Kinetic analysis of yeast-yeast interactions in oenological conditions

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Abstract

Fermentation by microorganisms is a key step in the production of traditional food products such as bread, cheese, beer and wine. In these fermentative ecosystems, microorganisms interact in various ways (competition, predation, commensalism and mutualism). Traditional wine fermentation is a complex microbial process performed by different yeast species which can be classified in two groups: *Saccharomyces* and non-*Saccharomyces* species. To better

understand the different interactions occurring within wine fermentation, isolated cultures were compared to cultures involving one reference strain of *S. cerevisiae* species and one strain of five non-*Saccharomyces* yeast species out of five (*Metschnikowia pulcherrima, Metschnikowia fructicola, Torulaspora delbrueckii, Hanseniaspora opuntiae and Hanseniaspora uvarum*). In each case, we studied the population dynamics, the resources consumption and the production of metabolites of central carbon metabolism. This deep phenotyping of competition kinetics allowed us to identify the main mechanisms of interaction. *T. delbrueckii* and *S. cerevisiae* compete for resources with comparable fitness in our experimental conditions. *S. cerevisiae* and *H. uvarum* and *H. opuntiae* are also competing for resources although both *Hanseniaspora* strains are characterized by a strong mortality in isolated and mixed fermentations. *M. pulcherrima* and *M. fructicola* have a negative interaction with *S. cerevisiae*, provoking a decrease in viability in co-culture, probably caused by the synthesis of a killer toxin. Overall, this work highlights the interest of measuring the population and metabolites kinetics in order to understand yeast-yeast interactions. These results are a first step for ecological engineering and the intelligent design of optimal multi-starter consortiums using modeling tools.

Importance

Most oenological fermentations are using today fermentation starters composed of a *Saccharomyces cerevisiae* single species. Recently the interest of other yeast species has increased in order to diversify the organoleptic qualities of the wine. However, these other species cannot finish the fermentation and are therefore most often associated with *S. cerevisiae* strain in multi-species starters. Until now, there is a lack of knowledge on the interaction mechanisms that support these multi-species starters. The goal of this work is to increase our knowledge of yeast-yeast interaction in winemaking fermentation. Furthermore, acquired data will be used in the future to build a mathematical model predicting the production dynamics of

mixed fermentations. This knowledge is of great importance to guide the design of optimized yeast consortium to increase wine quality and therefore has a great economic potential.

Introduction

In natural or anthropized environments, microbial species are part of an ecosystem and interact positively or negatively in a complex network. Until recently, process optimization in agriculture or food processing was mostly based on the selection of single strains. However, this paradigm is now being challenged and the scientific community is increasingly seeking to exploit and optimize consortia consisting of several strains and/or species. Indeed, many studies have shown that more diverse anthropized environments have many advantages in terms of resilience, disease resistance or yield of production (1). Efforts are now being made to design optimal consortia of various species and strains whose interactions will be exploited to maximize a given criterion.

In oenology which is both an economical and societal important food ecosystem, the addition of 'starters' composed of selected yeasts at the beginning of the fermentation process is common. It is estimated that 80% of the oenological fermentations in the world are conducted with starters (2, 3). Most often, these fermentation starters are composed of a single *Saccharomyces cerevisiae* strain selected for its ability to complete the fermentation. Indeed, numerous experiments have shown that *S. cerevisiae* species, which is rare at the beginning of the fermentation, most often becomes predominant at the end of the fermentation, demonstrating their superior fermentation abilities (4–7). However, in recent years, multi-species starters have emerged with the aim to increase the aromatic complexity of wines. These new starters most often combine one strain of *S. cerevisiae* allowing to complete the

74 fermentation and one other species strain, often from a different genus than *Saccharomyces*,
75 bringing a greater variety of flavors (4, 5, 7, 8).
76 There are numerous experiments and even industrial products that mix *Saccharomyces* and non-
77 *Saccharomyces* strains to improve the organoleptic qualities of wine (5, 9, 10). Non-
78 *Saccharomyces* strains used in these experiments are very diverse with more than 23 different
79 species (5) including among others *Torulaspora delbrueckii*, *Metschnikowia pulcherrima*,
80 *Metschnikowia fructicola*, *Hanseniaspora opuntiae* and *Hanseniaspora uvarum*. *T. delbrueckii*
81 is one of the few species that is capable to finish the fermentation and has shown to increase
82 wine quality in mixed cultures with *S. cerevisiae* (9, 11, 12). Bely et al (11) have shown that a
83 mixed culture *S. cerevisiae* and *T. delbrueckii* improves the analytical profile of sweet wines
84 notably by decreasing volatile acidity and acetaldehyde production (with 53% less in volatile
85 acidity and 60% less acetaldehyde than a pure culture of *S. cerevisiae*) (11). On the opposite,
86 species from the *Metschnikowia* genus ferments poorly in oenological conditions but also can
87 have interesting attributes (4). In conjunction with *S. cerevisiae*, the presence of *M. pulcherrima*
88 can reduce ethanol concentrations (4, 13), can increase 'citrus/grape fruit' and 'pear' attributes
89 (14), as well as the persistence of 'smocky' and 'flowery' attributes (15). *M. pulcherrima* also
90 has an amensalism effect on *S. cerevisiae* by depletion of iron via the production of
91 pulcherriminic acid (16). *M. fructiola* has been less studied and never in conjunction with *S.*
92 *cerevisiae* but still presents an interesting capacity to inhibit *Botrytis* growth (17). At last, the
93 *Hanseniaspora* genus also has also been studied in sequential or simultaneous fermentation
94 with *S. cerevisiae* and has shown to increase volatile compound production during winemaking
95 (4). It has been shown to increase 'tropical fruit', 'berry', 'floral', and 'nut aroma' character
96 (18) linked to higher concentrations of acetate esters, esters of MCFAs, isoamyl alcohol, 2-
97 phenylethanol and α-terpineol (19).

98

99 Despite these diverse studies, the composition and the protocol of inoculation of these multi-
100 strains starters are still very empirical and only based on input/output balance without
101 considering the dynamics of the microbial populations and their interactions. This lack of
102 knowledge about the yeast-yeast interactions prevents improving a rationale design of multi-
103 strain starters (20). To address this problem, we precisely followed population dynamics and
104 metabolite productions during oenological fermentations realized in unique or mixed cultures
105 of yeasts. This detailed analysis of the fermentation dynamics will enhance our understanding
106 of the interaction mechanisms and of the influence of yeasts-yeasts interactions on the
107 composition of the final product.
108

109 Results

110 In this work we compared the performances in winemaking conditions of isolated cultures of
111 six different yeast species (*Saccharomyces cerevisiae*, *Torulaspora delbrueckii*, *Metschnikowia*
112 *pulcherrima*, *Metschnikowia fructicola*, *Hanseniaspora opuntiae* and *Hanseniaspora uvarum,*
113 one strain by species) with paired co-cultures mixing each of the five non-*Saccharomyces*
114 strains with one GFP marked *Saccharomyces cerevisiae* strain. Obtained results will make it
115 possible to evaluate the intensity of yeast-yeast interactions. By comparing the output of
116 isolated and mixed cultures, we evaluated the intensity of yeast-yeast interactions and/or its
117 consequences on ecosystem services production.
118

119 We first investigated the consequences of co-culture onthe dynamic of CO_2 production which
120 is a good indicator of the fermentation progress. The values of the maximum rate of CO_2
121 production (***Vmax***, Fig. 1a, Table. 1) and of the maximum CO_2 produced were estimated
122 (***CO_2max***, Fig. 1b, Table. 1). The ***Vmax*** was highly dependent on the species (p.value < 0.001).
123 *S. cerevisiae* cultures (***Sc***) displayed the maximum ***Vmax*** (0.99 ± 0.02 $g.L^{-1}.h^{-1}$) followed by *T.*

124 *delbrueckii* (***Td***, 0.51 ± 0.05 $g.L^{-1}.h^{-1}$), followed by *Hanseniaspora* species (***Hu*** 0.33 ± 0.04 $g.L^{-1}.h^{-1}$, and ***Ho*** 0.42 ± 0.02 $g.L^{-1}.h^{-1}$) and finally *Metschnikowia* species (***Mp*** 0.165 ± 0.02 $g.L^{-1}.h^{-1}$and ***Mf*** 0.17 ± 0.04 $g.L^{-1}.h^{-1}$). The five mixed cultures had an intermediate ***Vmax*** values between those of ***Sc*** and ***Td*** isolated cultures (Fig. 1a). Mixed culture containing ***Mp***, ***Td*** or ***Mf*** species had significantly higher ***Vmax*** than those containing ***Hu*** or ***Ho*** species. we considered two types of cultures, those where the fermentation was finished within xx hours (***CO2max*** > 80$g.L^{-1}$) and those where it was not (Fig. 1b). All mixed cultures and the Sc cultures were able to finish the fermentation while all non-*Saccharomyces* isolated cultures did not. ***Td*** isolated cultures had the highest $\boldsymbol{CO_2max}$ among non-*Saccharomyces* species (65 ± 4 $g.L^{-1}$), followed by *Hanseniaspora* species (***Hu*** 30 ± 0.4 $g.L^{-1}$, and ***Ho*** 46 ± 0.6 $g.L^{-1}$) and finally *Metschnikowia* species (***Mp*** 22 ± 0.4 $g.L^{-1}$ and ***Mf*** 20 ± 1 $g.L^{-1}$). For these two parameters, the mixed cultures can be grouped with the ***Sc*** indicating a prevalent impact of the *S. cerevisiae* species on the fermentation kinetics.

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138 We then looked at the population dynamics for each fermentation (Fig. 2) and determined the maximum growth rate of the population (***mu***), the maximum population size, also called the carrying capacity (***K***) and the relative frequency of each species at the end of mixed cultures (Table. 1). The fermentations conducted by *S. cerevisiae* alone goes through an exponential growth rate ($\boldsymbol{mu_{Sc}}$ = 5.6 ± 0.8 10^6cells.$mL^{-1}.h^{-1}$) and reaches a maximum population around 1.5 10^8cells.mL^{-1} ($\boldsymbol{K_{Sc}}$ = 1.56 ± 0.16 10^8cells.mL^{-1}) that remains constant until the end of the fermentation. ***Td*** fermentations have dynamics comparable to those of ***Sc*** with a similar growth rate but a smaller carrying capacity ($\boldsymbol{K_{Td}}$ = 1.23 ± 0.05 10^8cells.mL^{-1}). The fermentations mediated by *Hanseniaspora* species alone had as similar growth dynamics similar to ***Sc*** at the beginning of the fermentation with though a higher growth rate ($\boldsymbol{mu_{Ho}}$ = 7.7 ± 2.3 10^6cells.$mL^{-1}.h^{-1}$, $\boldsymbol{mu_{Hu}}$ = 13.4 ± 4.3 10^6cells.$mL^{-1}.h^{-1}$). On the opposite, their stationary phase is quite

149 different from that of ***Sc***; characterized by a higher cell mortality with a population drop of
150 about 70% by the end of the fermentation. The fermentation mediated by *Metschnikowia*
151 species have growth dynamics globally similar to ***Sc*** fermentations: a similar growth rate ($\boldsymbol{mu_{mp}}$
152 $= 2.3 \pm 0.4\ 10^6 cells.mL^{-1}.h^{-1}$, $\boldsymbol{mu_{Mf}} = 3.4 \pm 0.7\ 10^6 cells.mL^{-1}.h^{-1}$), a much smaller carrying
153 capacity ($\boldsymbol{K_{Mp}} = 0.6 \pm 0.01\ 10^6 cells.mL^{-1}$, $\boldsymbol{K_{Mf}} = 0.8 \pm 0.05\ 10^6 cells.mL^{-1}$) and no mortality
154 during the stationary phase. In most cases, the mixed cultures display an intermediated pattern
155 between the two corresponding isolated cultures (Fig. 2). However, the mixed cultures with
156 *Metschnikowia* species harbor a cell mortality during the stationary phase contrary to their
157 respective isolated cultures. In the case of ***ScvsMp*** fermentation, only *S. cerevisiae* population
158 decreases significantly during the stationary phase, while in ***ScvsMf*** fermentation; both sub
159 populations significantly diminish. As a measure of fitness, we can also follow the variations
160 of *S. cerevisiae* frequency along the fermentation. In all mixed cultures, *S. cerevisiae*
161 significantly increased during the fermentation from 10% initially to frequencies varying
162 between 32% and 96% at the end of the culture depending on species (Table. 1). In all cases,
163 except the mix with the *T. delbrueckii* ($prop_{ScvsTd} = 0.32 \pm 0.02$), *S. cerevisiae* finishes by
164 dominating the mixed cultures (Table. 1).
165
166 We then looked at resources consumption (glucose, fructose and sources of assimilable
167 nitrogen) and estimated the final concentration of sugars (fructose and glucose) and amino-
168 acids (Fig. 3, Table 1). The ***Sc*** fermentations consumed all sugars leaving less than 0.1% of the
169 initial concentration (Fig. 3a). Among isolated non-sacc, ***Td*** fermentations was the one
170 consuming the highest fraction of the initial sugars leaving around 31% of sugars followed by
171 ***Ho*** (45%), ***Hu*** (67%), ***Mf*** (68%) and ***Mp*** (71%). Furthermore, all species but *Hanseniaspora*
172 *opuntiae* have consumed more glucose than fructose. The mixed cultures consumed more
173 sugars than all isolated non-*Saccharomyces* fermentations but less than ***Sc*** fermentations with

174 a preference for glucose, indicating the major impact of the *S. cerevisiae* strain in sugar
175 consumption (consistent with the CO_2 production).
176 The consumption of nitrogen assimilable sources (NAS, amino-acids and ammonium) harbours
177 the same pattern (Fig. 3b). NAS was consumed almost entirely in ***Sc*** isolated culture and in all
178 mixed populations whereas the fraction of NAS consumed varied between 84% and 94% in
179 non-*saccharomyces* isolated culture. However, the preference for the different sources of
180 nitrogen are different for each species (Fig. 3c). Glycine and histidine are the least consumed
181 by ***Td***. ***Ho*** and ***Hu*** have similar behaviors: they both consume only half of the ammonium, 90%
182 of histidine and 89% and 79% of arginine respectively. In the same way, both *Metschnikowia*
183 species present a similar pattern; the non-exhausted NAS are identical for the two species:
184 ammonium, glycine, aspartate, arginine tyrosine, phenylalanine and alanine. Therefore, we can
185 classify NAS in function of their global preference by all tested yeasts. The obtained ranking
186 by order of preference is: glutamine, methionine, glutamate, valine, threonine, serine,
187 tryptophan, alanine, histidine, arginine, aspartate, glycine and surprisingly ammonium.
188
189 In parallel of the monitoring of the must resources consumption, we also investigated the
190 production of metabolites from the CCM (Central Carbon Metabolism): ethanol, glycerol,
191 succinate, pyruvate, acetate and alpha-ketoglutarate (Table 1). Theses productions has been
192 measured at the end of the cultures where sugar consumption being quite different from one
193 fermentation to another. Therefore, it appears more appropriate to first compare the production
194 yields (total production / sugar consumption) instead of the total productions. Moreover, we
195 decide to compare yields of production relatively to the ***Sc*** yield that is our reference (Fig. 4).
196 In the case of ethanol, only ***Mf*** fermentations has a yield of production statistically than the ***Sc***
197 inferior (-32%). For glycerol, all non-*saccharomyces* grown alone but ***Td*** had a greater yield
198 than ***Sc***. The mixed fermentations glycerol yield was not significantly different from the Sc one.

Regarding acetate, only *Hanseniaspora* species grown alone or in mixed culture harbored a greater yield than Sc (Fig. 4). For succinate, there could be a negative interaction between *Sc* and *Hanseniaspora* or *Sc* and *Metschnikowia*, as yields in mixed culture were inferior than in the corresponding isolated cultures. However, these differences were not significant after the correction for multiple tests. At last, we did not observe any significant variation of alpha-ketoglutarate yield of production, linked to its lower concentration and difficulty to be precisely measured.

The total production of each fermentation is the result of the combination of species yields, total sugar consumption and respective population dynamics during the fermentation. Therefore, differences observed in the total productions of mixed cultures result from additive or subtractive effects between these 3 parameters. Regarding ethanol, the total production is directly linked to the consumption of resources and all mixed cultures are equivalent to ***Sc*** fermentation. The case of glycerol is more interesting. Indeed, even if the sugar consumption for ***ScvsHu*** and ***ScvsMp*** mixed cultures are lower than ***Sc*** isolated culture, the total production of glycerol is superior to the corresponding isolated cultures ($Glycerol_{SCvsMp}$ = 6.1±0.1 $g.L^{-1}$, $Glycerol_{Sc}$ = 5.3±0.4 $g.L^{-1}$, $Glycerol_{Mp}$ = 3.7±0.2 $g.L^{-1}$). This result comes from the positive combination of the superior yield of glycerol production of *Hanseniaspora* and *Metschnikowia cells* and the population dynamics. In all other cases, even if the total production does not lie between the production bonds of the corresponding isolated cultures for some mixed cultures, these differences are never statistically significant.

Discussion

This manuscript presents one of the first works focusing on the kinetics of yeast-yeast interactions between two species during the fermentative process in oenology. Indeed, so far, most of the work presented in the literature focus on input-output balances (4, 21).

First, this work provides additional information about the population dynamics in winemaking conditions of non-saccharomyces yeasts that are still poorly known. In agreement with previous results (12, 22, 23), we found that *T. delbrueckii* has a very similar dynamic than *S. cerevisiae* and is able to finish the fermentation on its own, while not being as efficient or as fit as *S. cerevisiae*. However, other studies suggest that this result is not constant and depends on strains and environmental conditions. The *T. delbrueckii* strain used in Belda et al (14) was less performant than their *S. cerevisiae* strain. Our results also show that *T. delbrueckii* contrary to *S. cerevisiae* does not consume all NAS (the limiting nutrient, (23), leaving half of the glycine unconsumed. This difference in the consumption of nitrogen sources may explain the ability of *S. cerevisiae* to invade mixed culture (12) despite its low inoculation rate. Despite differences of carbon metabolites production between the isolated cultures of *T. delbrueckii* and *S. cerevisiae* (linked to the differences in resources consumption), the yields are not statistically different. The mixed cultures are identical to *S. cerevisiae* pure cultures because of *S. cerevisiae* invasion. Globally the interaction mechanism between these two species seems to be a simple competition for resources without much potential for optimization.

The two species, *H. uvarum* and *H. opuntiae* both present a very different pattern in fermentation from *S. cerevisiae*. Their population dynamics are characterized by a faster growth rate (reaching a maximum population similar to *S. cerevisiae*), followed by a long phase of mortality where viability drops to 30% (24). Consequently, these two strains are not able to finish the fermentation and leave most of the sugars (24). Nitrogen consumption is also specific

248 to this genus; only half of available ammonium is used. Unlike with *T. delbrueckii*, the yield of
249 production of carbon metabolites are very different than with *S. cerevisiae* and moreover the
250 mixed fermentations display cases of transgressive interactions: their glycerol production is
251 higher than both isolated cultures, despite a lower sugar consumption. These overproductions
252 of glycerol can be explained by the conjunction of the population dynamics, resource
253 consumptions (the ***Ho*** and ***Hu*** fermentation leaving ⅔ of sugars) and the individual species
254 yields of glycerol production which are from two to three times greater than that of *S. cerevisiae*.
255 However, this interaction it is not found in previous works (24) and, in this present work, seems
256 to be related to competition for resources without any change in the population dynamic and
257 metabolism of each strain between the mixed and pure cultures.
258
259 At last, the two *Metschnikowia* strains have unexpected fermentation dynamics. The ***Mp*** and
260 ***Mf*** isolated cultures are characterized by a short growth phase reaching a small carrying
261 capacity and followed by a long stationary phase without mortality. Nevertheless, in spite of
262 this very high viability, the measured resources (sugars and nitrogen) are not entirely consumed.
263 Why the cells stop growing remains unclear. A possible explanation could be linked to
264 unmeasured resources. The third most important resource of the medium is the lipids that are
265 present in the medium under the form of phytosterols. It has also been shown that some
266 *Metschnikowia* species are unable to consume lipids from the medium and therefore are limited
267 to their own production (see Fig S1). For all yeast species, lipid synthesis requires oxygen and
268 is therefore impossible in anaerobic conditions. From these elements, one can hypothesize that
269 *Metschnikowia* cells are producing their own lipids from oxygen at the beginning of the
270 fermentation and once the oxygen is exhausted they stop growing. Interestingly, despite the fact
271 that isolated cultures of *S. cerevisiae*, *M. pulcherrima* and *M. fructicola* did not present any
272 mortality, their corresponding mixed cultures (***ScvsMp*** and ***ScvsMf***) present 30% mortality.

273 Moreover, this mortality seems to impact differently both species. In the mixed ***ScvsMp***
274 fermentation only the *S. cerevisiae* cells are dying; in ***ScvsMf*** both species are impacted. To
275 explain the survival of *M. pulcherrima* cells compared to *S. cerevisiae* cells, it can be
276 hypothesized that *Metschnikowia* cells produce toxins (a ferric salt of pulcherriminic acid, (25)
277 that kill *S. cerevisiae* cells (16, 26, 27). In ***ScvsMf*** cultures, the mortality of both species
278 suggests an interaction mechanism different from the one observed between *M. pulcherrima*
279 and *S. cerevisiae*. A two-phase interaction could explain such pattern : *M. fructicola* synthesizes
280 a toxin that kills only *S. cerevisiae* cells and thereafter *M. fructicola* cells dies for another
281 reason, like ethanol sensibility. Indeed, the production of ethanol is almost four time greater in
282 mixed cultures than in isolated *Metschnikowia* fermentations. The reason why no loss of
283 viability is observed for *M. pulcherrima* when placed in mixed culture with *S. cerevisiae* is
284 probably a better tolerance to ethanol stress compared to *M. fructicola*. As a conclusion, the
285 interaction mechanism between the *Metschnikowia* species and *S. cerevisiae* appears to be very
286 different from that between *S. cerevisiae* and the other studied non-saccharomyces species. It
287 is not a competition for resources in sugars or nitrogen but rather the possibility of a killer toxin
288 synthesized by the *Metschnikowia* strains or a competition for a non-measured resource (lipids,
289 vitamins, minerals).
290
291 If we look globally, we can first note that all mixed cultures finished the fermentation. No major
292 antagonistic phenomena were identified between the *S. cerevisiae* strain chosen and the "non-
293 Saccharomyces" yeasts studied. Moreover, despite the presence of differences in yield and
294 interactions between strains, the rapid dominance of *S. cerevisiae* (representing however only
295 10% of the population at t0) results at mixed cultures that are overall little different from *S.*
296 *cerevisiae* pure cultures. This result confirms the good adaptation of *S. cerevisiae* to the
297 winemaking conditions (4–6, 21). Nevertheless, interesting data appear in our research work.

298 Indeed, despite the pre-eminence of *S. cerevisiae*, we have already highlighted transgressive
299 interactions between *S. cerevisiae* and *H. uvarum* and *M. pulcherrima* leading to the
300 overproduction of glycerol in mixed cultures. This observation opens the door for potential
301 more interesting results: when *S. cerevisiae* is inoculated at lower frequencies (0.01, 0.001), the
302 fact that production yields are different between the species can lead to more important
303 transgressive interactions (lower ethanol production for example) in mixed cultures.
304 Furthermore, most of detected interactions can be explained by competition for resources or the
305 production of toxins without indication of an alteration of individual cell behavior linked to the
306 presence of other species in the same medium. This observation linked to our deep phenotyping
307 of population dynamics could allow the modeling of mixed oenological fermentations. Using
308 such model, transgressive interactions linked to consumption of resources could be investigated
309 and inform the choice of couple of species and the protocol of inoculations (specifically by
310 changing the *S. cerevisiae* starting frequency). It could be used to predict mixed cultures
311 dynamics from data on isolated cultures, potentially saving a lot of time and money and open
312 the possibility of ecological engineering. The development of such mathematical model will
313 only be possible thanks to a deep tracking of population dynamics to understand underlying
314 mechanisms of growth and mortality. Therefore, to better identify and understand yeast-yeast
315 interaction mechanisms, we should extend our deep phenotyping of mixed population dynamics
316 to additional species and investigate intra-specific variability as well as interactions with culture
317 environments.
318

319 Mat & Met

320 In this work, we used one strain of 6 different species (one strain by species): *Saccharomyces*
321 *cerevisiae* (***Sc***), *Torulaspora delbrueckii* (***Td***), *Metschnikowia pulcherrima* (***Mp***),

322 *Metschnikowia fructicola* (***Mf***), *Hanseniaspora uvarum* (***Hu***) and *Hanseniaspora opuntiae*
323 (***Ho***). The *S. cerevisiae* strain is a haploid strain marked with GFP (59A-GFP, (2)). The 5 non-
324 *Saccharomyces* strains are natural strains isolated from viognier and sauvignon must in
325 different regions of France. For each species, 3 replicates of isolated cultures were performed
326 (except for *S. cerevisiae* that have a total of 8 replicates in different blocks). In addition, for
327 each non-*Saccharomyces*, 3 replicates of a mixed culture with the Sc strain was performed. In
328 all mixed fermentations, the starting proportion of *S.cerevisiae* cells was 10%. In this text, the
329 fermentations are referred by the species that perform them. The isolated cultures are referred
330 as: ***Sc***, ***Td***, ***Mp***, ***Mf***, ***Hu*** and ***Ho*** and the mixed cultures as ***ScvsTd, ScvsMp***, ***ScvsMf***, ***ScvsHu***
331 and ***ScvsHo***.
332 Initial cultures (12 h, in 50 ml YPD medium, 28 °C) were used to inoculate fermentation at a
333 density of 10^6 cells/ml therefore for mixed culture the density of *S. cerevisiae* cells was $0.1x10^6$
334 cells/mL and the density of non-sacc cells was $0.9x10^6$ cells/mL. Fermentations were carried
335 out in synthetic medium (SM) mimicking standard grape juice (28). The SM used in this study
336 contained 200 g/L of sugar (100 g/L of glucose and 100 g/L of fructose) and 200 mg/L of
337 assimilable nitrogen (as a mixed of ammonium chloride and amino acids). The concentrations
338 of weak acids, salts and vitamins were identical to those described by Seguinot et al. (29). The
339 pH of the medium was adjusted to 3.3 with 10M of NaOH. The SM medium was first saturated
340 with bubbling air during 40 minutes, then it was supplemented with 5 mg/L of phytosterols
341 (85451, Sigma Aldrich) to satisfy the lipid requirements of yeast cells during anaerobic growth.
342 Fermentation took place in 1.1-liter fermentors equipped with fermentation locks to maintain
343 anaerobiosis, at 20 °C, with continuous magnetic stirring (500 rpm) during approximately 200h.
344 CO_2 release was followed by automatic measurements of fermentor weight loss every 20 min.
345 The amount of CO_2 released allowed us to monitor the progress of the fermentation and evaluate
346 the maximum of released CO_2 (***CO2max***) as well as the maximum rate of CO_2 released (***Vmax***).

347 Samples were harvested after 6h, 12h and 24h, then every 12h during the first week and every
348 24h during the second week of fermentation. At each sample, the population density, the
349 viability and the proportion of *S. cerevisiae* cells were determined using a flow cytometer BD
350 Accuri™ C6 Plus as described in Delobel et al. (30). From these population data and by fitting
351 a growth population model (with the grofit package of R, (31), the carrying capacity (***K***) and
352 the maximum growth rate (***mu***) for each fermentation were estimated.
353 The final concentration of carbon metabolites in the medium (acetate, succinate, glycerol,
354 alpha-ketoglutarate, pyruvate, ethanol, glucose and fructose) with high-pressure liquid
355 chromatography (32). From these metabolite concentrations, we first calculated the consumed
356 sugar concentration as the difference between the final and the initial concentration of either
357 glucose or fructose. Then we calculated the yield of metabolite production by dividing the final
358 concentration by the corresponding consumed sugar concentration. Finally, we report these
359 yields to the yield of *S. cerevisiae* isolated cultures that we consider as our reference.
360 At last, the ammonium concentration after 100h of fermentation was determined enzymatically
361 with R-Biopharm (Darmstadt, Germany) and the free amino acid content of the must was
362 estimated with cation exchange chromatography with post-column ninhydrin derivatization
363 (33).
364 The experimental work was performed in 5 different blocks. Each block was composed of three
365 replicates of non-*Saccharomyces* fermentations (for example ***Td***), three replicates of the
366 corresponding mixed fermentations with *S. cerevisiae* (for example ***ScvsTd***) and one or two
367 fermentations of isolated *S. cerevisiae* cells (***Sc***). The block effect was evaluated on the
368 parameters of the ***Sc*** fermentation. For most of the studied parameters the block effect was not
369 significant. For the parameters where there was a block effect (mu and K), a statistical
370 correction for the block effect did not modify our results. Therefore, for simplification, we
371 consider that there was no block effect and compare all fermentations without any correction

372 for block effect. For each measured parameters, an anova was performed to evaluated the type
373 of fermentation (***Sc***, ***Td***, ***Mp***, ***Mf***, ***Hu***, ***Ho***, ***ScvsTd***, ***ScvsMp***, ***ScvsMf***, ***ScvsHu*** and ***ScvsHo***)
374 effect and then a Tukey-test was performed to determine statistical groups and two by two
375 statistical differences. All statistical analyses were performed using R (34) and Rstudio (35).
376 All data, analysis and figures scripts can be found in this github adresse:
377 https://github.com/tnidelet/Harle-et-al-2018.
378

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476

Tables

Table 1

Parameter values (mean ± standard deviation) for each type of fermentation.

	Mu	K	prop	Vmax	MaxCO2	Sugar	NAS	Ethanol	Glycerol	Succinate	Acetate	Pyruvate	Alpha
Sc	5.6±0.8^{cd}	1.6±0.2^{bc}	NA	0.99±0.03^{a}	88.2±2.2^{a}	0.2±0.2^{f}	10.4±8.6^{d}	95.2±8.4^{a}	5.3±0.4^{c}	1.05±0.28^{a}	0.63±0.06^{d}	0.12±0.08^{b}	0.08±0.04^{ab}
Ho	7.7±2.3^{bc}	1.6±0.1^{bc}	NA	0.42±0.02^{d}	46.4±0.6^{d}	92.3±1.4^{b}	122.2±3.5^{bc}	50.1±0.2^{c}	5.2±0.0^{c}	0.35±0.00^{b}	0.99±0.02^{b}	0.13±0.05^{ab}	0.09±0.1^{ab}
ScvsHo	5.7±0.3^{bcd}	**2.3±0.1**^{a}	0.83±0.02^{b}	0.57±0.01^{c}	82.7±0.6^{b}	23.3±1.3^{de}	**6.4±2.4**^{d}	87.2±0.8^{d}	**6.0±0.0**^{abc}	0.42±0.04^{b}	0.84±0.01^{c}	**0.08±0.00**^{b}	**0.03±0.0**^{b}
Hu	15.6±2.9^{a}	1.4±0.1^{cd}	NA	0.34±0.04^{d}	30.7±0.5^{e}	134.3±11.2^{a}	173.3±24.8^{b}	34.9±1.1^{d}	5.5±0.2^{bc}	0.66±0.03^{b}	1.15±0.03^{a}	0.17±0.04^{ab}	0.14±0.09^{ab}
ScvsHu	8.7±0.6^{b}	**1.7±0.1**^{b}	0.76±0.01^{c}	0.53±0.04^{c}	82.1±1.5^{b}	16.5±6.5^{e}	**4.6±4.2**^{d}	89.5±3.8^{d}	**6.6±0.3**^{a}	**0.60±0.06**^{b}	0.86±0.07^{bc}	0.16±0.04^{ab}	0.09±0.06^{ab}
Mf	3.4±0.8^{de}	0.8±0.05^{e}	NA	0.17±0.04^{e}	20.6±1.2^{f}	135.8±9.7^{a}	256.6±2.9^{a}	20.6±0.3^{d}	2.8±0.1^{e}	0.29±0.01^{b}	0.13±0.01^{g}	0.10±0.01^{b}	0.10±0.00^{ab}
ScvsMf	**3.1±0.2**^{de}	1.3±0.03^{d}	0.96±0.02^{a}	0.76±0.03^{b}	86.8±0.7^{ab}	9.9±2.1^{ef}	**1.8±0.1**^{d}	92.1±3.2^{d}	**6.0±0.1**^{abc}	0.58±0.02^{b}	0.46±0.02^{e}	0.12±0.01^{b}	0.08±0.01^{ab}
Mp	2.4±0.4^{e}	0.6±0.01^{e}	NA	0.16±0.02^{e}	21.8±0.4^{f}	142±3.7^{a}	305.5±5.9^{a}	23.4±1.8^{d}	3.7±0.2^{d}	0.26±0.03^{b}	0.07±0.06^{g}	0.29±0.04^{ab}	0.13±0.01^{ab}
ScvsMp	2.8±0.1^{de}	1.3±0.01^{d}	0.50±0.02^{d}	0.68±0.04^{b}	83.3±2.2^{b}	20.8±6.0^{de}	**2.1±0.2**^{d}	86.5±3.4^{ab}	**6.1±0.1**^{ab}	0.52±0.04^{b}	0.46±0.02^{e}	0.24±0.01^{ab}	**0.19±0.01**^{ab}
Td	5.3±1.0^{cde}	1.2±0.05^{d}	NA	0.51±0.06^{c}	65.7±4.0^{c}	63.5±11.5^{c}	112.6±21.1^{c}	56±5.3^{c}	4.0±0.3^{d}	0.42±0.04^{b}	0.17±0.03^{fg}	0.14±0.06^{ab}	0.02±0.01^{b}
ScvsTd	**4.2±0.6**^{de}	1.5±0.01^{bcd}	0.32±0.02^{e}	0.72±0.02^{b}	83.9±3.2^{ab}	35.8±5.2^{d}	**1.8±0.1**^{d}	76.8±3.7^{b}	5.2±0.2^{c}	0.63±0.06^{b}	0.27±0.06^{f}	**0.20±0.11**^{ab}	0.05±0.03^{b}

Mu : 10^6cells.mL^{-1}.h^{-1}; K : 10^8 cells.mL^{-1}, prop : no unity, Vmax : g.L^{-1}.h^{-1}, MaxCO2 : g.L^{-1}, Final Sugar : g.L^{-1}, Final NAS : mg.L^{-1}, Ethanol : g.L^{-1}, Glycerol : g.L^{-1}, Succinate : g.L^{-1}, Acetate : g.L^{-1}, Pyruvate : g.L^{-1}, Alpha : g.L^{-1}

Bold : values of mixed culture, that are not between the two values of corresponding isolated cultures, sign of transgressive interactions.

Superscript : Statistical groups obtain from a Tukey test

Figures

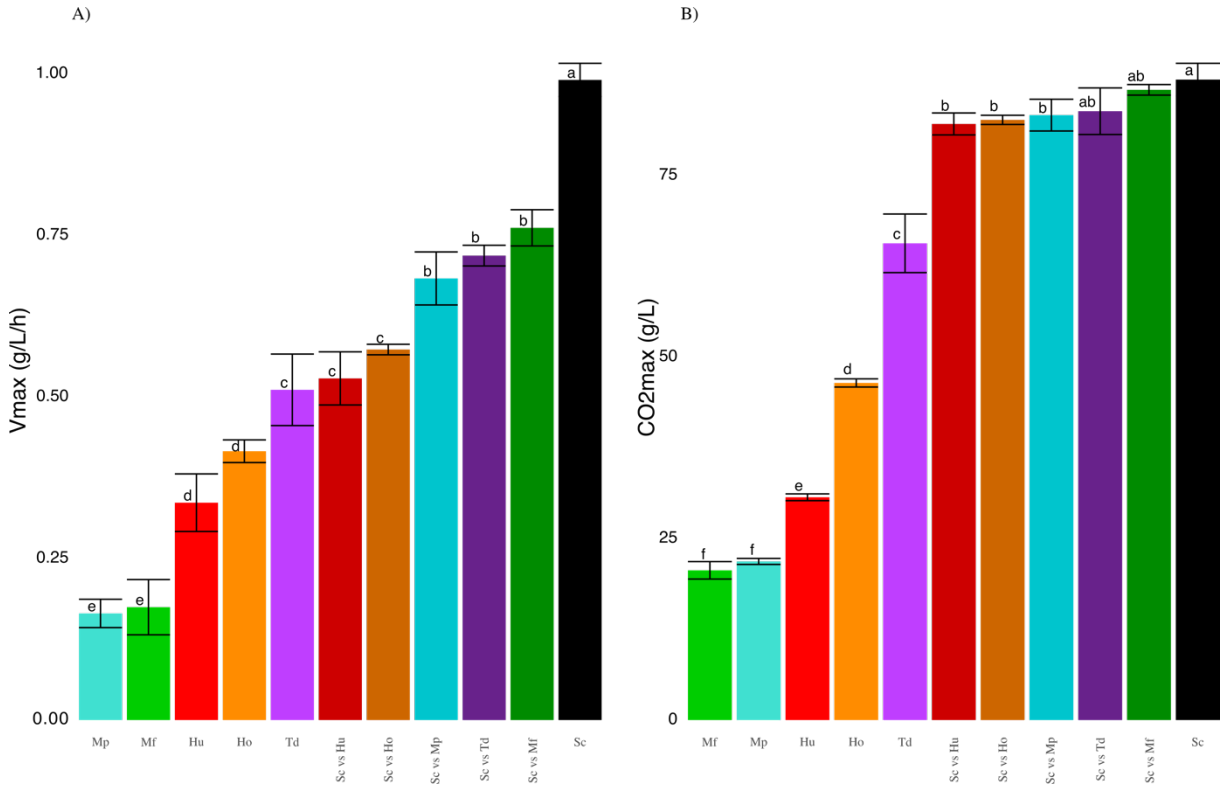
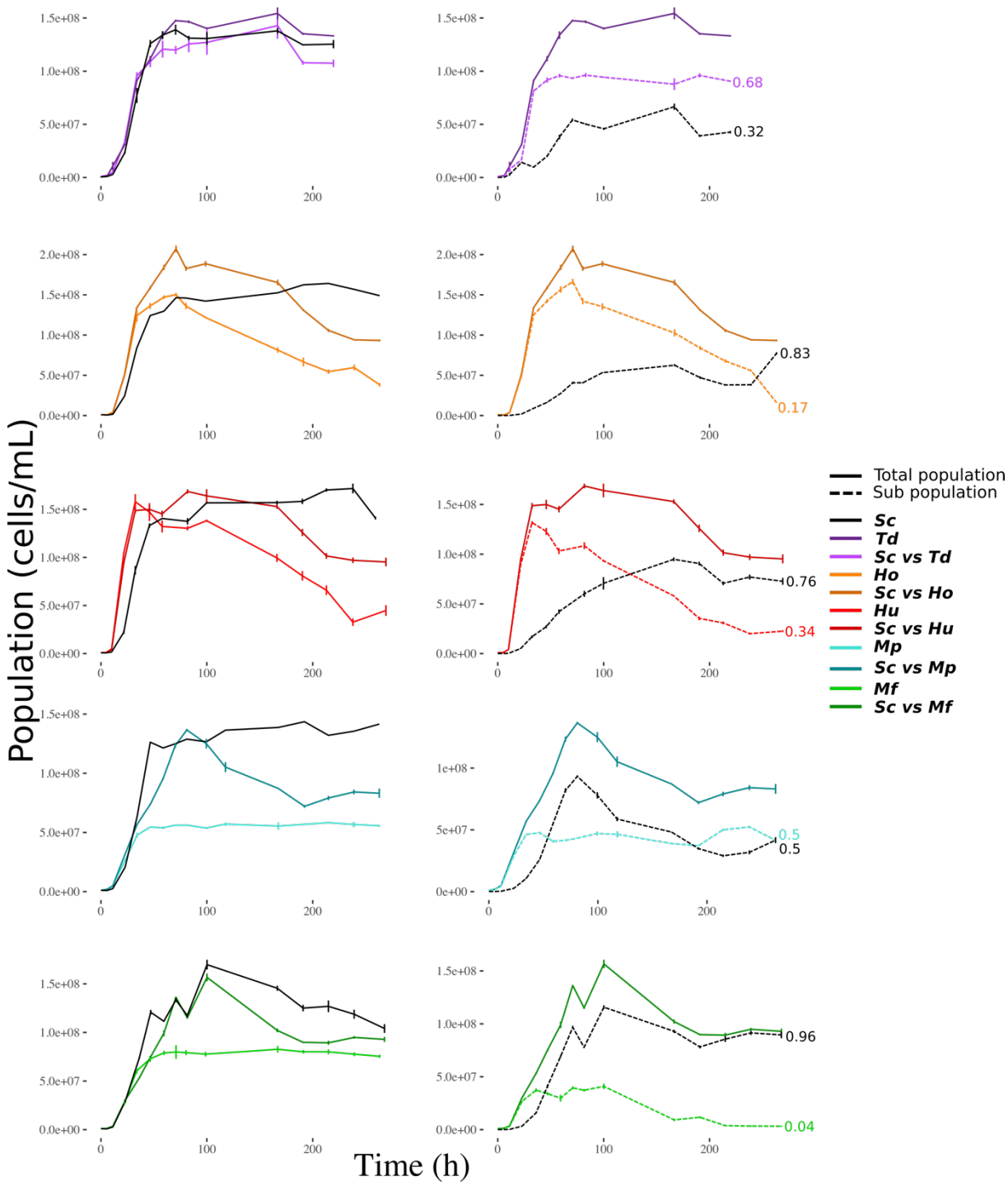


FIG 1

Representation of a) the maximum rate of CO_2 production (***Vmax***) and b) the total CO_2 produced in function of the species driving each fermentation (average ± standard deviation). The small letters indicate the statistical groups from a Tukey analysis.



508

509 **FIG2**

510 **Global monitoring of the kinetics of the total living population (left), and sub-population**

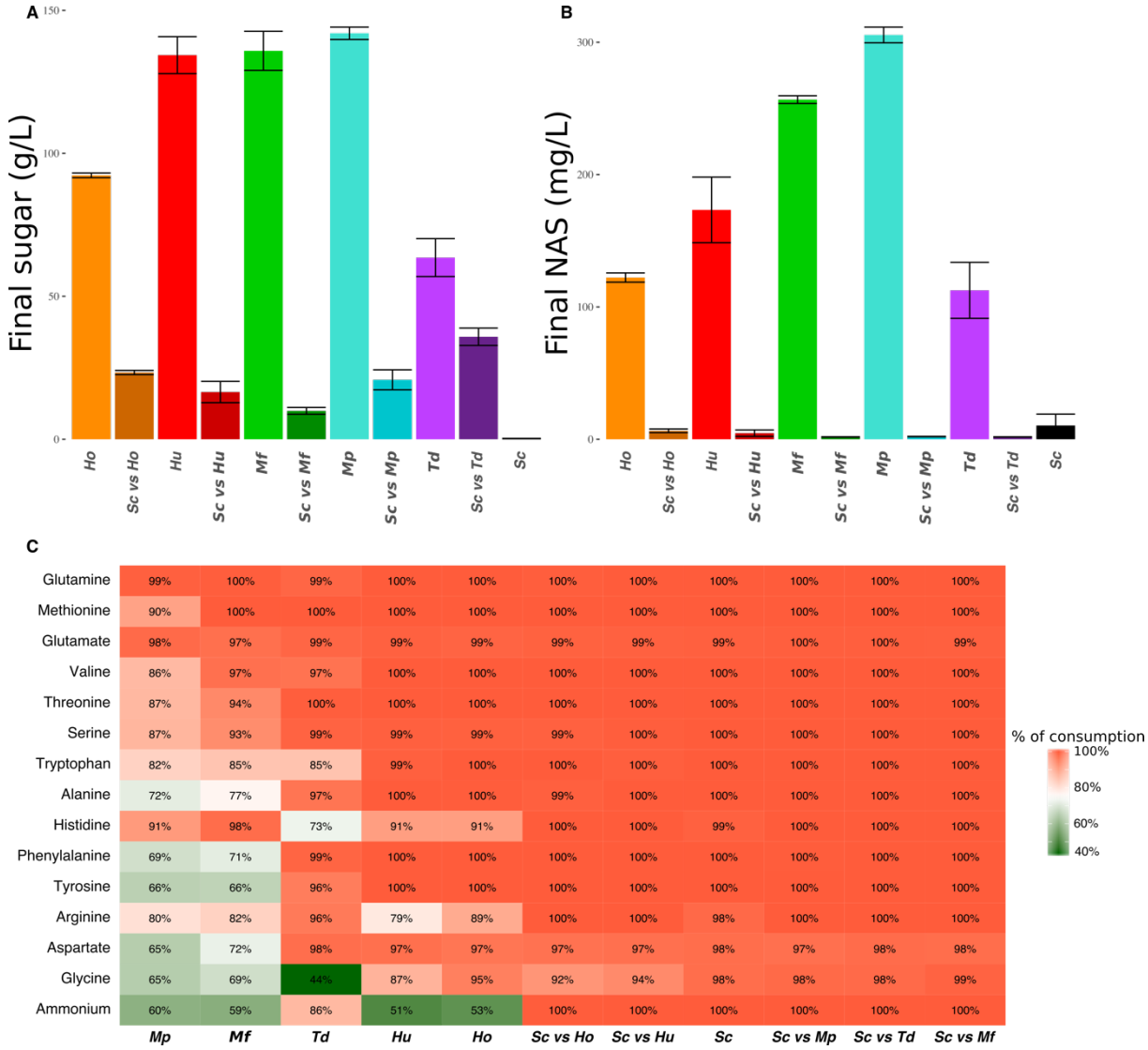
511 **in the mixed cultures (right) across the fermentation**. All points represent a sample (average

512 ± standard error). The full lines are for total population and the dashed lines for the two sub

513 populations into mixed cultures. At the end of dashed lines, the final proportion of both sub-

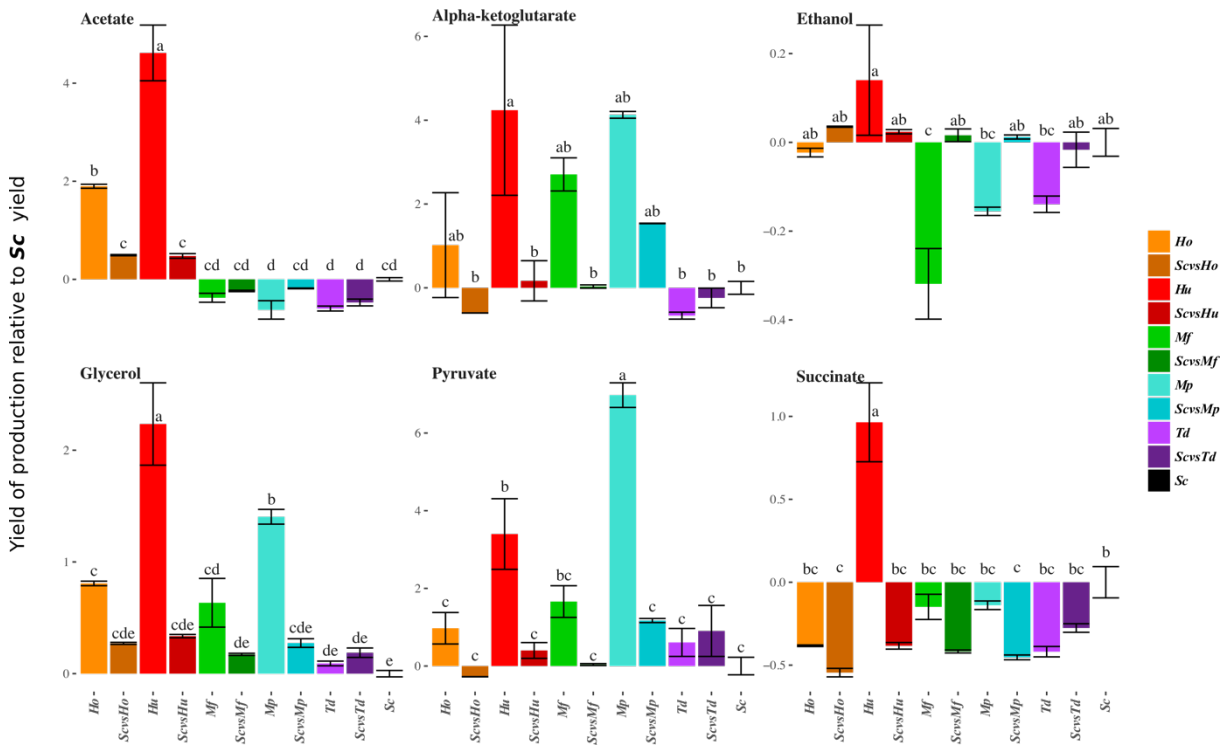
514 populations in mixed cultures is indicated. The light colors represent pure culture of 'non-

515 *Saccharomyces*' strains and the darker to the corresponding culture in competition with *S.*
516 *cerevisiae*. The isolated cultures of *S. cerevisiae* are represented in black. ***Sc, Mp, Mf, Ho, Hu,***
517 ***Td, ScvsMp, ScvsMf, ScvsHo, ScvsHu, ScvsTd*** correspond respectively to cultures with:
518 isolated *S. cerevisiae*, isolated *M. pulcherrima*, isolated *M. fructicola*, isolated *H. opuntiae*,
519 isolated *H.uvarum*, isolated *T. delbrueckii*, *M. pulcherrima* mixed with *S. cerevisiae*, *M.*
520 *fructicola* mixed with *S. cerevisiae*, *H. opuntiae* mixed with *S. cerevisiae*, *H. uvarum* mixed
521 with *S. cerevisiae* and *T. delbrueckii* mixed with *S. cerevisiae*.
522



523
524 **FIG3**

525 **Consumption of sugars and nitrogen assimilable sources (NAS) for each type of**
526 **fermentation.** A) Final concentration of sugar (average ± standard deviation). B) Final
527 concentration of NAS (average ± standard deviation). C) Percentage of consumption of each
528 NAS in each type of fermentation represented as a color gradient from green (<75 %) to red (>
529 75%). ***Sc, Mp, Mf, Ho, Hu, Td, ScvsMp, ScvsMf, ScvsHo, ScvsHu, ScvsTd*** correspond
530 respectively to cultures with: isolated *S. cerevisiae*, isolated *M. pulcherrima*, isolated *M.*
531 *fructicola*, isolated *H. opuntiae*, isolated *H.uvarum*, isolated *T. delbrueckii*, *M. pulcherrima*
532 mixed with *S. cerevisiae*, *M. fructicola* mixed with *S. cerevisiae*, *H. opuntiae* mixed with *S.*
533 *cerevisiae*, *H. uvarum* mixed with *S. cerevisiae* and *T. delbrueckii* mixed with *S. cerevisiae*.
534



535

536 **FIG4**

537 Yield (average ± standard deviation) of carbon metabolite production relative to the yield of
538 production of isolated *S. cerevisiae* cultures for acetate, alpha-ketoglutarate, ethanol, glycerol,
539 pyruvate and succinate and each type of fermentation. ***Sc, Mp, Mf, Ho, Hu, Td, ScvsMp,***
540 ***ScvsMf, ScvsHo, ScvsHu, ScvsTd*** correspond respectively to cultures with: isolated *S.*

541 *cerevisiae*, isolated *M. pulcherrima*, isolated *M. fructicola*, isolated *H. opuntiae*, isolated
542 *H.uvarum*, isolated *T. delbrueckii*, *M. pulcherrima* mixed with *S. cerevisiae*, *M. fructicola*
543 mixed with *S. cerevisiae*, *H. opuntiae* mixed with *S. cerevisiae*, *H. uvarum* mixed with *S.*
544 *cerevisiae* and *T. delbrueckii* mixed with *S. cerevisiae*.