Kinetic analysis of yeast-yeast interactions in oenological conditions

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keywords: winemaking fermentation, population dynamics, non-*Saccharomyces* species, transgressive interactions, ecosystems

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, namely competition, predation, commensalism and mutualism. Traditional wine fermentation is a complex microbial process performed by *Saccharomyces* and non-*Saccharomyces* yeast species. To better understand the different interactions occurring

within wine fermentation, isolated yeast cultures were compared with mixed co-cultures of one reference strain of *S. cerevisiae* with one strain of four non-*Saccharomyces* yeast species (*Metschnikowia pulcherrima, M. fructicola, Hanseniaspora opuntiae and H. uvarum*). In each case, we studied population dynamics, resource consumption and the production of metabolites from central carbon metabolism. This deep phenotyping of competition kinetics allowed us to identify the main mechanisms of interaction between species. *S. cerevisiae* competed with *H. uvarum* and *H. opuntiae* for resources although both *Hanseniaspora* species were characterized by a strong mortality either in isolated or mixed fermentations. *M. pulcherrima* and *M. fructicola* displayed a negative interaction with the *S. cerevisiae* strain tested, with a decrease in viability in co-culture, probably due to iron depletion via the production of pulcherriminic acid. Overall, this work highlights the importance of measuring cell populations and their metabolite kinetics to understand yeast-yeast interactions. These results are a first step towards ecological engineering and the rational design of optimal multi-species starter consortia using modeling tools.

Introduction

In natural or anthropized environments, microbial species are part of an ecosystem and interact positively or negatively, forming 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 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 (Barot et al., 2017). Efforts are now being made to design

optimal consortia of various species and strains whose interactions will be exploited to maximize given criteria such as fermentation quality, aromatic complexity or other organoleptic characteristics.

Wine fermentation is both an economically and societally important food ecosystem, where the addition of fermentation 'starters' composed of selected yeasts at the beginning of the fermentation process is common. In fact, around 80% of oenological fermentations worldwide are conducted with starters (Marsit and Dequin, 2015; Sablayrolles, 2008). Most often, these "starters" are only composed of a single *Saccharomyces cerevisiae* strain selected for its ability to complete fermentation. Indeed, numerous experiments have shown that *S. cerevisiae*, with an initially low population, most often becomes the predominant species at the end of the fermentation, demonstrating its superior fermentative abilities (García-Ríos et al., 2014; Jolly et al., 2014; Pinto et al., 2015; Varela, 2016). However, in recent years, multi-species starters have emerged, aiming at increasing the aromatic complexity of wines. They most often combine one strain of *S. cerevisiae* allowing to complete fermentation and another species, often from a different genus, contributing to a greater variety of flavors (García-Ríos et al., 2014; Ivey et al., 2013; Jolly et al., 2014; Varela, 2016).

Indeed, there are numerous experiments and even industrial products making use of such mixed starters to improve wine's organoleptic qualities (Jolly et al., 2014; P et al., 2008; Sadineni et al., 2012). The non-*Saccharomyces* strains used in these experiments are very diverse, with more than 23 different species (Jolly et al., 2014) including *Torulaspora delbrueckii*, *Metschnikowia pulcherrima*, *Metschnikowia fructicola*, *Hanseniaspora opuntiae* and *Hanseniaspora uvarum*. *S*pecies in the *Metschnikowia* genus ferment poorly in oenological conditions but can have interesting attributes (Varela, 2016): in conjunction with *S. cerevisiae*, *M. pulcherrima* can reduce ethanol concentrations (Contreras et al., 2014; Varela, 2016),

74 increase ‘citrus/grape fruit’ and ‘pear’ attributes (Belda et al., 2015), as well as allow the
75 persistence of ‘smocky’ and ‘flowery’ characteristics (Elena et al., 2015). *M. pulcherrima* also
76 has an amensalism effect on *S. cerevisiae* through iron depletion via the production of
77 pulcherriminic acid (Oro et al., 2014). *M. fructicola* has been less studied and never in
78 conjunction with *S. cerevisiae* although it presents the interesting ability to inhibit *Botrytis*
79 growth (Kurtzman and Droby, 2001). Last, the *Hanseniaspora* genus, studied in sequential or
80 simultaneous fermentation with *S. cerevisiae,* has been shown to increase volatile compound
81 production during winemaking (Varela, 2016). It notably increased the ‘tropical fruit’, ‘berry’,
82 ‘floral’ and ‘nut aroma’ characters (Hu et al., n.d.), that were linked to higher concentrations of
83 acetate esters, esters of MCFAs, isoamyl alcohol, 2-phenylethanol and α-terpineol (Tristezza et
84 al., 2016).

85

86 Despite these various studies, the composition and protocol of inoculation of these multi-strains
87 starters are still very empirical and only based on the input/output balance, without considering
88 the dynamics of the microbial populations or their interactions. This lack of knowledge about
89 yeast-yeast interactions prevents implementing a rational design of multi-strain starters (Song
90 et al., 2014). To address this problem, we decided to focus our study on population dynamics
91 and metabolite production during oenological fermentations performed in isolated or mixed
92 yeast cultures. Since our goal was not to obtain optimal mixes but to understand the mechanism
93 of microbial interaction, we chose to compare the population dynamics and yields between
94 isolated cultures of strains from five species (one *S. cerevisiae* and four non-*Saccharomyces*)
95 and four corresponding mixed cultures always including the *S. cerevisiae* strain as reference.
96 We were thus able to identify key microbial interaction mechanisms that are further discussed.
97

Results

In this work, we compared in winemaking conditions the performance of isolated cultures of five different strains from five yeast species (*Saccharomyces cerevisiae*, *Metschnikowia pulcherrima*, *Metschnikowia fructicola*, *Hanseniaspora opuntiae* and *Hanseniaspora uvarum*) and mixed co-cultures combining each of the four non-*Saccharomyces* species with one GFP-labelled *S. cerevisiae* strain representing 10% of the initial inoculate. As our research objective was not to evaluate the final product but to study interaction dynamics between yeast species, it was decided to stop the monitoring of fermentations at a given time, even if the sugar supply was not completely exhausted. For all fermentation with the *S. cerevisiae* reference strain, sugars were exhausted after around 200-220 h while in fermentations with isolated non-*Saccharomyces* strains, the sugar supply was still not exhausted after 400h. Here, we focused on the first 300 hours of fermentation.

By comparing the output of isolated and mixed cultures, we evaluated the intensity of yeast-yeast interactions and/or their consequences on ecosystem service production.

CO_2 kinetics

We first investigated the influence of species and co-culture on the dynamics of CO_2 production (proportional to sugar consumption), which is a good indicator of the fermentation progress. Indeed, CO_2 production is easy to monitor (based on weight measurement) and is directly proportional to ethanol synthesis and sugar consumption. The values of the maximum rate of CO_2 production (***Vmax***, Fig. 1a, Table. 1) and of the maximum CO_2 produced were estimated (***CO_2max***, Fig. 1b, Table. 1). ***Vmax*** was highly dependent on the species (p.value < 0.001): *S. cerevisiae* cultures (***Sc***) displayed the highest value ($\boldsymbol{Vmax_{Sc}}$ = 0.99 ± 0.02 $g.L^{-1}.h^{-1}$), followed by both *Hanseniaspora* species ($\boldsymbol{Vmax_{Hu}}$ = 0.33 ± 0.04 $g.L^{-1}.h^{-1}$, and $\boldsymbol{Vmax_{Ho}}$ = 0.42 ± 0.02 $g.L^{-1}.h^{-1}$) and finally both *Metschnikowia* species ($\boldsymbol{Vmax_{Mp}}$ = 0.165 ± 0.02 $g.L^{-1}.h^{-1}$and $\boldsymbol{Vmax_{Mf}}$

123 = 0.17 ± 0.04 $g.L^{-1}.h^{-1}$). The four mixed cultures had intermediate ***Vmax*** values between those
124 of ***Sc*** and the highest **Vmax** of all *non-sacc* cultures (Fig. 1a). Mixed cultures containing
125 *Metschnikowia* species had significantly higher ***Vmax*** values than those containing
126 *Hanseniaspora* species (Fig 1a). Although we did not monitor all cultures until the exhaustion
127 of glucose and fructose, it was however possible to estimate the capacity of a given species to
128 complete fermentation by estimating the amount of CO_2 produced during the first 300 hours.
129 ***Sc*** fermentations finished after around 220 hours with a $\boldsymbol{CO2max_{Sc}}$ = 88.2 ± 2.2 $g.L^{-1}$. We
130 therefore can make the hypothesis that all cultures that produced more than 80g $CO_2.L^{-1}$ (90%
131 of ***Sc*** maximum) within 300 hours will be able to complete fermentation. Under this
132 assumption, all mixed cultures, but not isolated non-*Saccharomyces* cultures, would eventually
133 complete fermentation. Among the latter cultures , both *Hanseniaspora* species had the highest
134 $\boldsymbol{CO_2max}$ (***Hu*** 30 ± 0.4 $g.L^{-1}$, and ***Ho*** 46 ± 0.6 $g.L^{-1}$) followed by *Metschnikowia* species (***Mp*** 22
135 ± 0.4 $g.L^{-1}$ and ***Mf*** 20 ± 1 $g.L^{-1}$).
136
137 **Population Kinetics**
138 We also looked at population dynamics in each culture (Fig. 2) and determined the maximum
139 growth rate of the population ($\boldsymbol{\mu}$), the maximum population size, also termed carrying capacity
140 (***K***) and the relative abundance of each species after 300 hours of mixed culture, corresponding
141 in our case to the end of the monitoring period (Table. 1). Fermentations with *S. cerevisiae*
142 alone went through an exponential growth rate ($\boldsymbol{\mu_{Sc}}$ = 0.15 ± 0.02 h^{-1}) and reached a maximum
143 population of around $1.5*10^8$cells.mL^{-1} ($\boldsymbol{K_{Sc}}$ = 1.55 ± 0.15 10^8cells.mL^{-1}) that remained constant
144 until the end of the fermentation. Fermentations with either *Hanseniaspora* species alone had a
145 growth dynamic similar to ***Sc*** at the beginning of the fermentation but a higher growth rate ($\boldsymbol{\mu_{Ho}}$
146 = 0.19 ± 0.03 h^{-1}, $\boldsymbol{\mu_{Hu}}$ = 0.62 ± 0.18 h^{-1}). On the opposite, their stationary phase was quite
147 different from that of ***Sc*** and characterized by a higher cell mortality with a population drop of

148 about 70% by the end of the process. Fermentations performed by *Metschnikowia* species in
149 isolation had growth dynamics mostly similar to ***Sc*** fermentations: a similar growth rate (μ_{mp} =
150 0.18 ± 0.03 h^{-1}, μ_{Mf} = 0.17 ± 0.2 h^{-1}), no mortality during the stationary phase but a much
151 reduced maximum population (K_{Mp} = 0.57 ± 0.01 10^6cells.mL^{-1}, K_{Mf} = 0.8 ± 0.05 10^6cells.mL^{-}
152 ^{1}). In most cases, mixed cultures displayed an intermediate pattern between the two
153 corresponding isolated cultures (Fig. 2). However, mixed or isolated cultures with
154 *Metschnikowia* displayed different cell mortality rates during the stationary phase: in the case
155 of ***ScvsMp*** fermentations, only the *S. cerevisiae* population decreased significantly during the
156 stationary phase, while in ***ScvsMf*** fermentations, both subpopulations significantly decreased.
157 As a measure of fitness, we also followed the variations of *S. cerevisiae* frequency along the
158 fermentation. In all mixed cultures, *S. cerevisiae* was found dominant (frequency > 50%) in the
159 end, increasing significantly during fermentation from 10% initially to frequencies varying
160 between 50% (***ScvsMp***) and 96% (***ScvsMf***) (Table. 1)

161 **Sugar and NAS consumption**

162 We then looked at the final concentration of resources: sugars (fructose and glucose) and NAS
163 (sum of all assimilable nitrogen sources, i.e. ammonium and amino-acids) (Fig. 3, Table 1). In
164 ***Sc*** fermentations, less than 0.1% of the initial concentration of both sugars remained (Fig. 3a).
165 As seen in the paragraph concerning CO_2 production, non-*Saccharomyces* species in
166 monocultures did not complete fermentation in the 300h period and left respectively 45% of
167 sugars for ***Ho***, 67% for ***Hu***, 68% for ***Mf*** and 71% for ***Mp***. Furthermore, all species except *H.*
168 *opuntiae* preferentially consumed glucose (see supplementary figure 1). Sugar consumption
169 was higher in mixed cultures than in single non-*Saccharomyces* species cultures (Table 1).
170 However, it was still lower than in ***Sc*** species cultures, also with a preference for glucose. This
171 indicates the major impact of *S. cerevisiae* on sugar consumption (consistent with the CO_2
172 production observed), compared to the other species studied.

173 The consumption of nitrogen assimilable sources (NAS, amino-acids and ammonium)
174 displayed the same pattern (Fig. 3b). NAS were almost entirely consumed both in ***Sc*** isolated
175 cultures and in all co-cultures, whereas in non-*Saccharomyces* isolated cultures the fraction of
176 NAS consumed varied between 84% and 94%. However, the preference for different nitrogen
177 sources varied with each species (Fig. 3c). Both *Hanseniaspora* species had similar behaviors,
178 consuming only half of the available ammonium, 90% of histidine and 89% or 79% of arginine
179 (Fig. 3c). *Metschnikowia* species presented a similar pattern. It was possible to classify these
180 non-*Saccharomyces* species preferences for the various NAS. The resulting ranking by order
181 of preference was glutamine, methionine, glutamate, valine, threonine, serine, tryptophan,
182 alanine, histidine, arginine, aspartate, glycine and, surprisingly last, ammonium.
183
184 **Metabolite production**
185 In parallel with must resources consumption monitoring, we also investigated the production
186 of metabolites from CCM (Central Carbon Metabolism): ethanol, glycerol, succinate, pyruvate,
187 acetate and alpha-ketoglutarate (Table 1). These measurements of metabolite production were
188 taken after 300 hours when sugars consumptions were quite different from one culture to
189 another depending on their dynamics. To allow figures comparison, we computed the
190 production yield (total production / sugar consumption) for each culture and, from these data,
191 we then estimated this yield relatively to that of ***Sc*** in isolated culture (Fig. 4).
192 In the case of ethanol, only ***Mf*** fermentations had a relative yield significantly inferior (-32%)
193 to 0 (0 being ***Sc*** yield). For glycerol, all isolated non-*Saccharomyces* fermentations had a
194 greater yield than ***Sc*** and mixed fermentations were intermediate between (but not significantly
195 different from) the corresponding isolated cultures. For acetate, only *Hanseniaspora* strains
196 displayed a higher yield (Fig. 4).

Finally, all mixed cultures seemed to have a lower succinate yield than both corresponding isolated cultures (but not significantly after correction for multiple tests).

For each fermentation, the total production of metabolites resulted from the combination of species yields, total sugar consumption and respective population dynamics during fermentation. Therefore, differences observed in the total productions of mixed cultures were the consequences of additive or subtractive effects observed for these 3 components. Considering ethanol, its total production was directly linked to the consumption of resources and all mixed cultures were equivalent to ***Sc*** fermentations (Table 1, supplementary figure S2). The case of glycerol was more interesting. Indeed, even if the average sugar consumption was lower in ***ScvsHu*** and ***ScvsMp*** mixed cultures than in isolated ***Sc*** culture, the total production of glycerol was significantly higher than that of 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 resulted from the positive combination of the greater glycerol yield by *Hanseniaspora* and *Metschnikowia* and their population dynamics. For all other metabolites, the total production of mixed cultures was not significantly different from the corresponding isolated cultures (Table 1).

Discussion

This study presents one of the first works focusing on the population dynamics and kinetics of yeast-yeast interactions between two species during the alcoholic fermentative process. By contrast, to our knowledge, most of the previous studies were concerned only with input-output balances (García et al., 2016; Varela, 2016). Firstly, it was possible to group the non-

221 *Saccharomyces* strains performance according to their genus (see PCA in supplementary
222 figures S3); *H. uvarum* and *H. opuntiae* both presented similar phenotypes. Their population
223 dynamics were characterized by a similarly high growth rate (reaching a maximum population
224 similar to *S. cerevisiae*), followed by a long mortality phase where viability dropped to 30%
225 (Moreira et al., 2008; Tristezza et al., 2016). Their NAS consumption was also similar, with
226 half of the available ammonium not being consumed. Even their yield and total production of
227 CCM metabolites were very similar.
228 For almost all these characteristics, *Hanseniaspora* mixed cultures presented intermediate
229 phenotypes compared to the corresponding isolated cultures. The only exception was the total
230 production of glycerol that seemed to be superior in mixed cultures whereas their sugar
231 consumption was inferior; this is characteristic of a transgressive interaction (often referred to
232 as over-yielding), i.e. a situation in which the ecosystem performance is higher than that of the
233 best-yielding species present (when cultivated alone). This glycerol overproduction in mixed
234 cultures has already been observed in previous works (Ciani and Ferraro, 1996; Tristezza et al.,
235 2016) but is not systematic depending on the strains and experimental conditions (Moreira et
236 al., 2008). In the present study, the glycerol overproduction and sugar consumption observed
237 in mixed cultures could be explained by the joint effect of population dynamics (*S. cerevisiae*
238 slowly dominating the population), resource consumptions (the ***Ho*** and ***Hu*** fermentation
239 leaving ⅔ of sugars) and the glycerol yields of *Hanseniaspora* strains that were two to three
240 times higher that of *S. cerevisiae*.
241
242 Regarding *Hanseniaspora* species, ***Mp*** and ***Mf*** isolated cultures were characterized by a short
243 growth phase reaching a small carrying capacity and followed by a long stationary phase with
244 no mortality. Nevertheless, despite this very high viability, the resources measured (sugars and
245 nitrogen) were not entirely consumed. The reason why the cells stopped growing remains

246 unclear. A possible explanation could be linked to unmonitored resources. For instance, the
247 third most important resource in the synthetic medium was lipids, available as phytosterols.
248 Concurrently, it has been shown that some *Metschnikowia* species were unable to import lipids
249 from the medium, depending instead on their own lipid synthesis (see Fig S1). For all yeast
250 species, lipid synthesis requires oxygen and is therefore impossible in anaerobic conditions.
251 From these data, one can hypothesize that *Metschnikowia* cells were able to produce their own
252 lipids in the aerobic conditions prevailing at the beginning of the fermentation. Then, with the
253 progress of fermentation and ensuing oxygen limitation, *Metschnikowia* cells became unable to
254 synthetize the necessary lipids and stopped multiplying.
255 Interestingly, even though *S. cerevisiae*, *M. pulcherrima* and *M. fructicola* mortality rates were
256 low in isolated cultures, the corresponding mixed cultures (***ScvsMp*** and ***ScvsMf***) presented 30%
257 mortality. Moreover, this mortality seemed to impact both species differently. In the mixed
258 ***ScvsMp*** culture, only *S. cerevisiae* cells eventually died, whereas in ***ScvsMf*** both species were
259 negatively affected. The survival of *M. pulcherrima* cells compared to *S. cerevisiae* cells could
260 be explained by the production of pulcherriminic acid by *M. pulcherrima* (MacDonald, 1965).
261 Indeed, pulcherriminic acid is known to deplete iron from the medium, which has a lethal effect
262 on *S. cerevisiae* cells (Oro et al., 2014; Sipiczki, 2006; Türkel and C, 2009). In ***ScvsMf*** cultures,
263 the mortality observed in both species suggests a more complex mechanism of interaction
264 (although it is not clear whether *M. fructicola* also produces pulcherriminic acid; Kurtzman and
265 Droby, 2001). To explain these results, we could hypothesize the conjunction of two different
266 mechanisms of interaction. It could be that *M. fructicola* synthesized a metabolite
267 (pulcherriminic acid?) impacting the viability of *S. cerevisiae* cells (through iron depletion?),
268 with *M. fructicola* cells dying thereafter for another reason such as sensitivity to ethanol.
269 Indeed, the production of ethanol was almost four times higher in mixed cultures than in
270 isolated *Metschnikowia* fermentations. Under such hypothesis, the reason why no loss of

viability was observed for *M. pulcherrima* in mixed culture with *S. cerevisiae* could probably be related to a better tolerance of *M. pulcherrima* to ethanol stress compared to *M. fructicola*. As in *Hanseniaspora*, we also observed an overproduction of glycerol in *Metschnikowia* mixed cultures. This also could be explained by the same conjunction of yield, sugar consumption and population dynamics. These overproductions of glycerol open the way for potentially more interesting results. In fact, one can hypothesize that, by exploiting this specific yield and population dynamics, for example by inoculating the S. *cerevisiae* strain at lower relative frequencies (0.01, 0.001, 0.0001), more important transgressive interactions (such as lower ethanol production) could be achieved in such mixed cultures.

For all *Metschnikowia* and *Hanseniaspora* mixed cultures, we only discussed transgressive interactions when mixed cultures over-produced (or under-produced) a given metabolite. Indeed, it was very difficult to identify interactions when the productions of mixed fermentations were within the range of isolated cultures productions. As the relative frequency of both strains in mixed cultures evolved during fermentation, it was difficult to link the final mix to the contributions of each species. It was even more difficult to assess whether these contributions combined additively or with interaction. New statistical developments will be needed to answer this question (Barot et al., 2017; Finckh and Mundt, 1992).

If we look more generally at *Metschnikowia* and *Hanseniaspora* mixed cultures with *S. cerevisiae*, we could not evidence any major antagonistic phenomena. For almost all assays, mixed cultures performance stood always between that of the corresponding isolated cultures (Fig 1, 2 & 3), with the exception of the total production of glycerol. Moreover, despite the differences in yield and interactions between species, the rapid dominance of *S. cerevisiae* (increasing from 10% to at least 50% during the fermentation) resulted in mixed cultures that

296 were overall not different from *S. cerevisiae* pure cultures. This result is in agreement with the
297 good adaptation of *S. cerevisiae* to winemaking conditions (García et al., 2016; Jolly et al.,
298 2014; Pinto et al., 2015; Varela, 2016) but could be different with a different set of strains. This
299 result is important in the context of ecological engineering. In fact, our results confirmed that
300 *S. cerevisiae* has a much better fitness than the non-*Saccharomyces* strains studied in this paper.
301 Therefore, if we want mixed culture behavior to deviate from that of *S. cerevisiae* monoculture,
302 it must be ensured that non-*Saccharomyces* cells dominate the culture as soon as possible. To
303 achieve this, two conceivable options are currently tested: either to reduce the proportion of *S.*
304 *cerevisiae* at t_0 or to successively inoculate (Tristezza et al., 2016) first the non-*Saccharomyces*
305 species , and only then the *S. cerevisiae* strain. These two options could be equivalent
306 depending on the type of interaction(s) that occurs. If strain behaviors in isolated or mixed
307 cultures are identical, then all interactions are mediated by the medium through the competition
308 for resources and the production of constitutive toxins such as ethanol (producing a toxin only
309 in mixed fermentation would be a behavior change) and could be qualified as "indirect". Indeed,
310 all the interactions observed in the present study could be explained by these types of
311 interactions.
312 Mathematical models could be designed from data on isolated cultures to predict the impact of
313 indirect interactions in mixed cultures. This would allow simulating numerous mixes of species
314 with various initial conditions and identify optimal strategies depending on one or several given
315 criteria. Using these approaches could limit the number of necessary tests, potentially saving a
316 lot of time and money and opening the way to a more methodical ecological engineering. The
317 development of such mathematical models will only be possible thanks to a deep tracking of
318 population dynamics to understand underlying mechanisms of growth and mortality.
319 Obviously, it is also critical to validate this approach by i) first extending the number of species
320 co-cultured with *S. cerevisiae*, ii) investigating intra-specific variability and strain-strain

interactions between species, iii) investigating the impact of the environment of culture (temperature, grape variety, etc.).

Mat & Met

Strains

In this work, we used one strain of 5 different species (one strain per species): *Saccharomyces cerevisiae* (***Sc***), *Metschnikowia pulcherrima* (***Mp***), *M. fructicola* (***Mf***), *Hanseniaspora uvarum* (***Hu***) and *H. opuntiae* (***Ho***). The *S. cerevisiae* strain is a haploid strain from EC1118 labelled with GFP (59A-GFP, Marsit and Dequin, 2015). The *Hanseniaspora uvarum* (CLIB 3221) *H. opuntiae* (CLIB 3093) and *Metschnikowia pulcherrima* (CLIB 3235) strains originated from the yeast CIRM (https://www6.inra.fr/cirm_eng/Yeasts/Strain-catalogue) and were isolated from grape musts. The *Metschnikowia fructicola strain was* from the Lallemand collection.

For each species, 3 replicates of isolated cultures were performed (except for *S. cerevisiae* that had a total of 8 replicates in different blocks). In addition, for each non-*Saccharomyces*, 3 replicates of a mixed culture with the **Sc** strain were performed. In all mixed fermentations, the starting proportion of *S. cerevisiae* cells was set at 10%. In this text, fermentations were referred to by the species that performed them, i.e. isolated cultures were referred to as: ***Sc***, ***Mp***, ***Mf***, ***Hu*** and ***Ho*** and mixed cultures as ***ScvsMp***, ***ScvsMf***, ***ScvsHu*** and ***ScvsHo***.

Medium

344 Initial cultures (12 h, in 50 ml YPD medium, 28 °C) were used to inoculate fermentation media
345 at a total density of 10^6 cells/mL; therefore, for mixed culture the *S. cerevisiae* cells density was
346 $0.1x10^6$ /mL and the non-*Saccharomyces* cells density was $0.9x10^6$ /mL. Fermentations were
347 carried out in a synthetic medium (SM) mimicking standard grape juice (Bely et al., 1990). The
348 SM used in this study contained 200 g/L of sugar (100 g glucose and 100 g fructose per liter)
349 and 200 mg/L of assimilable nitrogen (as a mix of ammonium chloride and amino acids). The
350 concentrations of weak acids, salts and vitamins were identical to those described by Seguinot
351 et al. (Seguinot et al., 2017). The pH of the medium was adjusted to 3.3 with 10M NaOH. The
352 SM medium was first saturated with bubbling air during 40 minutes, then it was supplemented
353 with 5 mg/L phytosterols (85451, Sigma Aldrich) to fulfill the lipid requirements of yeast cells
354 during anaerobic growth.

355 **Measurements**

356 Fermentation took place in 1.1-liter fermentors equipped with fermentation locks to maintain
357 anaerobiosis, at 20 °C, with continuous magnetic stirring (500 rpm) during approximately 300h.
358 CO_2 release was followed by automatic measurements of fermentor weight loss every 20 min.
359 The amount of CO_2 released allowed us to monitor the progress of the fermentation and evaluate
360 the maximum of released CO_2 (***CO2max***) as well as the maximum rate of CO_2 released (***Vmax***).
361 Samples were harvested after 6h, 12h and 24h, then every 12h during the first week and every
362 24h during the second week of fermentation. For each sample, the population density, viability
363 and proportion of *S. cerevisiae* cells were determined using a BD Accuri™ C6 Plus flow
364 cytometer as described in Delobel et al., (2012. From these population densities (without taking
365 into account viability), we fitted a growth population model (with the growthcurver package
366 in R, Sprouffske and Wagner, 2016), and determined the carrying capacity (***K***) and maximum
367 growth rate (***mu***) for each fermentation.

368 The final concentrations of carbon metabolites in the medium (acetate, succinate, glycerol,
369 alpha-ketoglutarate, pyruvate, ethanol, glucose and fructose) were determined with high-
370 pressure liquid chromatography (Camarasa et al., 2011). From these metabolite concentrations,
371 we first calculated the consumed sugar concentration as the difference between the final and
372 the initial concentration of either glucose or fructose. Then we calculated the yield of metabolite
373 production by dividing the final concentration by the corresponding consumed sugar
374 concentration. Finally, we compared these yields to the yield of *S. cerevisiae* isolated cultures
375 considered as reference.
376 Finally, the ammonium concentration after 100h of fermentation was determined enzymatically
377 with R-Biopharm (Darmstadt, Germany) and the free amino acid content of the must was
378 estimated through cation exchange chromatography with post-column ninhydrin derivatization
379 (Crépin et al., 2012).

380 **Statistical Analysis**

381 The experimental work was performed in 5 different blocks. Each block was composed of three
382 replicates of non-*Saccharomyces* fermentations (for example ***Hu***), three replicates of the
383 corresponding mixed fermentations with *S. cerevisiae* (for example ***ScvsHu***) and one or two
384 fermentations of isolated *S. cerevisiae* cells (***Sc***). The block effect was evaluated on the
385 parameters of the ***Sc*** fermentation. For most studied parameters, the block effect was not
386 significant. For those parameters where a block effect was observed (***mu*** and ***K***), a statistical
387 correction for block effect did not modify our results. Therefore, for simplification purposes,
388 we compared all fermentations without any correction for the block effect parameter. For each
389 measured parameter, an ANOVA was performed to evaluate the type of fermentation (***Sc***, ***Mp***,
390 ***Mf***, ***Hu***, ***Ho***, ***ScvsTd***, ***ScvsMp***, ***ScvsMf***, ***ScvsHu*** and ***ScvsHo***) effect and then a Tukey t-test was
391 performed to determine statistical groups and two-by-two statistical differences. All statistical
392 analyses were performed using R ("R Core Team (2016). R: A language and environment for

statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.," n.d.) and Rstudio ("RStudio (2012). RStudio: Integrated development environment for R (Version 0.96.122) [Computer software]. Boston, MA. Retrieved May 20, 2012. Available from http://www.rstudio.org/," n.d.). All data, analysis and figures scripts can be found in this github adress: https://github.com/tnidelet/Harle-et-al-2018.

Acknowledgments.

We thank the CIRM, the Lallemand company, Jean-Luc Legras, Virginie Galeote and Jean-Nicolas Jasmin for providing the strains used in this study. We thank also Christian Picou, Marc Perez, Faiza Macna for technical assistance and Delphine Sicard for advice.

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Tables

Table 1

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

	μ	K	prop	Vmax	MaxCO2	Sugar	NAS	Ethanol	Glycerol	Succinate	Acetate	Pyruvate	Alpha
Sc	0.15±0.02^{bc}	1.6±0.2^{bcd}	NA	0.99±0.03^{a}	88.2±2.2^{a}	0.2±0.2^{d}	10.4±8.6^{c}	95.2±8.4^{a}	5.3±0.4^{c}	1.05±0.28^{a}	0.63±0.06^{d}	0.12±0.08^{bc}	0.08±0.04^{ab}
Ho	0.19±0.03^{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^{b}	50.1±0.2^{b}	5.2±0.0^{c}	0.35±0.00^{b}	0.99±0.02^{b}	0.13±0.05^{bc}	0.09±0.1^{ab}
ScvsHo	**0.11±0.00**^{bc}	**2.3±0.1**^{a}	0.83±0.02^{b}	0.57±0.01^{c}	82.7±0.6^{bc}	23.3±1.3^{c}	**6.4±2.4**^{c}	87.2±0.8^{a}	**6.0±0.0**^{abc}	0.42±0.04^{b}	0.84±0.01^{c}	**0.08±0.00**^{c}	**0.03±0.0**^{b}
Hu	0.62±0.18^{a}	1.4±0.1^{cde}	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^{c}	5.5±0.2^{bc}	0.66±0.03^{b}	1.15±0.03^{a}	0.17±0.04^{abc}	0.14±0.09^{ab}
ScvsHu	0.24±0.01^{b}	**1.7±0.1**^{b}	0.76±0.01^{c}	0.53±0.04^{c}	82.1±1.5^{c}	16.5±6.5^{c}	**4.6±4.2**^{c}	89.5±3.8^{a}	**6.6±0.3**^{a}	**0.60±0.06**^{b}	0.86±0.07^{bc}	0.16±0.04^{abc}	0.09±0.06^{ab}
Mf	0.17±0.02^{bc}	0.8±0.05^{f}	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^{c}	2.8±0.1^{e}	0.29±0.01^{b}	0.13±0.01^{f}	0.10±0.01^{bc}	0.10±0.00^{ab}
ScvsMf	**0.09±0.01**^{bc}	1.3±0.03^{de}	0.96±0.02^{a}	0.76±0.03^{b}	86.8±0.7^{ab}	9.9±2.1^{cd}	**1.8±0.1**^{c}	92.1±3.2^{a}	**6.0±0.1**^{abc}	0.58±0.02^{b}	0.46±0.02^{e}	0.12±0.01^{bc}	0.08±0.01^{ab}
Mp	0.18±0.03^{bc}	0.6±0.01^{f}	NA	0.16±0.02^{e}	21.8±0.4^{f}	142±3.7^{a}	305.5±5.9^{a}	23.4±1.8^{c}	3.7±0.2^{d}	0.26±0.03^{b}	0.07±0.06^{f}	0.29±0.04^{a}	0.13±0.01^{ab}
ScvsMp	**0.09±0.00**^{c}	1.3±0.01^{e}	0.50±0.02^{d}	0.68±0.04^{b}	83.3±2.2^{bc}	20.8±6.0^{c}	**2.1±0.2**^{c}	86.5±3.4^{a}	**6.1±0.1**^{ab}	0.52±0.04^{b}	0.46±0.02^{e}	0.24±0.01^{ab}	**0.19±0.01**^{a}

Mu : 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 intermediate between the two values of the corresponding isolated cultures, a sign of transgressive interactions.

Superscript: Statistical groups obtained from a Tukey test

Figures

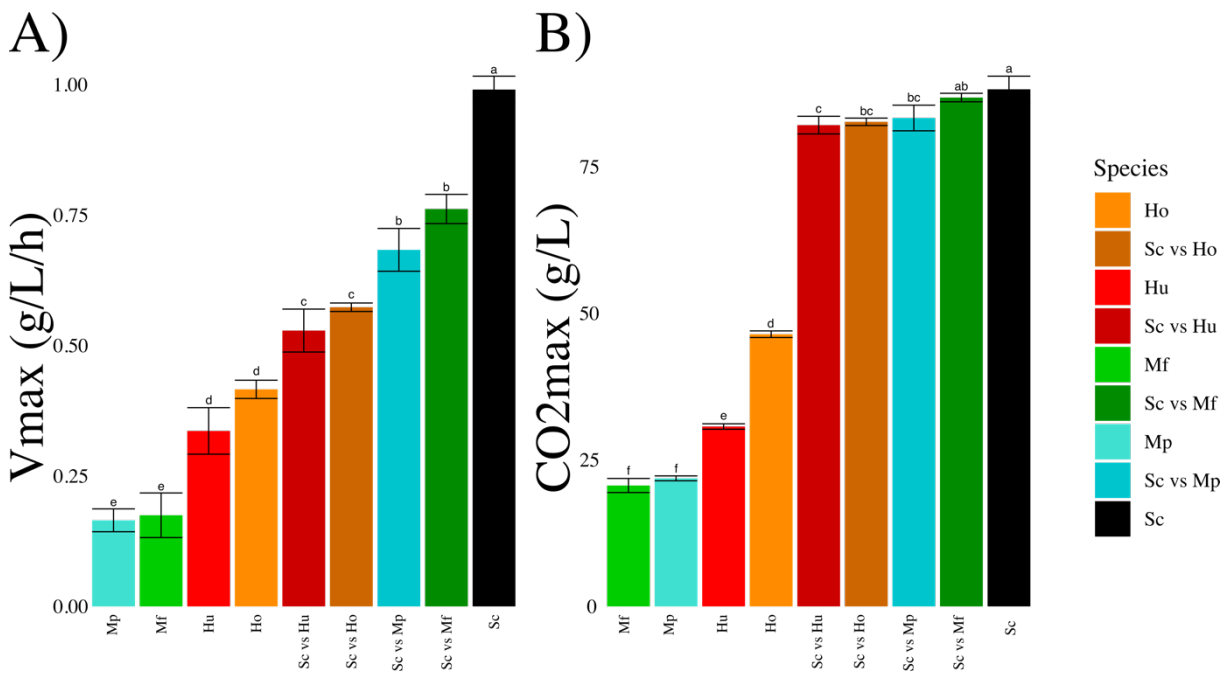


FIG 1

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.

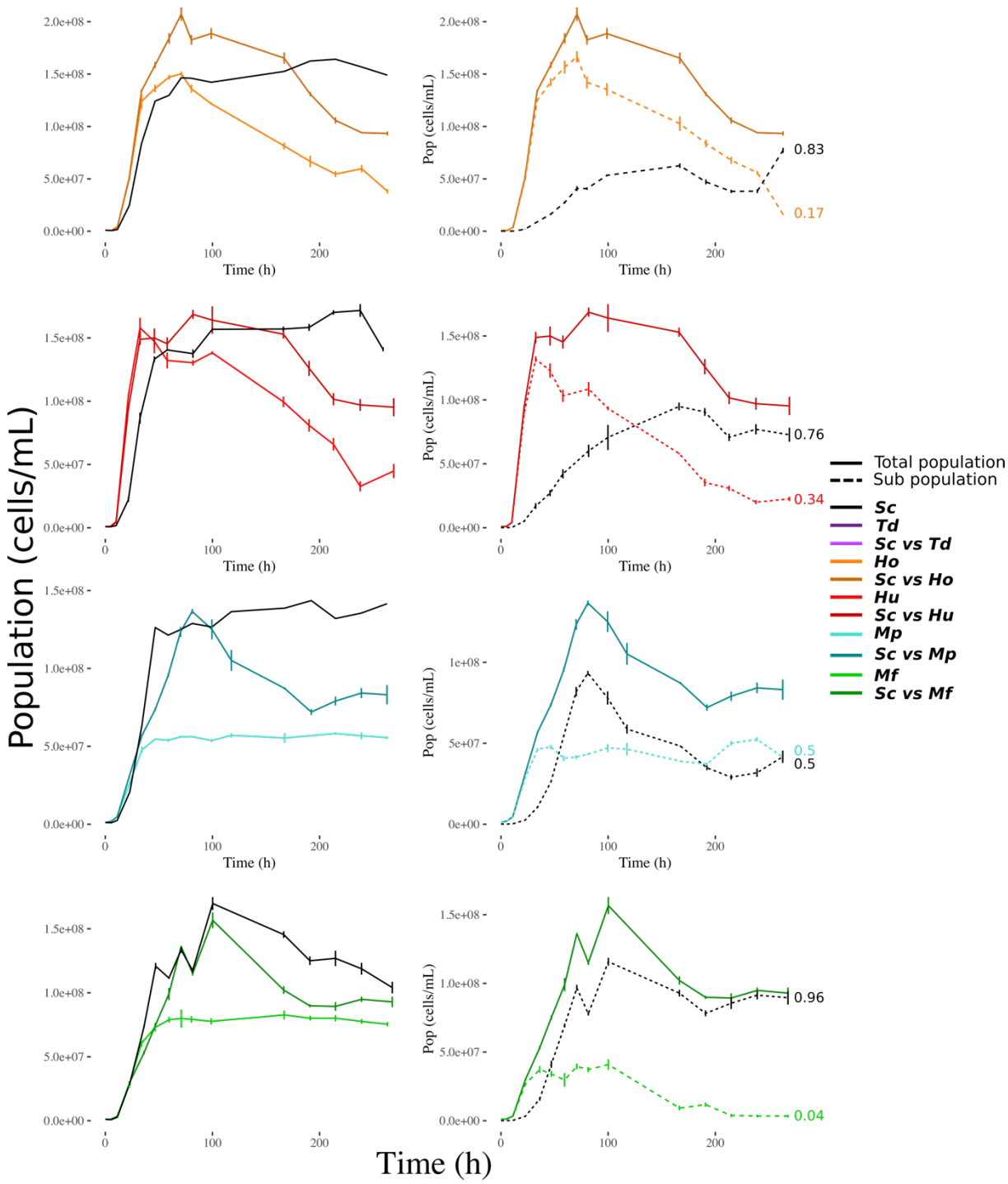
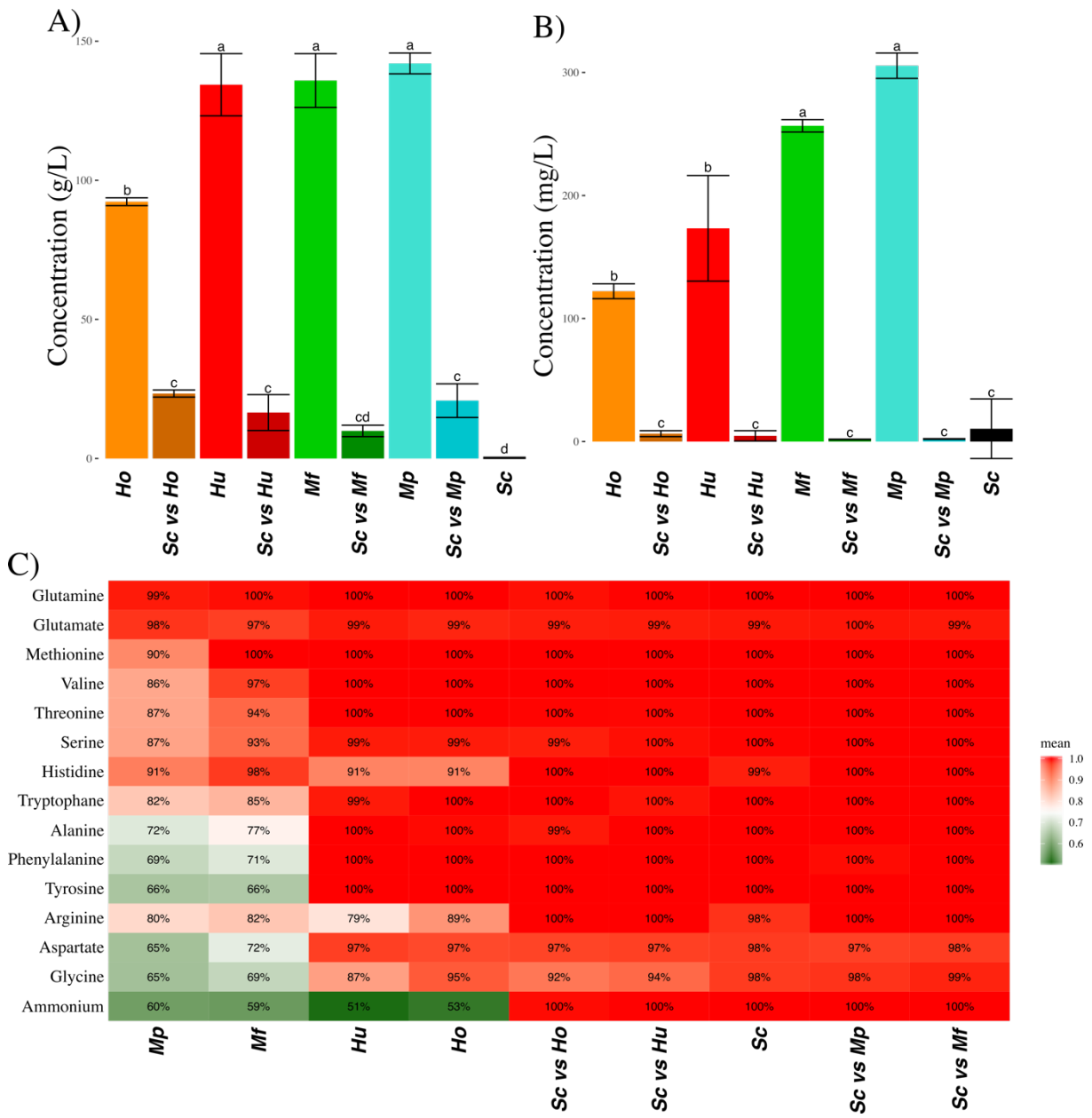


FIG2

Global monitoring of the kinetics of the total living population (left), and sub-population in the mixed cultures (right) across fermentation. Each point represents a sample (average ± standard error). full lines are for total population and dashed lines for the two sub populations in mixed cultures. At the end of dashed lines, the final proportion of both sub-populations in mixed cultures is indicated. The light colors represent pure cultures of 'non-*Saccharomyces*'

537 strains and dark ones to the corresponding culture in competition with *S. cerevisiae*. The
538 isolated cultures of *S. cerevisiae* are represented in black. ***Sc, Mp, Mf, Ho, Hu, ScvsMp,***
539 ***ScvsMf, ScvsHo*** and ***ScvsHu*** correspond respectively to cultures with: isolated *S. cerevisiae*,
540 isolated *M. pulcherrima*, isolated *M. fructicola*, isolated *H. opuntiae*, isolated *H.uvarum*, *M.*
541 *pulcherrima* mixed with *S. cerevisiae*, *M. fructicola* mixed with *S. cerevisiae*, *H. opuntiae*
542 mixed with *S. cerevisiae* and *H. uvarum* mixed with *S. cerevisiae*.

543

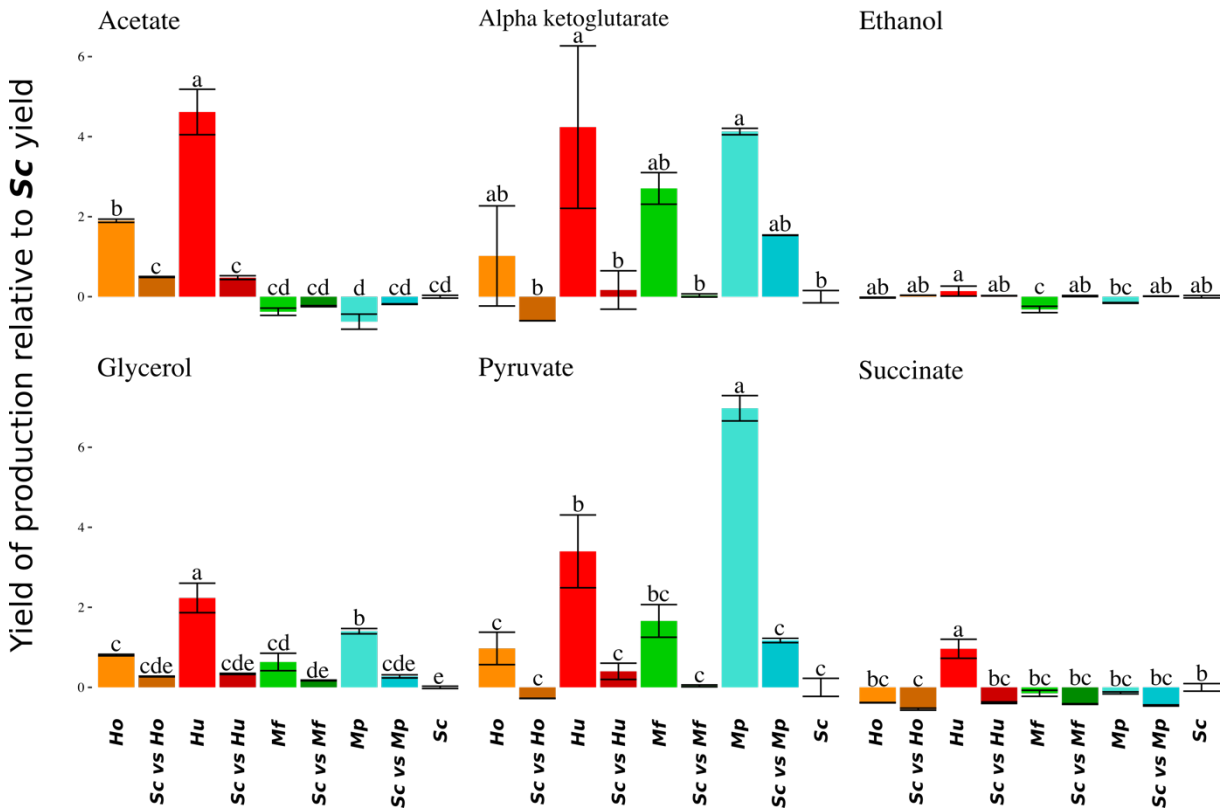


544

545 **FIG3**

546 **Consumption of sugars and nitrogen assimilable sources (NAS) for each type of**
547 **fermentation.** A) Final concentration of sugar (average ± standard deviation). B) Final
548 concentration of NAS (average ± standard deviation). C) Percentage of consumption of each
549 NAS in each type of fermentation represented as a color gradient from green (<75 %) to red (>
550 75%). ***Sc, Mp, Mf, Ho, Hu, ScvsMp, ScvsMf, ScvsHo*** and ***ScvsHu*** correspond respectively to
551 cultures with: isolated *S. cerevisiae*, isolated *M. pulcherrima*, isolated *M. fructicola*, isolated *H.*
552 *opuntiae*, isolated *H.uvarum*, *M. pulcherrima* mixed with *S. cerevisiae*, *M. fructicola* mixed
553 with *S. cerevisiae*, *H. opuntiae* mixed with *S. cerevisiae* and *H. uvarum* mixed with *S.*
554 *cerevisiae*.

555



556

557 **FIG4**

558 Yield (average ± standard deviation) of carbon metabolite production relative to the yield of
559 production of isolated *S. cerevisiae* cultures for acetate, alpha-ketoglutarate, ethanol, glycerol,
560 pyruvate and succinate and each type of fermentation. ***Sc, Mp, Mf, Ho, Hu, Td, ScvsMp,***

561 ***ScvsMf, ScvsHo*** and ***ScvsHu, ScvsTd*** correspond respectively to cultures with: isolated *S.*
562 *cerevisiae*, isolated *M. pulcherrima*, isolated *M. fructicola*, isolated *H. opuntiae*, isolated
563 *H.uvarum*, *M. pulcherrima* mixed with *S. cerevisiae*, *M. fructicola* mixed with *S. cerevisiae*, *H.*
564 *opuntiae* mixed with *S. cerevisiae* and *H. uvarum* mixed with *S. cerevisiae*.
565