lower ethanol than control experimental wine, as already found for co-inoculation (Figure 3A). The
308 lowest level of volatile acidity was found in experimental wine obtained by H9+Sc starter (0.69 mg/L), a
309 result very surprising as apiculate yeasts are known to be high producers of acetic acid, whereas in the
310 other samples volatile acidity ranged between 0.91 and 1.18 mg/L. Regarding acetaldehyde, this
311 compound was detected in the range between 64.38 (Zb1+Sc, well above the control fermentation) and
312 27.75 mg/L (St1+Sc), whereas the ethyl acetate content ranged from 6.85 mg/L (St1+Sc, below control)
313 to 28.14 mg/L (Db2+Sc, above control). All mixed cultures produced wines containing slightly higher
314 amount of n-propanol, than the wine from *S. cerevisiae* strain. Regarding the levels of the other higher
315 alcohols detected (isobutanol and amyl alcohols), control wine contained higher level of these
316 compounds than samples from mixed starters. Although these compounds represented the most
317 abundant groups in all the analysed samples, all the starters produced an amount of high alcohols lower
318 than 300 mg/L, which is within the acceptable level for these compounds.

319 The ability of mixed starter to reduce the ethanol content was calculated as ratio between ethanol
320 produced by *S. cerevisiae* pure culture and ethanol produced from each mixed starter (Fig. 3A). All the
321 mixed starters, in both inoculum modalities, determined an ethanol reduction, as already reported. The
322 reduction level was higher for simultaneous than for sequential inoculum for all the starters, except
323 Db2+Sc, in which a slightly higher reduction was found in sequential inoculum. In fermentations using

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simultaneous inoculations, the ethanol reduction ranged between 0.19 (Ha9+Sc) until 0.80 (Zb1+Sc), whereas in sequential inoculation the starter ability to reduce the ethanol content ranged between 0.09 (St2+Sc) and 0.45 (St8+Sc).

All the parameters determined in the experimental wines were submitted to Principal Component Analysis (PCA). The plot of all the experimental wines on the plane defined by the first two components is shown in Fig. 3B. The two principal components, PC1 and PC2, accounted for 58% of the total variance (36 and 22%, respectively). The PC1 was positively correlated mainly with D-amyl and isoamyl alcohols and negatively mainly associated with n-propanol, whereas the PC2 was mainly positively related to content of ethanol and residual fructose and negatively with total acidity and isobutanol. This analysis allowed to differentiate the experimental wines in function of inoculation modality; in fact, almost all the samples obtained with non-*Saccharomyces* strains inoculated simultaneously with *S. cerevisiae* strain are located in upper part of the scatterplot (except St8+Sc and Zb1+Sc, Figure 3B), whereas all the experimental wines obtained by sequential inoculum are grouped together in the lower part of the scatterplot. The only exception is represented by the two wines obtained with mixed starter including *Z. bailii* strain, which were located very near, in both the inoculation modalities. As expected, the experimental wines obtained with pure culture of EC1118 strain are located in the same quadrant of the plot. Furthermore, as regards the wines obtained by using the different *S. bacillaris* strains, in both the inoculation modalities the experimental wine grouped very near and far from the others, with exception of wine fermented with starter including St8 strain, which was located in a quadrant different from the wines fermented with mixed starters including St1, St2 and St5.

### 3.3. Physiological study of strains of interest

Next, the experiments were focused on two strains, *S. bacillaris* St8 and *Z. bailii* Zb1. These strains were selected as they determined relatively high ethanol reduction during laboratory scale fermentations and a big impact in overall metabolites after fermentation (Fig. 3). To further characterize them, they were tested in different growth conditions (Fig. 4), like different carbon sources (Fig. 4A). In

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standard, rich, glucose-containing medium YPD, all three strains grow fine, being *Z. baill*i a little bit slower. Similar results were obtained when sucrose (YPS) was used as carbon source. In such medium, glucose repression mechanisms can be tested, using a non-metabolizable glucose analog, 2-deoxyglucose (2DG); in this case a strong repression of growth was observed for *Z. bailli* and also (as expected) in *S. cerevisiae*. Interestingly enough, *S. bacillaris* is immune to this inhibitor, indicating that the mechanism of standard catabolite repression does not work for this yeast. When the carbon source was glycerol, a fully respiratory source, *S. cerevisiae* is the only one that grows well, and the other two strains struggle. This poor respiration may indicate poor management of oxygen radicals and oxidative stress sensitivity, but no high differences in tolerance to the oxidant $H_2O_2$ were found (Fig. 4B). In fact, *S. bacillaris* is slightly better equipped to deal with this stress than *S. cerevisiae*. Last, nitrogen sources were tested. In the standard minimal, ammonium-containing medium SD, all three strains were able to growth. Ammonium is a rich nitrogen source, but neither in poor proline (SPro), there are no big differences, although *S. bacillaris* is slightly delayed compared to ammonia.

Another tested condition was the growth of the three strains in sugar beet molasses (Fig. 4C), the media that is used for biomass propagation. In such conditions, *S. cerevisiae* proved to be the fastest one in terms of growth. Both non-*Saccharomyces* yeasts grow slower and reached a lower $OD_{600}$ at saturation point. However, they assimilate sucrose (that is the main sugar in molasses) well, as it disappeared for all strains after 24 h (data not shown). Ethanol produced can give us some ideas about their respiratory metabolism (Fig. 4D). The peak of ethanol was observed early, at the first day, when *S. cerevisiae* produce slightly more ethanol than the other two strains, after that *S. cerevisiae* and *Z. bailli* consume efficiently ethanol during the postdiauxic phase, while *S. bacillaris* does it more inefficiently, indicating again that is the one with a more distant phenotype in metabolic terms.

### 3.4. Molecular analysis of mixed fermentations in synthetic grape juice

Our next goal is to understand the molecular behaviour behind mixed fermentation, analysing gene expression by transcriptomic analysis and performing biochemical analysis of molecular markers

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of stress response and nutrient signalling. The goal was to evaluate the impact of the non-conventional yeasts on *S. cerevisiae*, so simultaneous inoculation was chosen to allow this interaction from the beginning, plus it induces a bigger ethanol difference (Fig. 3A), but with different inoculation ratios to have more *S. cerevisiae* biomass for RNA and protein extraction. *S. cerevisiae* was inoculated at concentration of $10^6$ cells/ml (higher than before to be able to have enough cells at shorter times), while *S. bacillaris* and *Z. bailli* were inoculated tenfold to be in excess, at $10^7$ cells/ml, so there is an initial excess of non-conventional yeasts, but the amount of *S. cerevisiae* is significant. The overall course of fermentation was followed by $CO_2$ production (Fig. 5A) and reducing sugar measurement (Fig. 5B). All fermentations finished, but the mixed fermentations are complete slightly faster than the one with only *S. cerevisiae*, probably due to the smaller inoculum of the latter. Again, experimental wines from mixed fermentation showed lower ethanol as seen in natural grape juice (Fig. 5C). Cells were diluted and spread in selective media to quantify the evolution of each one (Fig. 5D). *S. bacillaris* grows during the first 24 hours, and at this time *S. cerevisiae* reaches a similar cellular density. From that point, the viable numbers of *S. bacillaris* decline, and at 144 h there are no detection of this strain, while *S. cerevisiae* keeps it viability. *Z. bailli* has a different growth profile. It reaches the maximum population level later, at 48 hours, and then the viability is reduced, but it survives longer than *S. bacillaris* did. *S. cerevisiae* with *Z. bailli* grows vigorously and remain high, as was seen with *S. bacillaris*. In fact, both have a similar profile than *S. cerevisiae* alone, so the non-*Saccharomyces* do not offer a serious problem for growth as previously shown. Supplementary Table S1 shows the final concentration of metabolites of such fermentations.

Samples were taken at day 1, 2 and 3 to extract proteins and to check molecular markers (Fig. 6). First to check for stress response, the oxidative stress protein peroxiredoxin Tsa1 was detected using a specific antibody. A positive control was including consisting in cells grown in rich medium YPD and challenged with oxidative stress in the form of hydrogen peroxide. In those conditions, *S. cerevisiae* and *Z. bailli* showed a clear induction of the protein, indicating that it is reacting to that stress. However, *S. bacillaris* does not produce a reaction to this antibody, so its Tsa1 is not detected in this experiment. In

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the fermentation with *S. cerevisiae* alone, Tsa1 goes up until 48 h and then its levels are reduced. As *S. bacillaris* does not give signal, the profile in Sc+Sb (*S. cerevisiae* + *S. bacillaris*) comes only from *S. cerevisiae*. In this case, there is a higher level at longer times, indicating a more stressful environment in this mixed fermentation. In Sc+Zb (*S. cerevisiae* + *Z. bailii*) fermentation a strong Tsa1 signal was observed, similar to the oxidative stress cells, so one of them, or both, are in a non-optimal position. There is an antibody that reacts to sulfinilated Tsa1, indicating a hyperoxidaton caused by big stress, but this response was observed in presence of $H_2O_2$, which is not the case of grape juice fermentation, indicating that there is not a strong, irreversible oxidative stress during winemaking, including mixed fermentations. Next, an antibody against the phosphorylated form of AMPK kinase Snf1 was used. That marks the activity of kinase, due to starvation or stress. In this case, *S. bacillaris* gave also signal, but with a different size, in the oxidative stress test. *S. cerevisiae* and *Z. bailli* are again very similar. As seen before (Vallejo et al., 2020), during fermentation, Snf1 is activated very early and its activation decreases later on, at day 3. In the Sc+Sb mixed fermentation the pattern for *S. cerevisiae* is similar than the single fermentation, and in the case of *S. bacillaris'* band, the pattern is similar, but it goes down faster, at day 2. In Sc+Zb, both species cannot be discriminated, but the overall pattern is similar, with early induction and decrease over the next time points. However, an extra band appears suggesting some interactions causing some event of posttranscriptional modification that is altering Snf1 in possible one of those strains. Overall, *S. bacillaris* molecular markers are quite distinctive than the other two species.

### 3.5. Transcriptomic analysis of *S. cerevisiae* under mixed fermentations

Next, a transcriptomic analysis of *S. cerevisiae* gene expression in the presence of the other two yeast species was carried out, in order to see the common and differential impact of *Z. bailii and S. bacillaris*. Considering fermentation advance and growth in synthetic grape juice (Fig. 5), cells were collected at day 1 of fermentation. At this point, *S. cerevisiae* is actively growing and its gene expression

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427 machinery should be functioning at maximum capacity, while non-*Saccharomyces* have been present in
428 high numbers and also metabolically active, and the amount of all yeasts is similar (Fig. 5D). Three
429 samples were taken: *Z. bailii*+ *S. cerevisiae, S. bacillaris* + *S. cerevisiae* and *S. cerevisiae* alone. *S.*
430 *cerevisiae* genes levels were compared among the different samples, giving three comparisons,
431 represented as volcano plots in Fig. 7. At first glance, the presence of *S. bacillaris* (panel 7B) has a bigger
432 impact on *S. cerevisiae* than *Z. bailii* (panel 7A). Fig. 7C shows the genes that are differentially expresses
433 between both mixed fermentations. There are 498 *S. cerevisiae* genes upregulated at least two times
434 in Zb+Sc compared to Sc, and 179 genes downregulated (Supplementary Table S2). Among them, some
435 functional gene ontology categories of biological processes are overrepresented (Table 4 and
436 Supplementary Table S3). Many are involved in metabolism, like “de novo' NAD biosynthetic process
437 from tryptophan”, “glycolytic process”, “trehalose metabolic process”, “glycogen metabolic process”
438 and many more devoted to energy production and storage. Therefore, it seems that *Z. bailii* is competing
439 with *S. cerevisiae* for resources, causing a general activation of metabolism. Of particular interest is the
440 increase of glycolysis in a fermentative situation. Many glycolytic genes are up-regulated, for instance
441 all for glyceraldehyde-3-phosphate dehydrogenase isozymes, *TDH1*, *TDH3* and particularly *TDH2*.
442 Alcohol dehydrogenase I *ADH1* is also activated, so that would mean than *S. cerevisiae* is fully producing
443 ethanol, so any alcohol reduction is happening at a different level. “Cortical actin cytoskeleton
444 organization” and “fungal-type cell wall organization” indicate some structural stress in terms of
445 cytoskeleton and cell wall that have to be compensated. There are 179 genes downregulated 2-fold or
446 more. No GO category is over-represented among them, probably because 124 of them are of unknown
447 function, indicating that not so-well defined processes may be in place.

448 The impact of *S. bacillaris* in numerically *S. cerevisie* is more intense (Fig. 7B). There are 1063 genes
449 relatively up-regulated compared to the control fermentation. Again, genes involved in hexose
450 metabolism, glycolysis/gluconeogenesis, are up regulated, suggesting competition for sugars (Table 2).
451 Many biological function categories are ribosome related, like “rRNA export from nucleus” and
452 “cytoplasmic translation”, indicating that *S. bacillaris* is inducing an increase in protein synthesis in *S.*

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*cerevisiae* in a specific way. 1032 genes are downregulated, and interestingly there is overrepresentation of genes involved in intracellular transport, like "cytoplasm to vacuole transport by the Cvt pathway", "protein targeting to vacuole ", "endoplasmic reticulum to Golgi vesicle-mediated transport" and so on. Therefore, many aspects of the vesicular transport are repressed, so the increase of translation does not seem to be targeted for secretion or membrane targeting.

Next, a direct comparison between *Zb*+*Sc* and *Sb*+*Sc* was made (Fig. 7C). This way, the common effects caused by competition with any yeast will be ruled out, and specific effects will be highlighted. There are 773 *S. cerevisiae* genes that up-regulated in the presence of *Z. bailii* compared to *St. bacillaris* (or down-regulated in *St. bacillaris* compared to *Z. bailii*) (Supplementary Table S2). Conversely, there are 624 genes down-regulated in the presence of *Z. bailii vs St. bacillaris.* Regarding GO analysis, among the categories overrepresented in the up-regulated *S. cerevisiae* genes in the Zb vs Sb "late nucleophagy" and "autophagy of mitochondrion" (Supplementary Table S3) are present, including many *ATG* genes devoted to autophagy. Many of those genes were downregulated in Sb+ Sc vs Sc, so *S. bacillaris* has the ability to repress autophagy processes in *S. cerevisiae*. Amino acid metabolic categories as "homoserine metabolic process", "cysteine metabolic process" and "methionine biosynthetic process" are also present in the analysis. Finally, many related categories involving ion transport, particularly iron (iron ion transmembrane transport, copper ion transport, cellular iron ion homeostasis…) are present. These involved many siderophores (*ARN1, ARN2, SIT1*), ferric reductases (*FRE1, 2,3* and *5*), iron oxidase (*FET3*) and iron permease (*FTR1*). Siderophore genes are indeed down-regulated in the presence of *S. bacillaris*, while *Z. bailli* has no effect on them. Interestingly, there are two genes of cell wall mannoproteins involved in the retention of siderophore-iron in the cell wall, *FIT2* and *FIT3*, that are repressed in the presence of *S. bacillaris*, but induced in the presence of *Z. bailii*, so they show big differences when data are compared directly (Fig 7C). It seems that *S. bacillaris* provide an excess of iron in the form of siderophores that *S. cerevisiae* sense, reducing their mechanisms of uptake. Last, the 624 genes down-regulated in the presence of *Z. bailii vs S. bacillaris* are enriched in genes involved in rRNA metabolism and translation. The glycolytic genes did not appear in that kind of comparison, so it seems clear that its

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479 induction is a common feature in the presence of an alien yeast, probably due to nutrient competition
480 rather than direct interaction.

481

482 **4. Discussion**

483 Complex ecological interactions happen inside spontaneous grape juice fermentations. In this
484 work we tried to analyse such information simplifying the interaction between two yeast species in a
485 standardized growth medium that mimics grape juice, and allow us to perform global analysis, such
486 transcriptomic studies, and protein analysis in a controlled environment. An initial screen using eight
487 different strains and two inoculation strategies (simultaneous and sequential) allowed the selection of
488 strains and conditions. The presence of additional strains other than *S. cerevisiae* always delay
489 fermentation progression, even from the very beginning, when most nutrients are in theory not
490 exhausted and without a decrease in *S. cerevisiae* viability (Fig. 2 and 5). Therefore, metabolism should
491 be affected by the cohabitation with another kind of yeast. Analogous results on the time courses of
492 sequential fermentations were recently found by several authors. Englezos et al. (Englezos et al., 2019a)
493 reported that a sequential fermentation with *S. bacillaris* and *S. cerevisiae* in white grape must took 14
494 days to finish, while 9 days were needed for the single inoculation with *S. cerevisiae*. Although fast and
495 reliable completion of fermentation are of primary importance in the wine industry, the advantages of
496 the use of non-*Saccharomyces* yeasts in mixed fermentations is thought to compensate the slower
497 fermentation with the advantages in wine quality (Hranilovic et al., 2018). Moreover, a slow
498 fermentation kinetics could be considered as positive for a better retention of volatile compounds
499 (Medina et al., 2013). Our results confirmed that the use of non-*Saccharomyces* strains affected wine
500 characteristics; in fact, the experimental wines from mixed starters differed from single starter wine (Fig.
501 3). In the case of simultaneous inoculum, the wines obtained by using *S. bacillaris* and *Z. bailii* strains
502 differed more from single starter wine than *H. uvarum* (Fig. 3B). Viability loss could be the cause for *H.*
503 *uvarum* low impact (Fig. 2). This behaviour could be due to the low competition of these species, which

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was increased in the case of the delayed inoculation of the *S. cerevisiae* strain, as reported by other authors for different non-*Saccharomyces* species (Wang et al., 2016) (Gobbi et al., 2014). *H. uvarum* is more sensitive to death at the end of fermentation than *S. bacillaris* measured with direct *in situ* fluorescent hybridization (Wang et al., 2014). This phenomenon could have numerous explanations. The loss of viability of the non-*Saccharomyces* in the mixed fermentations can be related to production of yeast metabolites, such as ethanol, medium chain fatty acids and acetaldehyde. Direct cell-to-cell contact has been proved for the pair *S. bacillaris*-*S. cerevisiae* (Englezos et al., 2019b).

We have focused on two strains, a *S. bacillaris* and a *Z. bailli* for their deeper impact of fermentation and higher ethanol reduction (Fig. 3), in order to get a deeper understanding of the interaction between different yeast species. Both strains were able to reduce the ethanol level of the wine (Tables 2 and 3), but they are very different regarding other parameters. *S. bacillaris* loses viability at the end of fermentation much faster than *Z. bailii* during wine fermentation (Figure 2 and 5), it is insensitive to glucose repression (Fig. 4A) and both are less adapted to respiratory metabolism than *S. cerevisiae* (Fig. 4A). *Z. bailii* viability remains high (Fig. 2), but it is the strain that leads to a slower fermentation (Fig. 1). Therefore, these strains may induce low ethanol production by similar means or by mechanisms completely different. Genetically it is known that *S. bacillaris* lies further away than the two others (Masneuf-Pomarede et al., 2016). Molecular markers of *S. bacillaris* are less similar to *S. cerevisiae* than *Z. bailli* (Fig. 6). Glucose derepression kinase Snf1 is more similar in size between *S. cerevisiae* and *Z. bailli*, while in *S. bacillaris* it is smaller. However, the pattern of induction is quite similar, although targets may differ. Genomic analysis of two strains of *S. bacillaris* suggests a regulation of the fermentation/respiration processes that is partially different from that of *S. cerevisiae* (Lemos Junior et al., 2018), although *SNF1* gene is present. A reason for ethanol reduction could be the respiratory metabolism of non-*Saccharomyces* yeasts. However, *Z. bailli* has been described as a Crabtree positive yeast (Rodicio and Heinisch, 2009) and species of *Z. bailii* and *S. bacillaris* have be found to have a respiratory quotient higher than *S. cerevisiae* (Quirós et al., 2014). Therefore, it seems that those are not respiratory yeasts that lower ethanol by respiration. The fact that simultaneous fermentations

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530 generally lower ethanol more that sequential inoculations (Fig. 3A) may indicate that the main effect of
531 those yeast is the modulation of *S. cerevisiae* fermentative performance. Therefore, it was important to
532 see the effect on *S. cerevisiae* transcriptome.

533 The impact of *S. bacillaris* in *S. cerevisiae* transcriptome is more conspicuous than the one of *Z.*
534 *bailii* (Fig. 7). Interestingly, in the presence of both strains, *S. cerevisiae* increases the transcription of
535 genes related to hexose metabolism (Table 4), up-regulating most of glycolytic genes. This behaviour
536 may be seen being in contradiction with an ethanol decrease, but it may indicate that *S. cerevisiae* is
537 struggling to achieve all the energy required and it is gearing up this pathway. A similar behaviour in
538 glycolysis transcription has been seen in mixed fermentations with *M. pulcherrima* in aerobic conditions
539 (Mencher et al., 2021), although this yeast represses also aerobic respiration, unlike the two species
540 tested in synthetic grape juice fermentation. Stimulation of nutrient consumption seems to be a
541 common trend in co-cultivation with many non-conventional yeast (Curiel et al., 2017). However, there
542 are some molecular signatures that may indicate specific ways of regulation of carbon metabolism in
543 our selected strains. In the presence of *Z. bailli*, *S. cerevisiae* increase significantly the GO categories of
544 trehalose and glycogen synthesis, inducing the main genes involved in trehalose (trehalose synthase
545 genes *TPS1, TPS2, TSL1*, Supplementary Table S2) and glycogen (glycogen synthase genes *GSY1* and
546 *GSY2*). Genes involved in glycogen degradation like glycogen debranching *GDB1* and glycogen
547 phosphorylase *GPH1* are also induced, suggesting a potential futile cycle. If *S. cerevisiae* diverts part of
548 glucose to the accumulation of reserve and stress tolerance polysaccharides, that may reduce the
549 amount of glucose to be fermented into ethanol. In fact overexpression of trehalose synthesis in wine
550 yeasts leads to reduction of ethanol (Rossouw et al., 2013). *S. bacillaris* increases in a specific way the
551 genes of the GO "gluconeogenesis" (Table 4). Those are basically glycolytic genes, but gluconeogenic
552 specific genes like pyruvate carboxylase *PYC1*, phosphoenolpyruvate carboxykinase *PCK1* and fructose-
553 1,6-bisphosphatase *FBP1* are up-regulated, and that may be causing two futile cycles that may be
554 consuming energy with no gain, reducing the ethanol yield per glucose molecule. A common glycolytic
555 enzyme induced by the two yeasts is the glyceraldehyde 3-phosphate dehydrogenase coded by *TDH1-3*

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556 genes. This enzyme has been defined as a cell wall component (ML et al., 2001) and some of it fragments
557 have been described as antimicrobial peptides against a variety of yeasts (Branco et al., 2014), so it may
558 be behind death of the non-*Saccharomyces* yeast, particularly *S. bacillaris* by cell-to-cell interactions
559 (Englezos et al., 2019b).

560 The transcriptomic analysis reveals some specific interactions. For instance, the presence of *S.*
561 *bacillaris* repress genes involved in iron assimilation. Interestingly genes of siderophores (*ARN1, ARN2,*
562 *SIT1*) and particularly two genes of cell wall mannoproteins involved in the retention of siderophore-
563 iron in the cell wall, *FIT2* and *FIT3* (Fig. 7C) are among them. In synthetic grape juice there are no
564 siderophores, and it is known that *S. cerevisiae* do not produce them (Martínez-Pastor and Puig, 2020).
565 However, it has been reported the acquisition by some *Starmerella* yeasts of a bacterial operon to
566 synthetize the siderophore enterobactin by horizontal gene transfer (Kominek et al., 2019). The
567 presence of an excess of siderophore may be detrimental, as an excess of iron could trigger oxidative
568 stress, so *S. cerevisiae* prevents its import. The upregulation of *S. cerevisae*'s protein synthesis and down
569 regulation of vesicular transport by the presence of *S. bacillaris* are indicating a profound rearrangement
570 of biosynthetic processes that would require a careful and complex analysis.

571

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741 **Figure legends**

742 Fig. 1. Fermentation kinetics of mixed starters cultures of D. *polymorphus* (Db2), *H. uvarum* (Ha3 and
743 Ha9), *S. bacillaris* (St1, St8, St2 and St5) and *Z. bailii (*Zb1) strains simultaneously A) or sequentially B)
744 inoculated with *S. cerevisiae* EC1118 (Sc). Pure culture of S. cerevisiae EC1118 (Control Sc) was used as
745 a control. Data are means ± standard deviation of two independent experiments.

746

747 Fig. 2. Evolution of yeast populations in mixed fermentations inoculated with *S. cerevisiae* (Sc) and *D.*
748 *polymorphus* (Db2, A)), *H. uvarum* (Ha3, B)), *S. bacillaris* (St8, C)) and *Z. bailii* (Zb1, D)) in simultaneous

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749 and sequential modalities. Values are mean of two independent duplicates. Pure culture of *S. cerevisiae*
750 EC1118 (Control Sc) was used as a control. * indicate the inoculation time of *S. cerevisiae.*

751

752 Fig. 3. Metabolite analysis of mixed fermentations. A) Starter ability to reduce the ethanol content in
753 experimental wines obtained by simultaneous (green) and sequential (orange) inoculation as ratio
754 between ethanol produced by *S. cerevisiae* pure culture and ethanol produced from each mixed starter.
755 B) Principal component analysis (PCA) biplot of the oenological parameters and main volatile
756 compounds detected in experimental wines obtained by simultaneous (SiF) (black) and sequential (SeF)
757 (red) inoculum of selected non-*Saccharomyces* strains. Pure culture of *S. cerevisiae* EC1118 (Control Sc)
758 was used as control.

759

760 Fig. 4. Physiological study of strains of interest. A) Spot analysis of growth on different carbon and
761 nitrogen sources. *Z. bailii* (Zb), *S. bacillaris* (Sb) and *S. cerevisiae* (Sc) strains were grown overnight in
762 YPD liquid to stationary phase, serial dilutions were made and 5 µl drops were spotted in YPD, YPS
763 (sucrose), YPS containing 200 ng/ml 2-deoxyglucose (2DG), YPG (glycerol), SD (minimal medium with
764 ammonium as sole nitrogen source), SPro (minimal medium with proline as sole nitrogen source). B)
765 Oxidative analysis of those strains. Overnight cultures form YPD were plated on YPD plates and a paper
766 circle containing 5 µl drops of hydrogen peroxide was placed in the middle. Inhibition halos were
767 measured the next day. Experiments were carried in triplicate and mean and standard deviation is
768 shown. C) Growth in beet molasses followed by $OD_{600}$ measurement. D) Ethanol measurement from the
769 growth curve showed in panel C). Experiments carried out in triplicates and average and standard
770 deviation is shown.

771

772 Fig. 5. Mixed fermentation of selected strains for molecular analysis. *S. cerevisiae* (Sc) alone and mixed
773 fermentations with *S. bacillaris* (Sb+Sc) or *Z. bailii* (Zb+Sc) were carried out in synthetic grape juice. A)

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774 Weight loss of the fermentation. B) Reducing sugars during fermentation. C) Ethanol production at the
775 end of fermentation. D) Cell viability during fermentation.

776

777 Fig. 6. Western blot analysis of peroxirredoxin Tsa1 and glucose repression kinase Snf1 during mixed
778 fermentations. Samples from 1, 2 and 3 days of the fermentation described in Fig. 5 were taken and
779 proteins extracted. A control of oxidative stress was made in cells growing exponentially in YPD
780 challenged with 5 mM $H_2O_2$ for 30 min. Blots with the same amount of proteins were blotted and probed
781 with anti-Tsa1, anti- Tsa1-$SO_3$ and Snf1-P specific antibodies. Actin was used as loading control.

782

783 Fig. 7. Transcriptomic analysis of mixed fermentations. Volcano plot of: A) *Z.bailii* + *S. cerevisiae*
784 fermentation vs *S. cerevisiae* alone control. B) *S. bacillaris*+ ***S.*** *cerevisiae* fermentation vs *S. cerevisiae*
785 alone control. C) *Z.bailii* + S.cerevisiae fermentation vs *S. bacillaris*+ ***S.*** *cerevisiae* fermentation. $Log_2$ of
786 fold change and $log_2$ of p-value were plotted.

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795 **Table 1.** Main technological characteristics of the selected non-*Saccharomyces* strains.

| Strain code | Quantitative enzymatic activities | | EtOH[(b)] | $SO_2$[(b)] |
|---|---|---|---|---|
| | β-glucosidase[(a)] | β-xylosidase[(a)] | | |
| Db2 | 157.162 ± 25.98 | 108.182 ± 20.20 | 0.43 ± 0.07 | 0.11 ± 0.01 |
| Ha3 | 116.345 ± 14.43 | 106.141± 5.77 | 0.04 ± 0.00 | 0.01 ± 0.00 |
| Ha9 | 177.570 ± 14.43 | 51.039 ± 2.89 | 0.06 ± 0.01 | 0.01 ± 0.00 |
| St1 | 26.550 ± 8.66 | 0.00 ± 0.00 | 0.09 ± 0.01 | 0.03 ± 0.00 |
| St2 | 8.182 ± 5.77 | 0.00 ± 0.00 | 0.08 ± 0.01 | 0.02 ± 0.00 |
| St5 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.08 ± 0.02 | 0.03 ± 0.01 |
| St8 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.08 ± 0.00 | 0.02 ± 0.00 |
| Zb1 | 83.692 ± 8.66 | 22.468 ± 8.66 | 0.62 ± 0.05 | 0.03 ± 0.00 |

796 [a]Enzymatic activities reported as nmol pNP/mL/h

797 [b]Strain tolerance to ethanol and $SO_2$, expressed as the ratio between the growth in broth with (14% v/v

798 ethanol and 150 mg/L $SO_2$) and without the stress factor.

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**Table 2.** Oenological parameters and main volatile compounds of experimental wines obtained from mixed starters cultures of selected non-*Saccharomyces* strains (Db2, Ha3, Ha9, St1, St8, St2, St5 and Zb1) simultaneously inoculated with *S. cerevisiae* EC1118 (Sc). Pure culture of *S. cerevisiae* EC1118 (Control Sc) was used as control.

| **Oenological characteristics** | **Db2+Sc** | **Ha3+Sc** | **Ha9+Sc** | **St1+Sc** | **St2+Sc** | **St5+Sc** | **St8+Sc** | **Zb1+Sc** | **Control (Sc)** |
|---|---|---|---|---|---|---|---|---|---|
| Ethanol | 12.09±0.07$^{ab}$ | 11.96±0.01$^{ab}$ | 12.19±0.02$^{ac}$ | 11.84±0.06$^{ab}$ | 11.91±0.23$^{ab}$ | 11.93±0.21$^{ab}$ | 11.81±0.18$^{bc}$ | 11.58±0.20$^{b}$ | 12.38±0.05$^{a}$ |
| Fructose | 2.40±0.14$^{ab}$ | 2.45±0.21$^{b}$ | 2.20±0.14$^{ab}$ | 1.40±0.28$^{c}$ | 1.45±0.07$^{c}$ | 1.40±0.14$^{c}$ | 1.40±0.14$^{c}$ | 1.80±0.00$^{ac}$ | 1.80±0.14$^{ac}$ |
| Glucose | 1.50±0.00$^{a}$ | 1.40±0.00$^{a}$ | 1.55±0.07$^{a}$ | 0.90±0.00$^{a}$ | 1.05±0.07$^{a}$ | 0.85±0.21$^{a}$ | 1.05±0.07$^{a}$ | 5.70±0.71$^{b}$ | 1.45±0.07$^{a}$ |
| Total acidity | 5.22±0.14$^{ac}$ | 5.37±0.00$^{a}$ | 5.15±0.16$^{ac}$ | 4.89±0.01$^{c}$ | 5.00±0.11$^{ac}$ | 5.13±0.15$^{ac}$ | 5.18±0.08$^{ac}$ | 6.00±0.03$^{b}$ | 5.02±0.01$^{ac}$ |
| Volatile acidity | 1.13±0.06$^{a}$ | 0.88±0.04$^{b}$ | 0.71±0.00$^{bc}$ | 0.68±0.04$^{cd}$ | 0.61±0.03$^{c}$ | 0.65±0.02$^{c}$ | 0.83±0.02$^{bd}$ | 0.68±0.12$^{bc}$ | 0.86±0.01$^{bd}$ |
| Acetaldehyde | 48.22±11.25$^{ab}$ | 43.51±14.59$^{ab}$ | 65.95±5.28$^{a}$ | 29.26±0.30$^{b}$ | 30.62±2.76$^{bc}$ | 32.76±4.40$^{bc}$ | 25.13±4.16$^{b}$ | 38.72±7.91$^{ab}$ | 59.36±4.16$^{ac}$ |
| Ethyl acetate | 28.95±9.45$^{a}$ | 11.84±3.59$^{ab}$ | 17.44±5.66$^{ab}$ | 7.91±0.31$^{b}$ | 8.07±1.42$^{b}$ | 9.02±1.85$^{b}$ | 7.71±2.80$^{b}$ | 9.12±1.07$^{b}$ | 7.38±3.39$^{ab}$ |
| *n*-Propanol | 23.06±4.62 | 19.67±2.12 | 22.01±4.29 | 21.15±2.36 | 25.64±4.07 | 29.74±3.34 | 29.52±5.52 | 18.91±0.46 | 19.83±1.44 |
| Isobutanol | 19.22±0.42$^{ab}$ | 21.32±0.61$^{ab}$ | 18.69±2.83$^{ab}$ | 25.07±0.04$^{ab}$ | 19.91±1.47$^{ab}$ | 15.51±1.90$^{a}$ | 17.86±2.12$^{a}$ | 36.93±12.96$^{b}$ | 28.01±2.53$^{ab}$ |
| D-amyl alcohol | 38.52±1.01$^{a}$ | 36.80±0.46$^{ab}$ | 36.39±1.20$^{ab}$ | 25.24±0.71$^{ab}$ | 23.74±2.50$^{b}$ | 24.3±2.80$^{b}$ | 13.04±9.05$^{b}$ | 35.14±1.08$^{a}$ | 40.12±0.77$^{a}$ |
| Isoamyl alcohol | 96.41±8.22$^{a}$ | 91.70±0.98$^{a}$ | 95.48±5.62$^{a}$ | 53.34±2.26$^{b}$ | 51.51±6.21$^{b}$ | 47.91±2.28$^{b}$ | 49.86±1.85$^{b}$ | 87.59±3.54$^{ac}$ | 104.04±2.32$^{ac}$ |

Data are means ± standard deviation of two independent experiments. Different superscript letters in the same row correspond to statistically significant differences (Tukey's test, $p < 0.05$) between mixed and control fermentation. The oenological parameters are expressed as g/L, with exception of ethanol, expressed as % v/v; volatile compounds are expressed as mg/L.

**Table 3.** Oenological parameters and main volatile compounds of experimental wines obtained from mixed starters cultures of selected non-*Saccharomyces* strains (Db2, Ha3, Ha9, St1, St8, St2, St5 and Zb1) sequentially inoculated with *S. cerevisiae* EC1118 (Sc). Pure culture of *S. cerevisiae* EC1118 (Control Sc) was used as control.

| Oenological characteristics | Db2+Sc | Ha3+Sc | Ha9+Sc | St1+Sc | St2+Sc | St5+Sc | St8+Sc | Zb1+Sc | Control (Sc) |
|---|---|---|---|---|---|---|---|---|---|
| Ethanol | 11.46±0.08$^{a}$ | 11.61±0.07$^{a}$ | 11.66±0.05$^{a}$ | 11.37±0.01$^{ab}$ | 11.70±0.13$^{a}$ | 11.54±0.09$^{a}$ | 11.34±0.13$^{ab}$ | 11.53±0.00$^{a}$ | 11.79±0.20$^{a}$ |
| Fructose | 2.00±0.14$^{ab}$ | 1.45±0.07$^{a}$ | 1.40±0.00$^{a}$ | 1.35±0.07$^{a}$ | 1.45±0.21$^{a}$ | 1.35±0.07$^{a}$ | 1.40±0.00$^{a}$ | 1.75±0.07$^{a}$ | 2.80±0.71$^{b}$ |
| Glucose | 2.10±0.00$^{ad}$ | 1.05±0.07$^{bd}$ | 1.15±0.07$^{bc}$ | 1.10±0.14$^{bd}$ | 1.05±0.35$^{bd}$ | 1.00±0.14$^{bd}$ | 0.95± 0.07$^{bd}$ | 1.80±0.28$^{ac}$ | 1.70±0.14$^{cd}$ |
| Total acidity | 5.70±0.06$^{ab}$ | 5.88±0.03$^{ac}$ | 5.98±0.03$^{ac}$ | 5.35±0.16$^{bd}$ | 5.73±0.12$^{ab}$ | 5.52±0.03$^{ad}$ | 5.78±0.25$^{ab}$ | 6.30±0.09$^{c}$ | 5.18±0.12$^{d}$ |
| Volatile acidity | 1.18±0.01$^{a}$ | 1.03±0.04$^{ac}$ | 0.69±0.01$^{b}$ | 0.88±0.06$^{c}$ | 0.97±0.04$^{c}$ | 1.17±0.01$^{ad}$ | 1.16±0.01$^{ad}$ | 0.91±0.10$^{c}$ | 0.99±0.05$^{cd}$ |
| Acetaldehyde | 46.26±3.92$^{ab}$ | 48.42±8.63$^{ab}$ | 33.11±3.61$^{a}$ | 27.75±0.28$^{a}$ | 39.46±1.31$^{a}$ | 38.32±10.33$^{a}$ | 42.96±6.36$^{ab}$ | 64.38±2.09$^{b}$ | 39.25±2.85$^{a}$ |
| Ethyl acetate | 28.14±6.98$^{a}$ | 13.45±0.33$^{bc}$ | 17.81±2.33$^{c}$ | 6.85±0.20$^{b}$ | 6.91±0.06$^{b}$ | 8.43±0.44$^{bc}$ | 8.88±1.17$^{bc}$ | 9.97±0.01$^{bc}$ | 10.09±1.29$^{bc}$ |
| *n*-Propanol | 28.54±0.78$^{ab}$ | 28.68±0.37$^{ab}$ | 31.76±2.16$^{b}$ | 22.73±3.34$^{a}$ | 22.33±3.34$^{a}$ | 24.74±0.73$^{ab}$ | 20.69±2.24$^{a}$ | 22.13±2.83$^{a}$ | 20.24±0.67$^{a}$ |
| Isobutanol | 29.70±0.51 | 26.24±1.06 | 30.91±8.37 | 33.14±0.07 | 33.07±8.64 | 23.46±0.34 | 24.38±0.32 | 32.80±4.00 | 32.15±2.08 |
| amyl alcohol | 40.78±2.70$^{ac}$ | 43.50±0.50$^{a}$ | 46.81±4.65$^{a}$ | 29.46±5.72$^{bc}$ | 27.06±0.50$^{b}$ | 33.13±0.53$^{bd}$ | 33.39±3.10$^{bd}$ | 42.27±2.70$^{ad}$ | 48.53±0.39$^{a}$ |
| Isoamyl alcohol | 102.66±0.14$^{ab}$ | 94.42±2.55$^{ab}$ | 104.50±1.07$^{ab}$ | 80.75±12.44$^{a}$ | 79.04±21.57$^{a}$ | 82.30±6.57$^{a}$ | 83.04±9.35$^{a}$ | 115.62±7.14$^{ab}$ | 127.23±2.59$^{b}$ |

Data are means ± standard deviation of two independent experiments. Different superscript letters in the same row correspond to statistically significant differences (Tukey's test, $p < 0.05$) between mixed and control fermentation. The oenological parameters are expressed as g/L, with exception of ethanol, expressed as % v/v; volatile compounds are expressed as mg/L

822

823 **Table 4.** Main Biological Process GO categories over-represented in mixed fermentations. See Supplementary Table S3

824 for a full analysis.

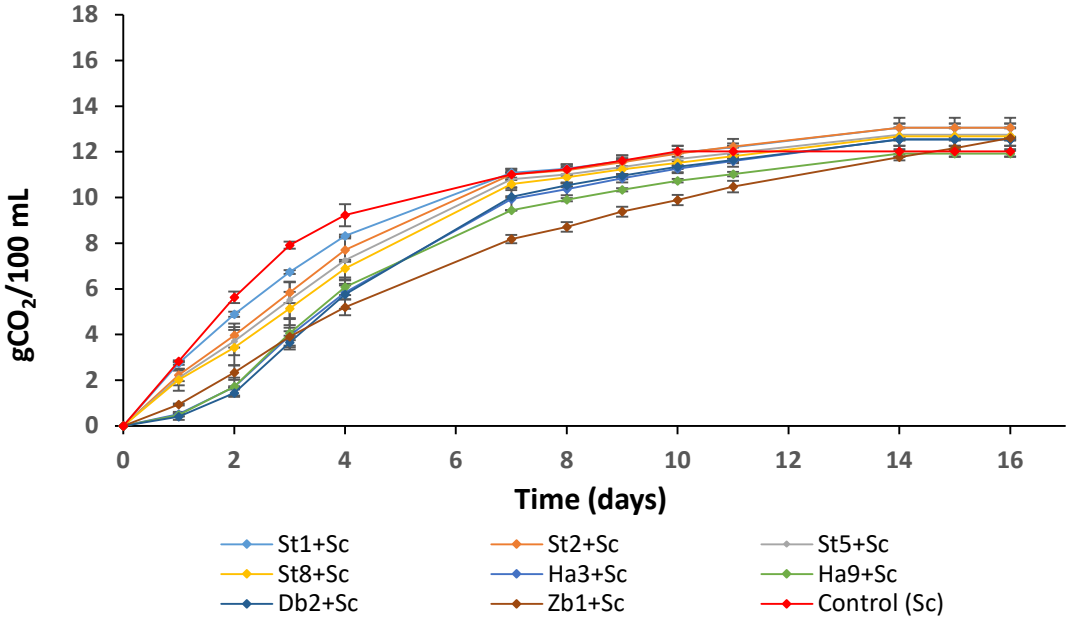
| Fermentation | GO categories | Fold enrichment |
|---|---|---|
| **Zb+Sc vs Sc, up-regulated** | 'de novo' NAD biosynthetic process | 13,91 |
| | glycolytic process | 10.43 |
| | trehalose metabolic process | 9.48 |
| | glycogen metabolic process | 5.62 |
| **Zb+Sc vs Sc, down-regulated** | None | |
| **Sb+Sc vs Sc, up-regulated** | gluconeogenesis (GO:0006094) | 7.62 |
| | rRNA transport | 6.38 |
| | cytoplasmic translation | 5.38 |
| **Sb+Sc vs Sc, down-regulated** | cytoplasm to vacuole transport by the Cvt pathway | 4.11 |
| | protein targeting to vacuole | 2.92 |
| **Zb+Sc vs Sb+Sc, up-regulated** | late nucleophagy | 7.01 |
| | homoserine metabolic process | 6.95 |
| | iron ion transmembrane transport | 6.95 |
| **Zb+Sc vs Sb+Sc, down-regulated** | rRNA export from nucleus | 9.62 |
| | cytoplasmic translation | 8.61 |

825

826

Fig. 1

A)


18
16
14
12
10
8
6
4
2
0
0
2
4
6
8
10
12
14
16
gCO2/100 mL
Time (days)
St1+Sc
St2+Sc
St5+Sc
St8+Sc
Ha3+Sc
Ha9+Sc
Db2+Sc
Zb1+Sc
Control (Sc)

B)

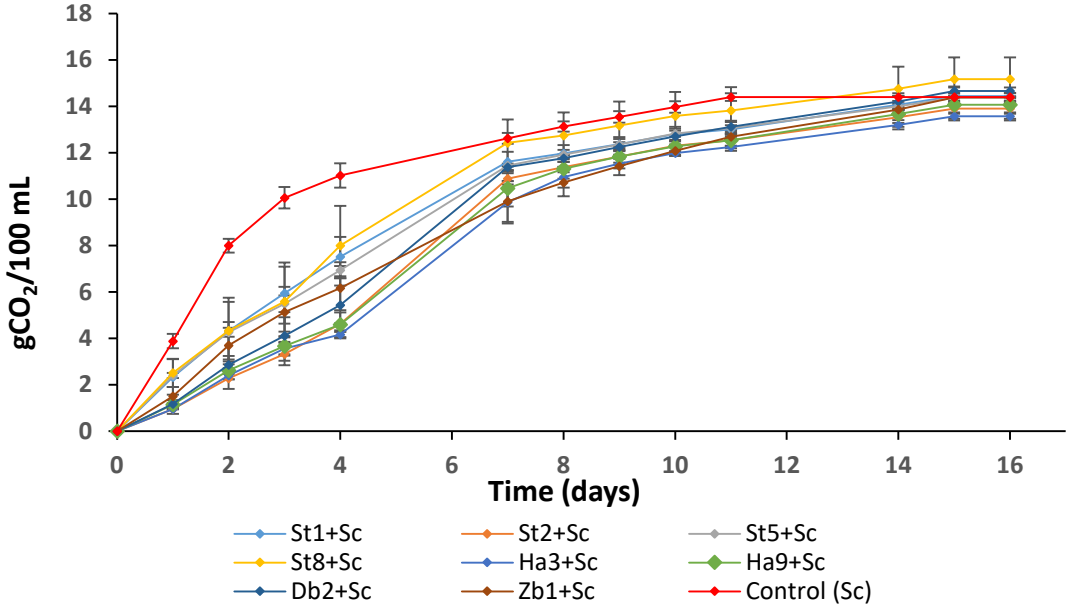

18
16
14
12
10
8
6
4
2
0
0
2
4
6
8
10
12
14
16
gCO2/100 mL
Time (days)
St1+Sc
St2+Sc
St5+Sc
St8+Sc
Ha3+Sc
Ha9+Sc
Db2+Sc
Zb1+Sc
Control (Sc)

Fig. 2

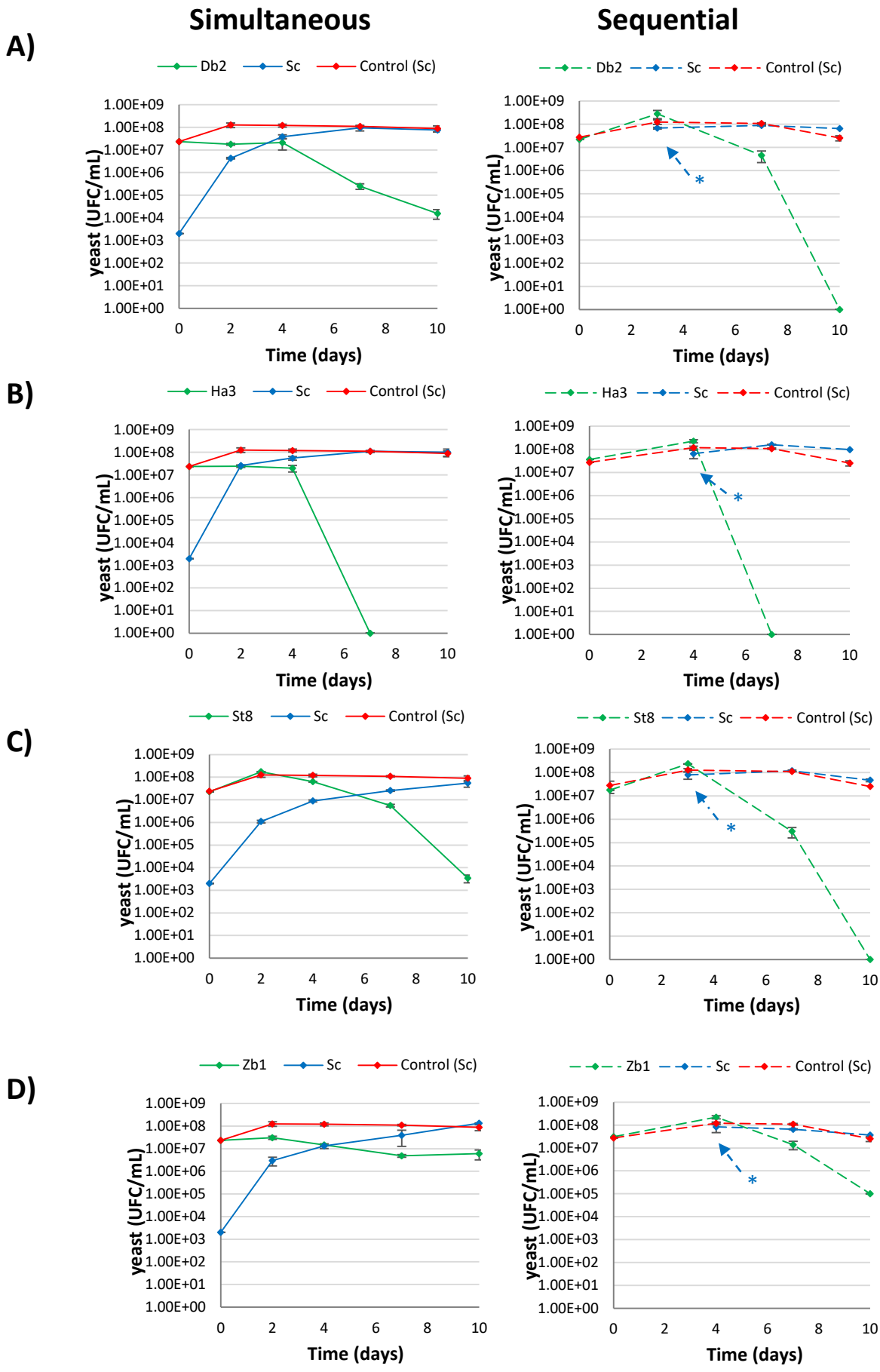
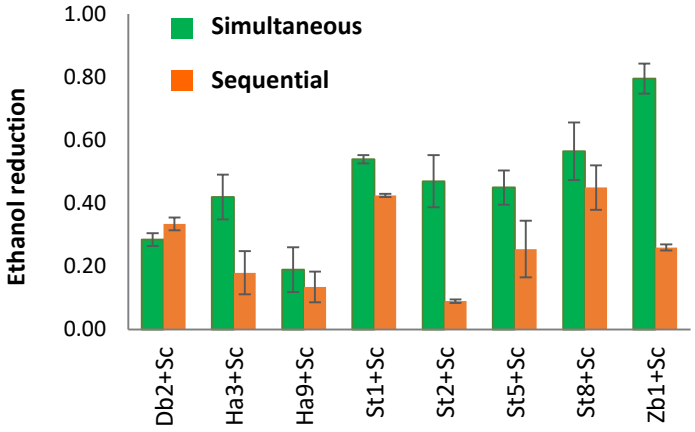

Simultaneous
Sequential
A)
Db2
Sc
Control (Sc)
B)
Ha3
C)
St8
D)
Zb1
yeast (UFC/mL)
Time (days)
1.00E+09
1.00E+08
1.00E+07
1.00E+06
1.00E+05
1.00E+04
1.00E+03
1.00E+02
1.00E+01
1.00E+00
0
2
4
6
8
10
*

**Fig. 3**

**A)**


Simultaneous
Sequential
Ethanol reduction
1.00
0.80
0.60
0.40
0.20
0.00
Db2+Sc
Ha3+Sc
Ha9+Sc
St1+Sc
St2+Sc
St5+Sc
St8+Sc
Zb1+Sc

**B)**

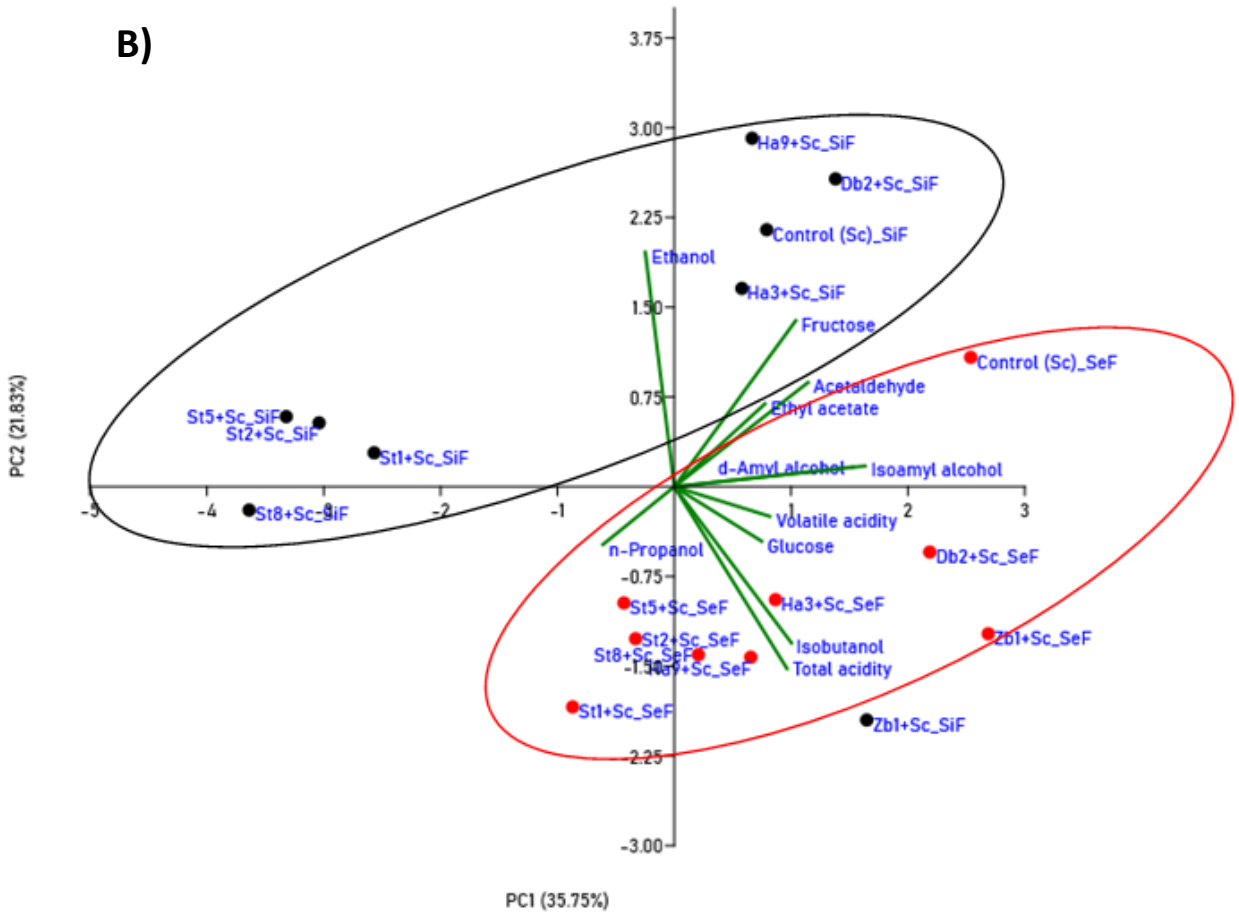
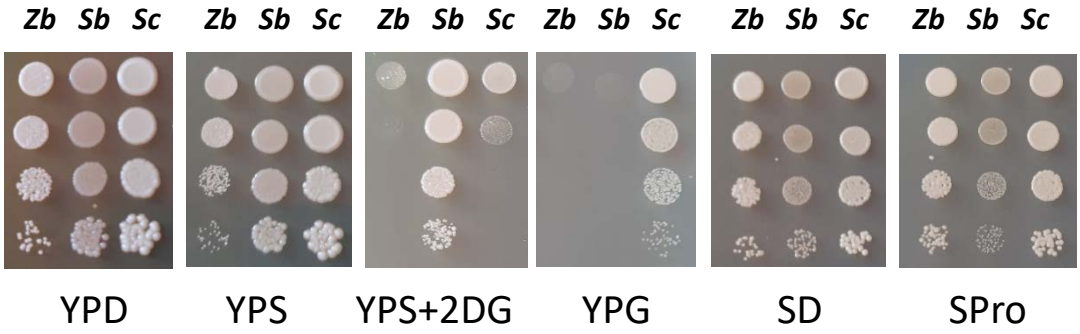
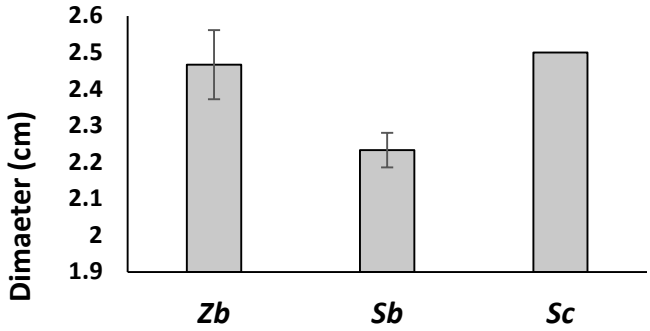

PC2 (21.83%)
PC1 (35.75%)
3.75
3.00
2.25
1.50
0.75
-0.75
-1.50
-2.25
-3.00
-5
-4
-3
-2
-1
1
2
3
Ha9+Sc_SiF
Db2+Sc_SiF
Control (Sc)_SiF
Ethanol
Ha3+Sc_SiF
Fructose
Control (Sc)_SeF
Acetaldehyde
Ethyl acetate
St5+Sc_SiF
St2+Sc_SiF
St1+Sc_SiF
d-Amyl alcohol
Isoamyl alcohol
St8+Sc_SiF
Volatile acidity
Glucose
n-Propanol
Db2+Sc_SeF
St5+Sc_SeF
Ha3+Sc_SeF
St2+Sc_SeF
Zb1+Sc_SeF
St8+Sc_SeF
Isobutanol
Total acidity
Ha9+Sc_SeF
St1+Sc_SeF
Zb1+Sc_SiF

Fig. 4

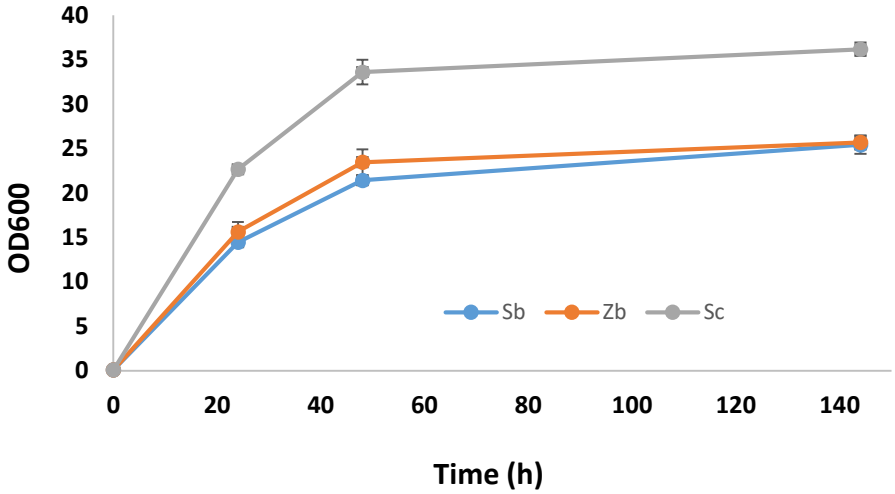
A)


Zb Sb Sc
Zb Sb Sc
Zb Sb Sc
Zb Sb Sc
Zb Sb Sc
Zb Sb Sc
YPD
YPS
YPS+2DG
YPG
SD
SPro

B)


Dimaeter (cm)
2.6
2.5
2.4
2.3
2.2
2.1
2
1.9
Zb
Sb
Sc

C)


OD600
40
35
30
25
20
15
10
5
0
0
20
40
60
80
100
120
140
Sb
Zb
Sc
Time (h)

D)

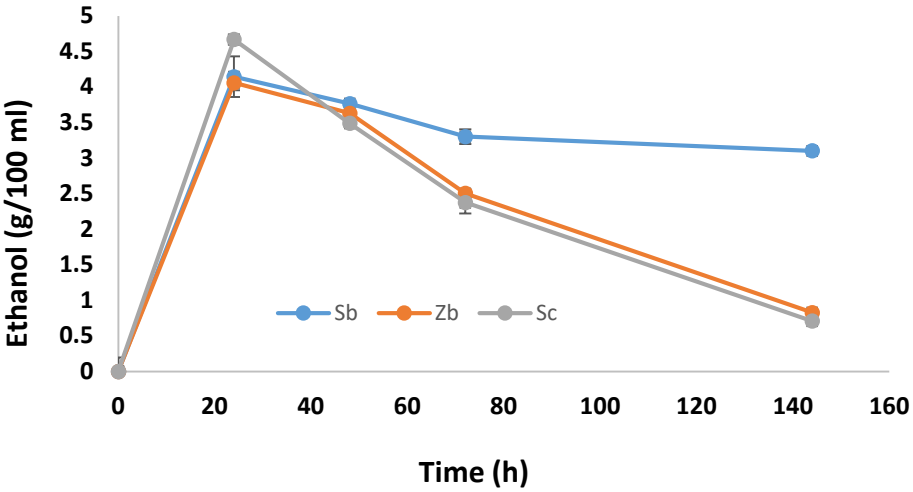
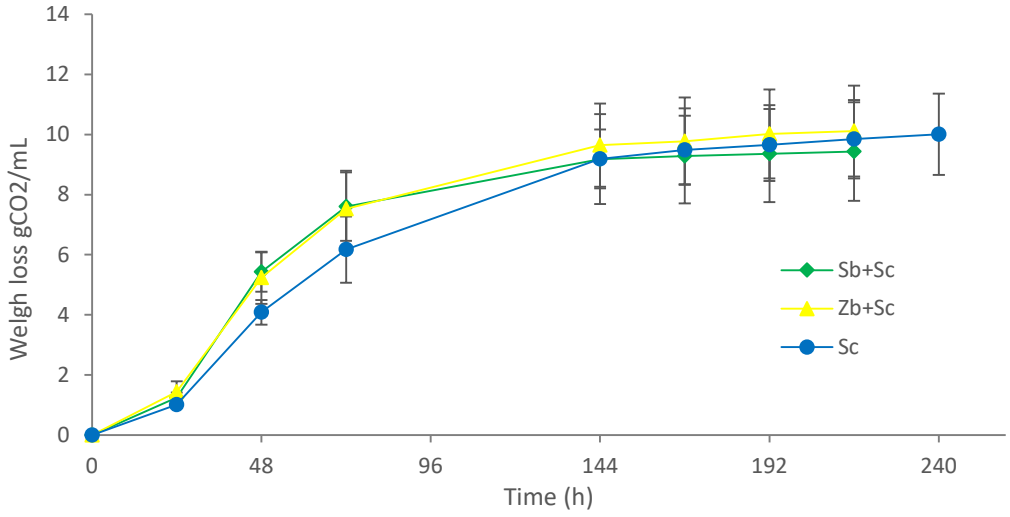
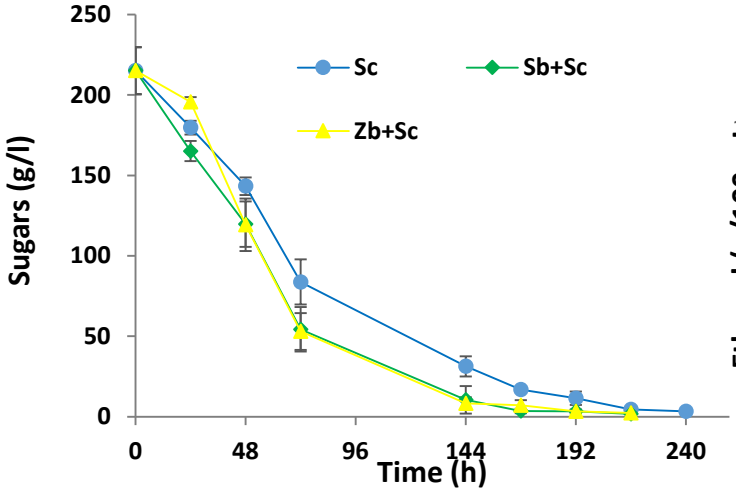

Ethanol (g/100 ml)
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
0
20
40
60
80
100
120
140
160
Sb
Zb
Sc
Time (h)

Fig. 5

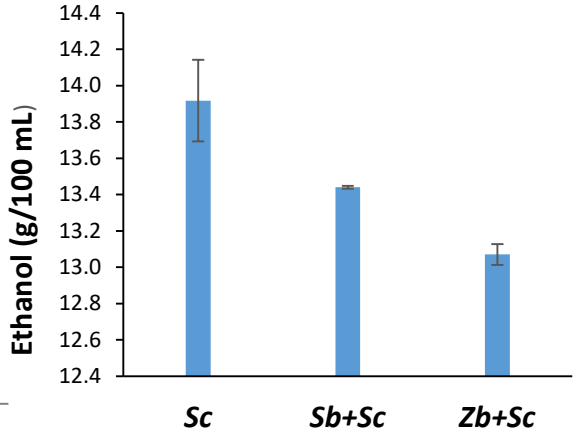
A)


Welgh loss gCO2/mL
Time (h)
Sb+Sc
Zb+Sc
Sc

B)


Sugars (g/l)
Time (h)
Sc
Sb+Sc
Zb+Sc

C)


Ethanol (g/100 mL)
Sc
Sb+Sc
Zb+Sc

D)

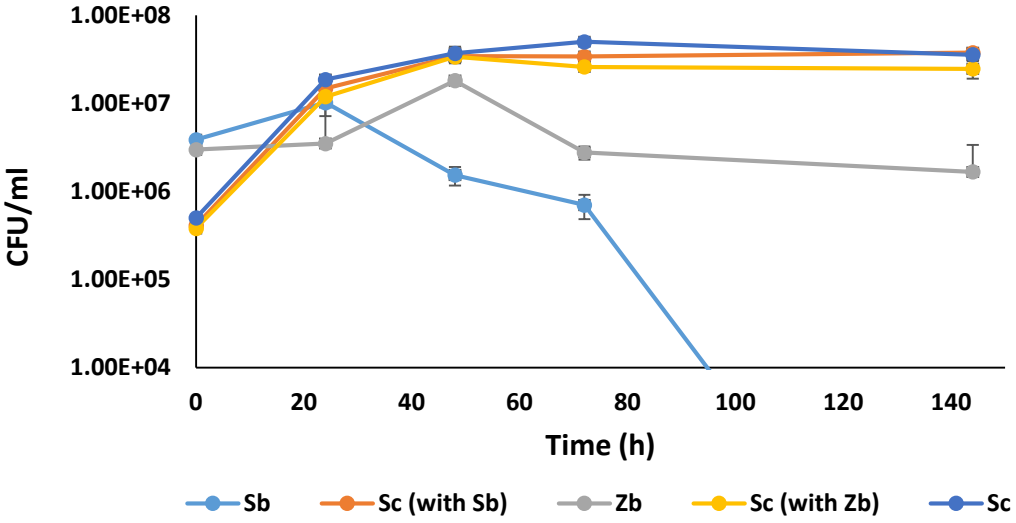

CFU/ml
Time (h)
Sb
Sc (with Sb)
Zb
Sc (with Zb)
Sc

Fig. 6

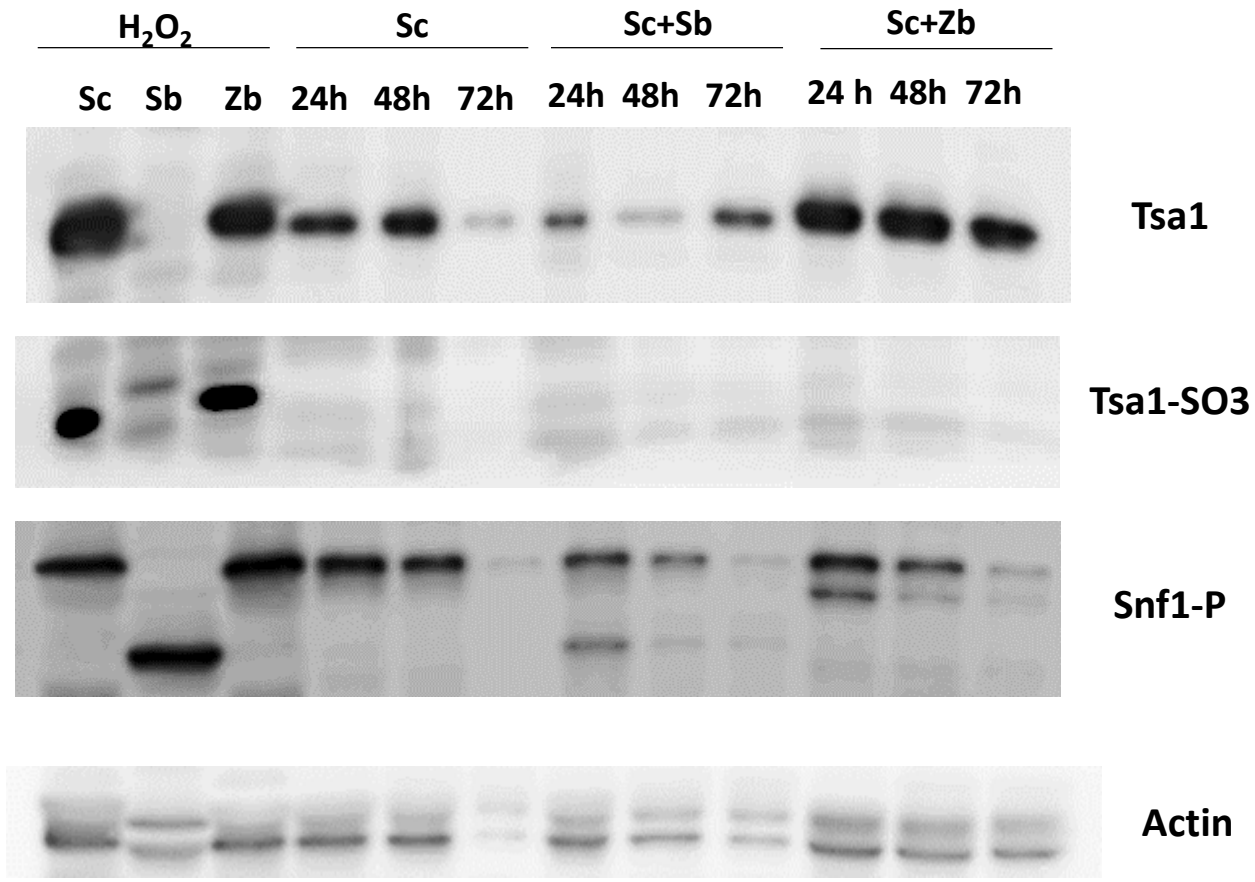
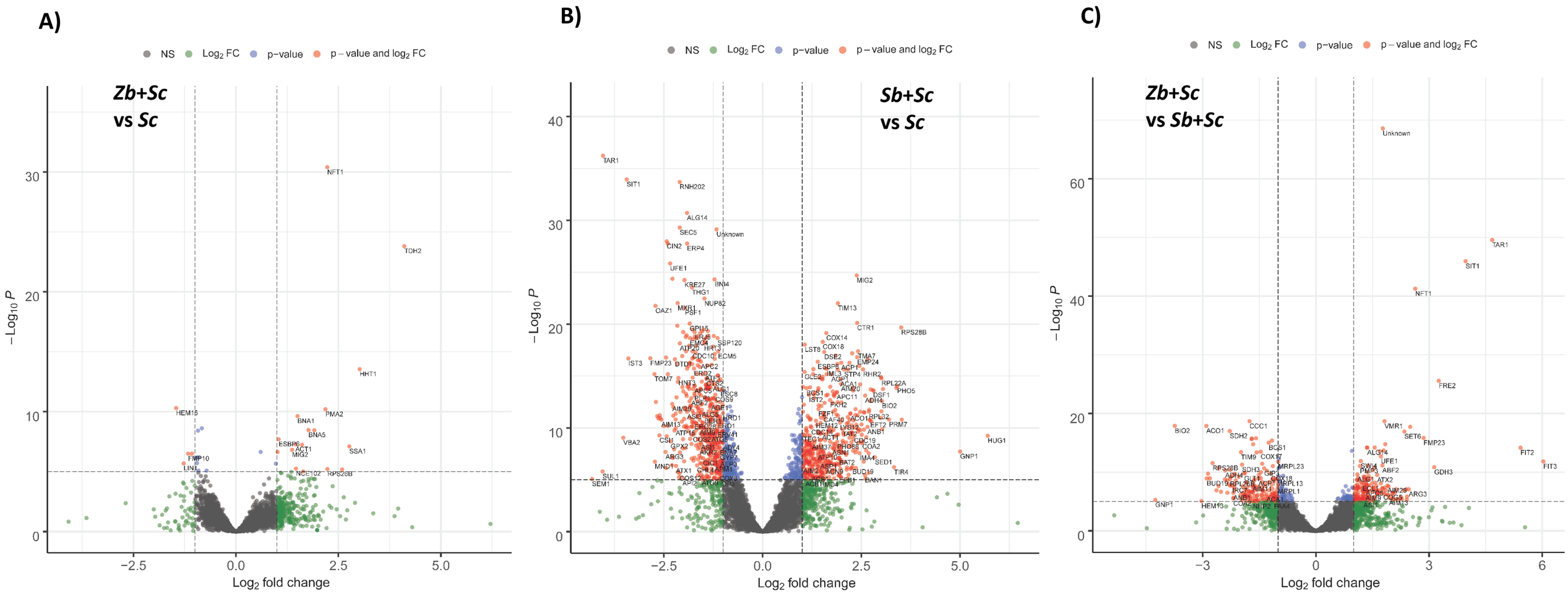

$H_2O_2$
Sc
Sc+Sb
Sc+Zb
Sc Sb Zb 24h 48h 72h 24h 48h 72h 24 h 48h 72h
Tsa1
Tsa1-SO3
Snf1-P
Actin

Fig. 7


A)
B)
C)
NS
Log2 FC
p−value
p − value and log2 FC
Zb+Sc
vs Sc
Sb+Sc
vs Sc
Zb+Sc
vs Sb+Sc
−Log10 P
Log2 fold change
NFT1
TDH2
HHT1
HEM15
PMA2
BNA1
BNA5
ESBP6
ACT1
MIG2
SSA1
FMP10
LIN1
NCE102
RPS26B
TAR1
SIT1
RNH202
ALG14
SEC5
Unknown
CIN2
ERP4
UFE1
KRE27
BNI4
THG1
NUP82
MXR1
OAZ1
PSF1
GPI15
ERG6
EMC4
SSP120
ATP20
HRT3
CDC10
ECM5
IST3
FMP23
DTD1
APC2
ERD2
TOM7
HNT3
CTS2
APC5
ALG1
ESC8
PHO5
AIM28
COS9
AGE1
ASI3
LCB5
HRD1
ERG28
ERD1
AIM13
ATP15
AIM9
ERV41
VBA2
CSI1
COS12
ATG8
GPX2
ASI1
ADH4
AKR2
BNA7
GYP1
ARG3
CIC1
AFR3
MND1
ATX1
CHL4
APM1
SUL1
SEM1
API2
ATG9
COX3
MIG2
TIM13
CTR1
RPS28B
COX14
COX18
LST8
DSE2
TMA7
EMP24
ESBP6
ACP1
GLE2
IML3
STP4
RHR2
AGP1
ACA1
AIM20
RPL22A
BGS1
APC11
DSF1
IST2
FRH2
BIO2
FET5
CAF40
ACO1
RPL32
HEM12
LYS12
EFT2
PRM7
CDC1
TAT2
ANB1
TEG1
ACT1
CDC19
HUG1
AIM37
RHO5
COA2
ASN1
IMA4
GNP1
ATP11
SED1
BAT2
ASF1
AIM2
ACB1
BUD19
TIR4
BAN1
MD4
FRE2
VMR1
SET6
FMP23
FIT2
FIT3
GDH3
SWI4
PMP3
ABF2
ALG1
ATX2
ECM1
AIM29
ARG3
AIM9
COQ5
AIM13
ASK1
NIP2
PAA4
HEM13
COA2
ACA1
ANB1
IRC7
AIM17
MRPL1
MRPL13
MRPL23
SDH3
SDH4
GLE1
ADH1
BUD19
RPL26B
RPS28B
SDH2
TIM9
COX17
BCS1
ACO1
BIO2
CCC1