# Kinetic analysis of yeast-yeast interactions in oenological conditions

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**Abstract** (250 words, concisely summarize the basic content of the paper without presenting extensive experimental details)

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** (150 words, should provide a nontechnical explanation of the significance of the study to the field)

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 fermentation and one other species strain, often from a different genus than *Saccharomyces*, bringing a greater variety of flavors (4, 5, 7, 8).

There are numerous experiments and even industrial products that mix *Saccharomyces* and non-*Saccharomyces* strains to improve the organoleptic qualities of wine (5, 9, 10). Non-*Saccharomyces* strains used in these experiments are very diverse with more than 23 different species (5) including among others *Torulaspora delbrueckii*, *Metschnikowia pulcherrima*, *Metschnikowia fructicola*, *Hanseniaspora opuntiae* and *Hanseniaspora uvarum*. *T. delbrueckii* is one of the few species that is capable to finish the fermentation and has shown to increase wine quality in mixed cultures with *S. cerevisiae* (9, 11, 12). Bely et al (11) have shown that a mixed culture *S. cerevisiae* and *T. delbrueckii* improves the analytical profile of sweet wines notably by decreasing volatile acidity and acetaldehyde production (with 53% less in volatile acidity and 60% less acetaldehyde than a pure culture of *S. cerevisiae*) (11). On the opposite, species from the *Metschnikowia* genus ferments poorly in oenological conditions but also can have interesting attributes (4). In conjunction with *S. cerevisiae*, the presence of *M. pulcherrima* can reduce ethanol concentrations (4, 13), can increase 'citrus/grape fruit' and 'pear' attributes (14), as well as the persistence of 'smocky' and 'flowery' attributes (15). *M. pulcherrima* also has an amensalism effect on *S. cerevisiae* by depletion of iron via the production of pulcherriminic acid (16). *M. fructiola* has been less studied and never in conjunction with *S. cerevisiae* but still presents an interesting capacity to inhibit *Botrytis* growth (17). At last, the *Hanseniaspora* genus also has also been studied in sequential or simultaneous fermentation

99 with *S. cerevisiae* and has shown to increase volatile compound production during winemaking
100 (4). It has been shown to increase 'tropical fruit', 'berry', 'floral', and 'nut aroma' character
101 (18) linked to higher concentrations of acetate esters, esters of MCFAs, isoamyl alcohol, 2-
102 phenylethanol and α-terpineol (19).

103

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

113

114

# 115 Results

116 In this work we compared the performances in winemaking conditions of isolated cultures of
117 six different yeast species (*Saccharomyces cerevisiae*, *Torulaspora delbrueckii*, *Metschnikowia*
118 *pulcherrima*, *Metschnikowia fructicola*, *Hanseniaspora opuntiae* and *Hanseniaspora uvarum,*
119 one strain by species) with paired co-cultures mixing each of the five non-*Saccharomyces*
120 strains with one GFP marked *Saccharomyces cerevisiae* strain. Obtained results will make it
121 possible to evaluate the intensity of yeast-yeast interactions. By comparing the output of

122 isolated and mixed cultures, we evaluated the intensity of yeast-yeast interactions and/or its
123 consequences on ecosystem services production.
124
125 We first investigated the consequences of co-culture onthe dynamic of $CO_2$ production which
126 is a good indicator of the fermentation progress. The values of the maximum rate of $CO_2$
127 production (***Vmax***, Fig. 1a, Table. 1) and of the maximum $CO_2$ produced were estimated
128 (***$CO_2$max***, Fig. 1b, Table. 1). The ***Vmax*** was highly dependent on the species (p.value < 0.001).
129 *S. cerevisiae* cultures (***Sc***) displayed the maximum ***Vmax*** (0.99 ± 0.02 $g.L^{-1}.h^{-1}$) followed by *T.*
130 *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
133 between those of ***Sc*** and ***Td*** isolated cultures (Fig. 1a). Mixed culture containing ***Mp***, ***Td*** or ***Mf***
134 species had significantly higher ***Vmax*** than those containing ***Hu*** or ***Ho*** species. we considered
135 two types of cultures, those where the fermentation was finished within xx hours (***CO2max*** >
136 80$g.L^{-1}$) and those where it was not (Fig. 1b). All mixed cultures and the Sc cultures were able
137 to finish the fermentation while all non-*Saccharomyces* isolated cultures did not. ***Td*** isolated
138 cultures had the highest ***$CO_2$max*** among non-*Saccharomyces* species (65 ± 4 $g.L^{-1}$), followed
139 by *Hanseniaspora* species (***Hu*** 30 ± 0.4 $g.L^{-1}$, and ***Ho*** 46 ± 0.6 $g.L^{-1}$) and finally *Metschnikowia*
140 species (***Mp*** 22 ± 0.4 $g.L^{-1}$ and ***Mf*** 20 ± 1 $g.L^{-1}$). For these two parameters, the mixed cultures
141 can be grouped with the ***Sc*** indicating a prevalent impact of the *S. cerevisiae* species on the
142 fermentation kinetics.
143
144 We then looked at the population dynamics for each fermentation (Fig. 2) and determined the
145 maximum growth rate of the population (***mu***), the maximum population size, also called the
146 carrying capacity (***K***) and the relative frequency of each species at the end of mixed cultures

147 (Table. 1). The fermentations conducted by *S. cerevisiae* alone goes through an exponential
148 growth rate ($\boldsymbol{mu_{Sc}}$ = 5.6 ± 0.8 $10^6$cells.mL$^{-1}$.h$^{-1}$) and reaches a maximum population around 1.5
149 $10^8$cells.mL$^{-1}$ ($\boldsymbol{K_{Sc}}$ = 1.56 ± 0.16 $10^8$cells.mL$^{-1}$) that remains constant until the end of the
150 fermentation. ***Td*** fermentations have dynamics comparable to those of ***Sc*** with a similar growth
151 rate but a smaller carrying capacity ($\boldsymbol{K_{Td}}$ = 1.23 ± 0.05 $10^8$cells.mL$^{-1}$). The fermentations
152 mediated by *Hanseniaspora* species alone had as similar growth dynamics similar to ***Sc*** at the
153 beginning of the fermentation with though a higher growth rate ($\boldsymbol{mu_{Ho}}$ = 7.7 ± 2.3 $10^6$cells.mL$^{-}$
154 $^{1}$.h$^{-1}$, $\boldsymbol{mu_{Hu}}$ = 13.4 ± 4.3 $10^6$cells.mL$^{-1}$.h$^{-1}$). On the opposite, their stationary phase is quite
155 different from that of ***Sc***; characterized by a higher cell mortality with a population drop of
156 about 70% by the end of the fermentation. The fermentation mediated by *Metschnikowia*
157 species have growth dynamics globally similar to ***Sc*** fermentations: a similar growth rate ($\boldsymbol{mu_{mp}}$
158 = 2.3 ± 0.4 $10^6$cells.mL$^{-1}$.h$^{-1}$, $\boldsymbol{mu_{Mf}}$ = 3.4 ± 0.7 $10^6$cells.mL$^{-1}$.h$^{-1}$), a much smaller carrying
159 capacity ($\boldsymbol{K_{Mp}}$ = 0.6 ± 0.01 $10^6$cells.mL$^{-1}$, $\boldsymbol{K_{Mf}}$ = 0.8 ± 0.05 $10^6$cells.mL$^{-1}$) and no mortality
160 during the stationary phase. In most cases, the mixed cultures display an intermediated pattern
161 between the two corresponding isolated cultures (Fig. 2). However, the mixed cultures with
162 *Metschnikowia* species harbor a cell mortality during the stationary phase contrary to their
163 respective isolated cultures. In the case of ***ScvsMp*** fermentation, only *S. cerevisiae* population
164 decreases significantly during the stationary phase, while in ***ScvsMf*** fermentation; both sub
165 populations significantly diminish. As a measure of fitness, we can also follow the variations
166 of *S. cerevisiae* frequency along the fermentation. In all mixed cultures, *S. cerevisiae*
167 significantly increased during the fermentation from 10% initially to frequencies varying
168 between 32% and 96% at the end of the culture depending on species (Table. 1). In all cases,
169 except the mix with the *T. delbrueckii* ($\text{prop}_{\text{ScvsTd}}$ = 0.32 ± 0.02), *S. cerevisiae* finishes by
170 dominating the mixed cultures (Table. 1).
171

172 We then looked at resources consumption (glucose, fructose and sources of assimilable
173 nitrogen) and estimated the final concentration of sugars (fructose and glucose) and amino-
174 acids (Fig. 3, Table 1). The ***Sc*** fermentations consumed all sugars leaving less than 0.1% of the
175 initial concentration (Fig. 3a). Among isolated non-sacc, ***Td*** fermentations was the one
176 consuming the highest fraction of the initial sugars leaving around 31% of sugars followed by
177 ***Ho*** (45%), ***Hu*** (67%), ***Mf*** (68%) and ***Mp*** (71%). Furthermore, all species but *Hanseniaspora*
178 *opuntiae* have consumed more glucose than fructose. The mixed cultures consumed more
179 sugars than all isolated non-*Saccharomyces* fermentations but less than ***Sc*** fermentations with
180 a preference for glucose, indicating the major impact of the *S. cerevisiae* strain in sugar
181 consumption (consistent with the $CO_2$ production).
182 The consumption of nitrogen assimilable sources (NAS, amino-acids and ammonium) harbours
183 the same pattern (Fig. 3b). NAS was consumed almost entirely in ***Sc*** isolated culture and in all
184 mixed populations whereas the fraction of NAS consumed varied between 84% and 94% in
185 non-*saccharomyces* isolated culture. However, the preference for the different sources of
186 nitrogen are different for each species (Fig. 3c). Glycine and histidine are the least consumed
187 by ***Td***. ***Ho*** and ***Hu*** have similar behaviors: they both consume only half of the ammonium, 90%
188 of histidine and 89% and 79% of arginine respectively. In the same way, both *Metschnikowia*
189 species present a similar pattern; the non-exhausted NAS are identical for the two species:
190 ammonium, glycine, aspartate, arginine tyrosine, phenylalanine and alanine. Therefore, we can
191 classify NAS in function of their global preference by all tested yeasts. The obtained ranking
192 by order of preference is: glutamine, methionine, glutamate, valine, threonine, serine,
193 tryptophan, alanine, histidine, arginine, aspartate, glycine and surprisingly ammonium.
194
195 In parallel of the monitoring of the must resources consumption, we also investigated the
196 production of metabolites from the CCM (Central Carbon Metabolism): ethanol, glycerol,

197 succinate, pyruvate, acetate and alpha-ketoglutarate (Table 1). Theses productions has been
198 measured at the end of the cultures where sugar consumption being quite different from one
199 fermentation to another. Therefore, it appears more appropriate to first compare the production
200 yields (total production / sugar consumption) instead of the total productions. Moreover, we
201 decide to compare yields of production relatively to the ***Sc*** yield that is our reference (Fig. 4).
202 In the case of ethanol, only ***Mf*** fermentations has a yield of production statistically than the ***Sc***
203 inferior (-32%). For glycerol, all non-*saccharomyces* grown alone but ***Td*** had a greater yield
204 than ***Sc***. The mixed fermentations glycerol yield was not significantly different from the Sc one.
205 Regarding acetate, only *Hanseniaspora* species grown alone or in mixed culture harbored a
206 greater yield than Sc (Fig. 4). For succinate, there could be a negative interaction between *Sc*
207 and *Hanseniaspora* or *Sc* and *Metschnikowia*, as yields in mixed culture were inferior than in
208 the corresponding isolated cultures. However, these differences were not significant after the
209 correction for multiple tests. At last, we did not observe any significant variation of alpha-
210 ketoglutarate yield of production, linked to its lower concentration and difficulty to be precisely
211 measured.
212
213 The total production of each fermentation is the result of the combination of species yields,
214 total sugar consumption and respective population dynamics during the fermentation.
215 Therefore, differences observed in the total productions of mixed cultures result from additive
216 or subtractive effects between these 3 parameters. Regarding ethanol, the total production is
217 directly linked to the consumption of resources and all mixed cultures are equivalent to ***Sc***
218 fermentation. The case of glycerol is more interesting. Indeed, even if the sugar consumption
219 for ***ScvsHu*** and ***ScvsMp*** mixed cultures are lower than ***Sc*** isolated culture, the total production
220 of glycerol is superior to the corresponding isolated cultures ($Glycerol_{SCvsMp}$ = 6.1±0.1 $g.L^{-1}$,
221 $Glycerol_{Sc}$ = 5.3±0.4 $g.L^{-1}$, $Glycerol_{Mp}$ = 3.7±0.2 $g.L^{-1}$). This result comes from the positive

222 combination of the superior yield of glycerol production of *Hanseniaspora* and *Metschnikowia*
223 *cells* and the population dynamics. In all other cases, even if the total production does not lie
224 between the production bonds of the corresponding isolated cultures for some mixed cultures,
225 these differences are never statistically significant.
226
227

# 228 Discussion

229 This manuscript presents one of the first works focusing on the kinetics of yeast-yeast
230 interactions between two species during the fermentative process in oenology. Indeed, so far,
231 most of the work presented in the literature focus on input-output balances (4, 21).
232
233 First, this work provides additional information about the population dynamics in winemaking
234 conditions of non-saccharomyces yeasts that are still poorly known. In agreement with previous
235 results (12, 22, 23), we found that *T. delbrueckii* has a very similar dynamic than *S. cerevisiae*
236 and is able to finish the fermentation on its own, while not being as efficient or as fit as *S.*
237 *cerevisiae*. However, other studies suggest that this result is not constant and depends on strains
238 and environmental conditions. The *T. delbrueckii* strain used in Belda et al (14) was less
239 performant than their *S. cerevisiae* strain. Our results also show that *T. delbrueckii* contrary to
240 *S. cerevisiae* does not consume all NAS (the limiting nutrient, (23), leaving half of the glycine
241 unconsumed. This difference in the consumption of nitrogen sources may explain the ability of
242 *S. cerevisiae* to invade mixed culture (12) despite its low inoculation rate. Despite differences
243 of carbon metabolites production between the isolated cultures of *T. delbrueckii* and *S.*
244 *cerevisiae* (linked to the differences in resources consumption), the yields are not statistically
245 different. The mixed cultures are identical to *S. cerevisiae* pure cultures because of *S. cerevisiae*

246 invasion. Globally the interaction mechanism between these two species seems to be a simple
247 competition for resources without much potential for optimization.
248
249 The two species, *H. uvarum* and *H. opuntiae* both present a very different pattern in
250 fermentation from *S. cerevisiae*. Their population dynamics are characterized by a faster growth
251 rate (reaching a maximum population similar to *S. cerevisiae*), followed by a long phase of
252 mortality where viability drops to 30% (24). Consequently, these two strains are not able to
253 finish the fermentation and leave most of the sugars (24). Nitrogen consumption is also specific
254 to this genus; only half of available ammonium is used. Unlike with *T. delbrueckii*, the yield of
255 production of carbon metabolites are very different than with *S. cerevisiae* and moreover the
256 mixed fermentations display cases of transgressive interactions: their glycerol production is
257 higher than both isolated cultures, despite a lower sugar consumption. These overproductions
258 of glycerol can be explained by the conjunction of the population dynamics, resource
259 consumptions (the ***Ho*** and ***Hu*** fermentation leaving ⅔ of sugars) and the individual species
260 yields of glycerol production which are from two to three times greater than that of *S. cerevisiae*.
261 However, this interaction it is not found in previous works (24) and, in this present work, seems
262 to be related to competition for resources without any change in the population dynamic and
263 metabolism of each strain between the mixed and pure cultures.
264
265 At last, the two *Metschnikowia* strains have unexpected fermentation dynamics. The ***Mp*** and
266 ***Mf*** isolated cultures are characterized by a short growth phase reaching a small carrying
267 capacity and followed by a long stationary phase without mortality. Nevertheless, in spite of
268 this very high viability, the measured resources (sugars and nitrogen) are not entirely consumed.
269 Why the cells stop growing remains unclear. A possible explanation could be linked to
270 unmeasured resources. The third most important resource of the medium is the lipids that are

271 present in the medium under the form of phytosterols. It has also been shown that some
272 *Metschnikowia* species are unable to consume lipids from the medium and therefore are limited
273 to their own production (see Fig S1). For all yeast species, lipid synthesis requires oxygen and
274 is therefore impossible in anaerobic conditions. From these elements, one can hypothesize that
275 *Metschnikowia* cells are producing their own lipids from oxygen at the beginning of the
276 fermentation and once the oxygen is exhausted they stop growing. Interestingly, despite the fact
277 that isolated cultures of *S. cerevisiae*, *M. pulcherrima* and *M. fructicola* did not present any
278 mortality, their corresponding mixed cultures (***ScvsMp*** and ***ScvsMf***) present 30% mortality.
279 Moreover, this mortality seems to impact differently both species. In the mixed ***ScvsMp***
280 fermentation only the *S. cerevisiae* cells are dying; in ***ScvsMf*** both species are impacted. To
281 explain the survival of *M. pulcherrima* cells compared to *S. cerevisiae* cells, it can be
282 hypothesized that *Metschnikowia* cells produce toxins (a ferric salt of pulcherriminic acid, (25)
283 that kill *S. cerevisiae* cells (16, 26, 27). In ***ScvsMf*** cultures, the mortality of both species
284 suggests an interaction mechanism different from the one observed between *M. pulcherrima*
285 and *S. cerevisiae*. A two-phase interaction could explain such pattern : *M. fructicola* synthesizes
286 a toxin that kills only *S. cerevisiae* cells and thereafter *M. fructicola* cells dies for another
287 reason, like ethanol sensibility. Indeed, the production of ethanol is almost four time greater in
288 mixed cultures than in isolated *Metschnikowia* fermentations. The reason why no loss of
289 viability is observed for *M. pulcherrima* when placed in mixed culture with *S. cerevisiae* is
290 probably a better tolerance to ethanol stress compared to *M. fructicola*. As a conclusion, the
291 interaction mechanism between the *Metschnikowia* species and *S. cerevisiae* appears to be very
292 different from that between *S. cerevisiae* and the other studied non-saccharomyces species. It
293 is not a competition for resources in sugars or nitrogen but rather the possibility of a killer toxin
294 synthesized by the *Metschnikowia* strains or a competition for a non-measured resource (lipids,
295 vitamins, minerals).

296

297 If we look globally, we can first note that all mixed cultures finished the fermentation. No major
298 antagonistic phenomena were identified between the *S. cerevisiae* strain chosen and the "non-
299 Saccharomyces" yeasts studied. Moreover, despite the presence of differences in yield and
300 interactions between strains, the rapid dominance of *S. cerevisiae* (representing however only
301 10% of the population at t0) results at mixed cultures that are overall little different from *S.*
302 *cerevisiae* pure cultures. This result confirms the good adaptation of *S. cerevisiae* to the
303 winemaking conditions (4–6, 21). Nevertheless, interesting data appear in our research work.
304 Indeed, despite the pre-eminence of *S. cerevisiae*, we have already highlighted transgressive
305 interactions between *S. cerevisiae* and *H. uvarum* and *M. pulcherrima* leading to the
306 overproduction of glycerol in mixed cultures. This observation opens the door for potential
307 more interesting results: when *S. cerevisiae* is inoculated at lower frequencies (0.01, 0.001), the
308 fact that production yields are different between the species can lead to more important
309 transgressive interactions (lower ethanol production for example) in mixed cultures.
310 Furthermore, most of detected interactions can be explained by competition for resources or the
311 production of toxins without indication of an alteration of individual cell behavior linked to the
312 presence of other species in the same medium. This observation linked to our deep phenotyping
313 of population dynamics could allow the modeling of mixed oenological fermentations. Using
314 such model, transgressive interactions linked to consumption of resources could be investigated
315 and inform the choice of couple of species and the protocol of inoculations (specifically by
316 changing the *S. cerevisiae* starting frequency). It could be used to predict mixed cultures
317 dynamics from data on isolated cultures, potentially saving a lot of time and money and open
318 the possibility of ecological engineering. The development of such mathematical model will
319 only be possible thanks to a deep tracking of population dynamics to understand underlying
320 mechanisms of growth and mortality. Therefore, to better identify and understand yeast-yeast

interaction mechanisms, we should extend our deep phenotyping of mixed population dynamics to additional species and investigate intra-specific variability as well as interactions with culture environments.

# Mat & Met

In this work, we used one strain of 6 different species (one strain by species): *Saccharomyces cerevisiae* (***Sc***), *Torulaspora delbrueckii* (***Td***), *Metschnikowia pulcherrima* (***Mp***), *Metschnikowia fructicola* (***Mf***), *Hanseniaspora uvarum* (***Hu***) and *Hanseniaspora opuntiae* (***Ho***). The *S. cerevisiae* strain is a haploid strain marked with GFP (59A-GFP, (2)). The 5 non-*Saccharomyces* strains are natural strains isolated from viognier and sauvignon must in different regions of France. For each species, 3 replicates of isolated cultures were performed (except for *S. cerevisiae* that have a total of 8 replicates in different blocks). In addition, for each non-*Saccharomyces*, 3 replicates of a mixed culture with the Sc strain was performed. In all mixed fermentations, the starting proportion of *S.cerevisiae* cells was 10%. In this text, the fermentations are referred by the species that perform them. The isolated cultures are referred as: ***Sc***, ***Td***, ***Mp***, ***Mf***, ***Hu*** and ***Ho*** and the mixed cultures as ***ScvsTd***, ***ScvsMp***, ***ScvsMf***, ***ScvsHu*** and ***ScvsHo***.

Initial cultures (12 h, in 50 ml YPD medium, 28 °C) were used to inoculate fermentation at a density of $10^6$ cells/ml therefore for mixed culture the density of *S. cerevisiae* cells was $0.1x10^6$ cells/mL and the density of non-sacc cells was $0.9x10^6$ cells/mL. Fermentations were carried out in synthetic medium (SM) mimicking standard grape juice (28). The SM used in this study contained 200 g/L of sugar (100 g/L of glucose and 100 g/L of fructose) and 200 mg/L of assimilable nitrogen (as a mixed of ammonium chloride and amino acids). The concentrations of weak acids, salts and vitamins were identical to those described by Seguinot et al. (29). The

345 pH of the medium was adjusted to 3.3 with 10M of NaOH. The SM medium was first saturated
346 with bubbling air during 40 minutes, then it was supplemented with 5 mg/L of phytosterols
347 (85451, Sigma Aldrich) to satisfy the lipid requirements of yeast cells during anaerobic growth.
348 Fermentation took place in 1.1-liter fermentors equipped with fermentation locks to maintain
349 anaerobiosis, at 20 °C, with continuous magnetic stirring (500 rpm) during approximately 200h.
350 $CO_2$ release was followed by automatic measurements of fermentor weight loss every 20 min.
351 The amount of $CO_2$ released allowed us to monitor the progress of the fermentation and evaluate
352 the maximum of released $CO_2$ (***CO2max***) as well as the maximum rate of $CO_2$ released (***Vmax***).
353 Samples were harvested after 6h, 12h and 24h, then every 12h during the first week and every
354 24h during the second week of fermentation. At each sample, the population density, the
355 viability and the proportion of *S. cerevisiae* cells were determined using a flow cytometer BD
356 Accuri™ C6 Plus as described in Delobel et al. (30). From these population data and by fitting
357 a growth population model (with the grofit package of R, (31), the carrying capacity (***K***) and
358 the maximum growth rate (***mu***) for each fermentation were estimated.
359 The final concentration of carbon metabolites in the medium (acetate, succinate, glycerol,
360 alpha-ketoglutarate, pyruvate, ethanol, glucose and fructose) with high-pressure liquid
361 chromatography (32). From these metabolite concentrations, we first calculated the consumed
362 sugar concentration as the difference between the final and the initial concentration of either
363 glucose or fructose. Then we calculated the yield of metabolite production by dividing the final
364 concentration by the corresponding consumed sugar concentration. Finally, we report these
365 yields to the yield of *S. cerevisiae* isolated cultures that we consider as our reference.
366 At last, the ammonium concentration after 100h of fermentation was determined enzymatically
367 with R-Biopharm (Darmstadt, Germany) and the free amino acid content of the must was
368 estimated with cation exchange chromatography with post-column ninhydrin derivatization
369 (33).

370 The experimental work was performed in 5 different blocks. Each block was composed of three
371 replicates of non-*Saccharomyces* fermentations (for example ***Td***), three replicates of the
372 corresponding mixed fermentations with *S. cerevisiae* (for example ***ScvsTd***) and one or two
373 fermentations of isolated *S. cerevisiae* cells (***Sc***). The block effect was evaluated on the
374 parameters of the ***Sc*** fermentation. For most of the studied parameters the block effect was not
375 significant. For the parameters where there was a block effect (mu and K), a statistical
376 correction for the block effect did not modify our results. Therefore, for simplification, we
377 consider that there was no block effect and compare all fermentations without any correction
378 for block effect. For each measured parameters, an anova was performed to evaluated the type
379 of fermentation (***Sc***, ***Td***, ***Mp***, ***Mf***, ***Hu***, ***Ho***, ***ScvsTd***, ***ScvsMp***, ***ScvsMf***, ***ScvsHu*** and ***ScvsHo***)
380 effect and then a Tukey-test was performed to determine statistical groups and two by two
381 statistical differences. All statistical analyses were performed using R (34) and Rstudio (35).
382 All data, analysis and figures scripts can be found in this github adresse: XXX.
383

384 # Acknowledgments.

385

386 # References


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# 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$^{bcd}$ | 1.6±0.2$^{bcd}$ | NA | 0.99±0.03$^{a}$ | 88.2±2.2$^{a}$ | 0.2±0.2$^{a}$ | 10.4±8.6 | 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$^{e}$ | 122.2±3.5 | 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 | 0.57±0.01$^{c}$ | 82.7±0.6$^{b}$ | 23.3±1.3$^{bc}$ | **6.4±1.4** | 87.2±0.8$^{ab}$ | **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*** | 13.4±4.3$^{a}$ | 1.5±0.2$^{bcde}$ | NA | 0.34±0.04$^{d}$ | 30.7±0.5$^{e}$ | 134.3±11.2$^{f}$ | 173.3±24.8 | 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 | 0.53±0.04$^{c}$ | 82.1±1.5$^{b}$ | 16.5±6.5$^{b}$ | **4.6±2.4** | 89.5±3.8$^{ab}$ | **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*** | 3.7±0.8$^{d}$ | 0.8±0.05$^{f}$ | NA | 0.17±0.04$^{e}$ | 20.6±1.2$^{f}$ | 135.8±9.7$^{f}$ | 256.6±2.9 | 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**$^{d}$ | 1.3±0.03$^{cde}$ | 0.96±0.02 | 0.76±0.03$^{b}$ | 86.8±0.7$^{ab}$ | 9.9±2.1$^{ab}$ | **1.8±0.1** | 92.1±3.2$^{a}$ | **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$^{d}$ | 0.6±0.01$^{f}$ | NA | 0.17±0.02$^{e}$ | 21.8±0.4$^{f}$ | 142±3.7$^{f}$ | 305.5±5.9 | 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$^{d}$ | 1.3±0.01$^{de}$ | 0.50±0.02 | 0.68±0.04$^{b}$ | 83.3±2.2$^{b}$ | 20.8±6.0$^{bc}$ | **2.1±0.2** | 86.5±3.4$^{ab}$ | **6.1±0.1**$^{abc}$ | 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$^{bcd}$ | 1.2±0.05$^{e}$ | NA | 0.51±0.06$^{c}$ | 65.7±4.0$^{c}$ | 63.5±11.5$^{d}$ | 112.6±21.1 | 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**$^{cd}$ | 1.5±0.01$^{bcde}$ | 0.32±0.02 | 0.72±0.02$^{d}$ | 83.9±3.2$^{ab}$ | 35.8±5.2$^{c}$ | **1.8±0.1** | 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 : $10^6$cells.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**

Boxplot 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. The small letters indicate the statistical groups from a Tukey analysis.

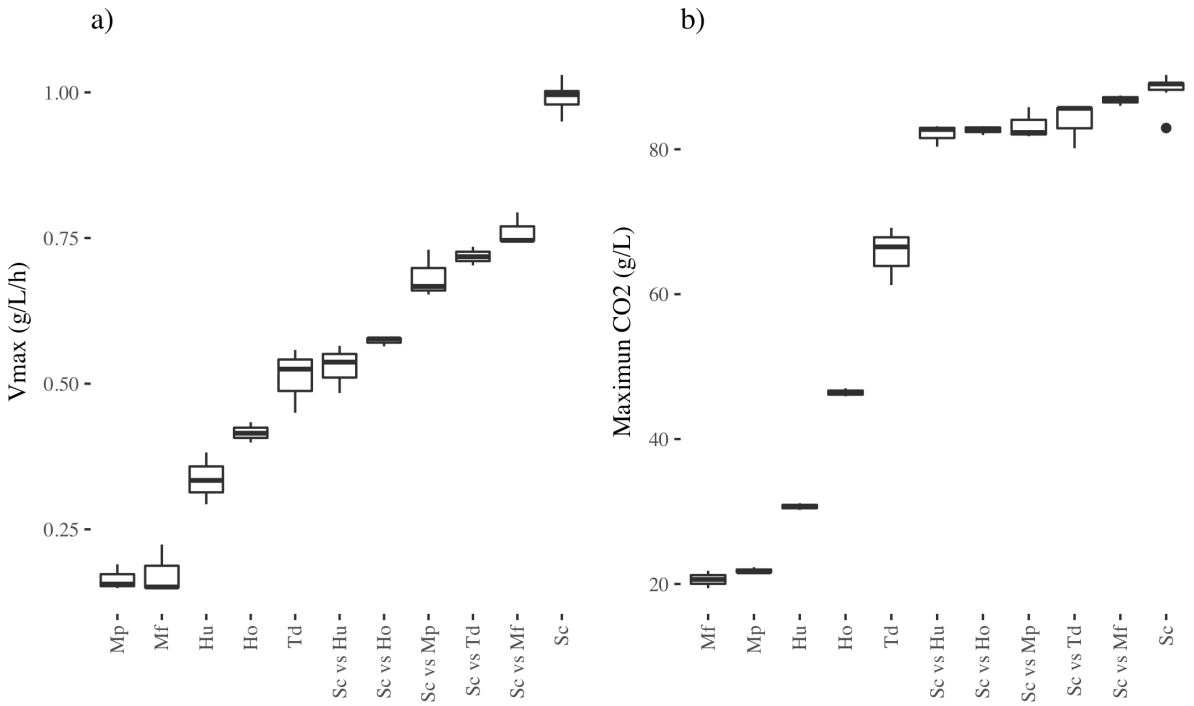
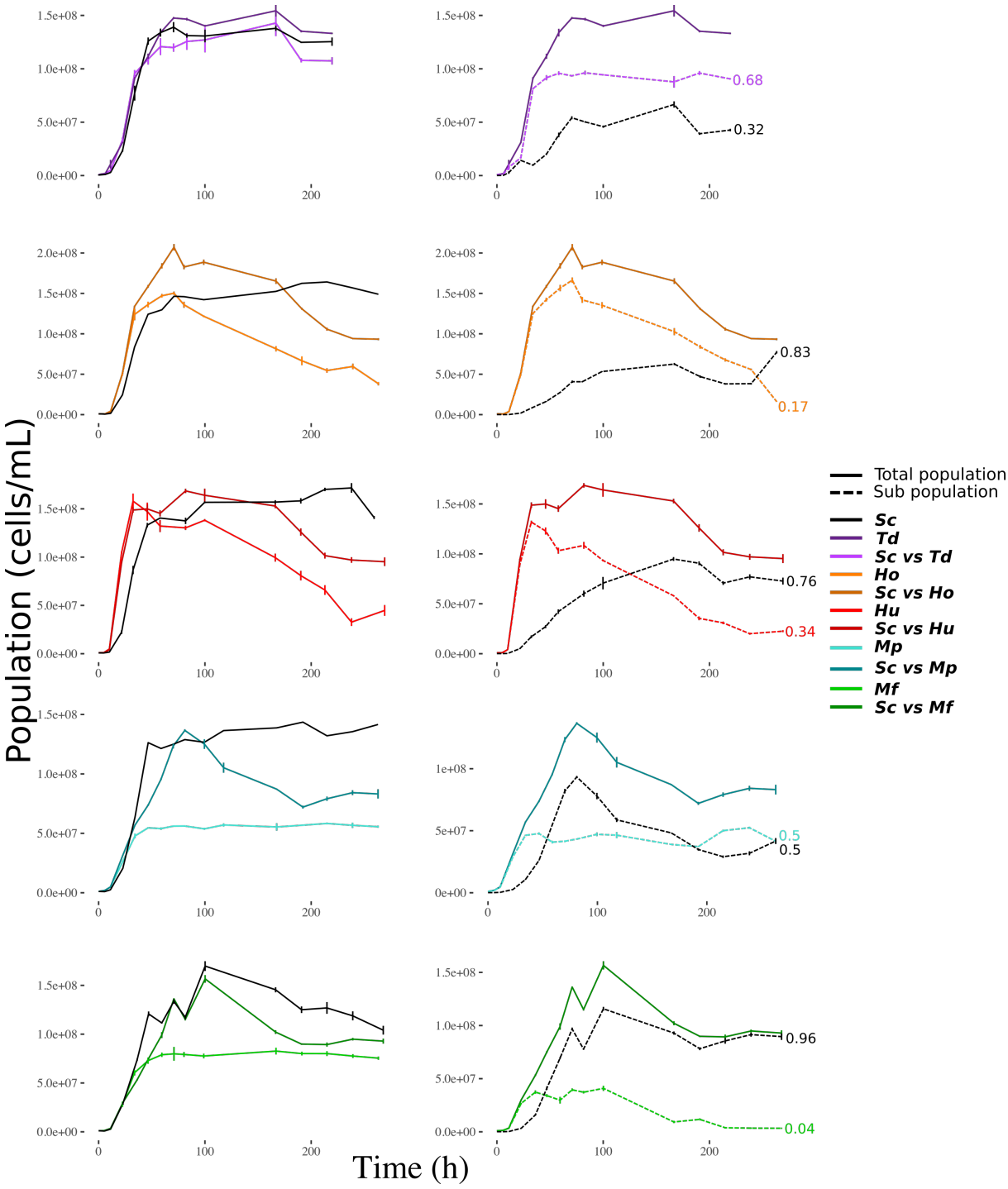



**FIG2**

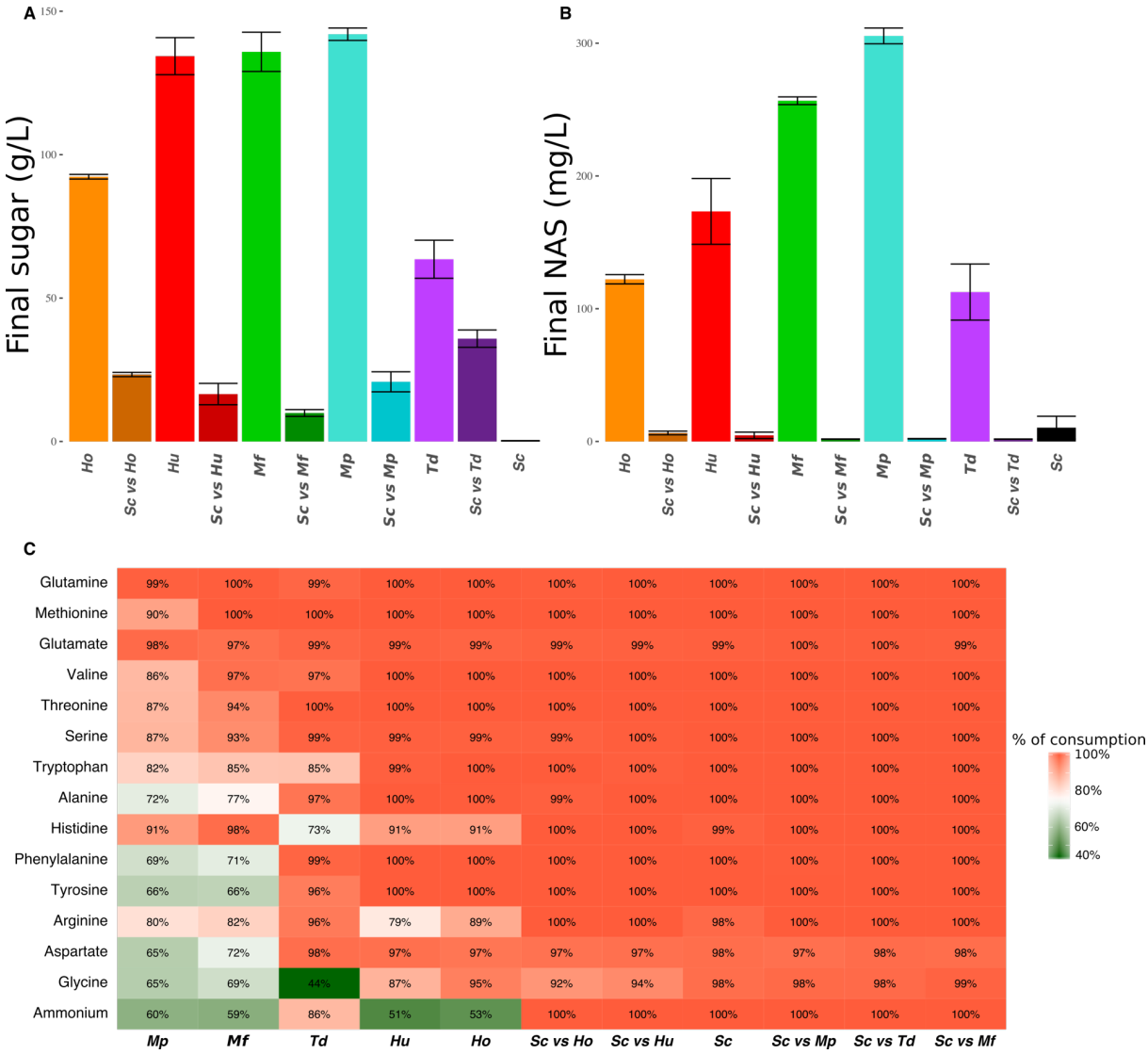
**Global monitoring of the kinetics of the total living population (left), and sub-population in the mixed cultures (right) across the fermentation**. All points represent a sample (average ± standard error). The full lines are for total population and the dashed lines for the two sub populations into 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 culture of 'non-*Saccharomyces*' strains and the darker to the corresponding culture in competition with *S.*

*cerevisiae*. The isolated cultures of *S. cerevisiae* are represented in black. ***Sc, Mp, Mf, Ho, Hu, Td, ScvsMp, ScvsMf, ScvsHo, ScvsHu, ScvsTd*** correspond respectively to cultures with: isolated *S. cerevisiae*, isolated *M. pulcherrima*, isolated *M. fructicola*, isolated *H. opuntiae*, isolated *H.uvarum*, isolated *T. delbrueckii*, *M. pulcherrima* mixed with *S. cerevisiae*, *M. fructicola* mixed with *S. cerevisiae*, *H. opuntiae* mixed with *S. cerevisiae*, *H. uvarum* mixed with *S. cerevisiae* and *T. delbrueckii* mixed with *S. cerevisiae*.



526 **FIG3**

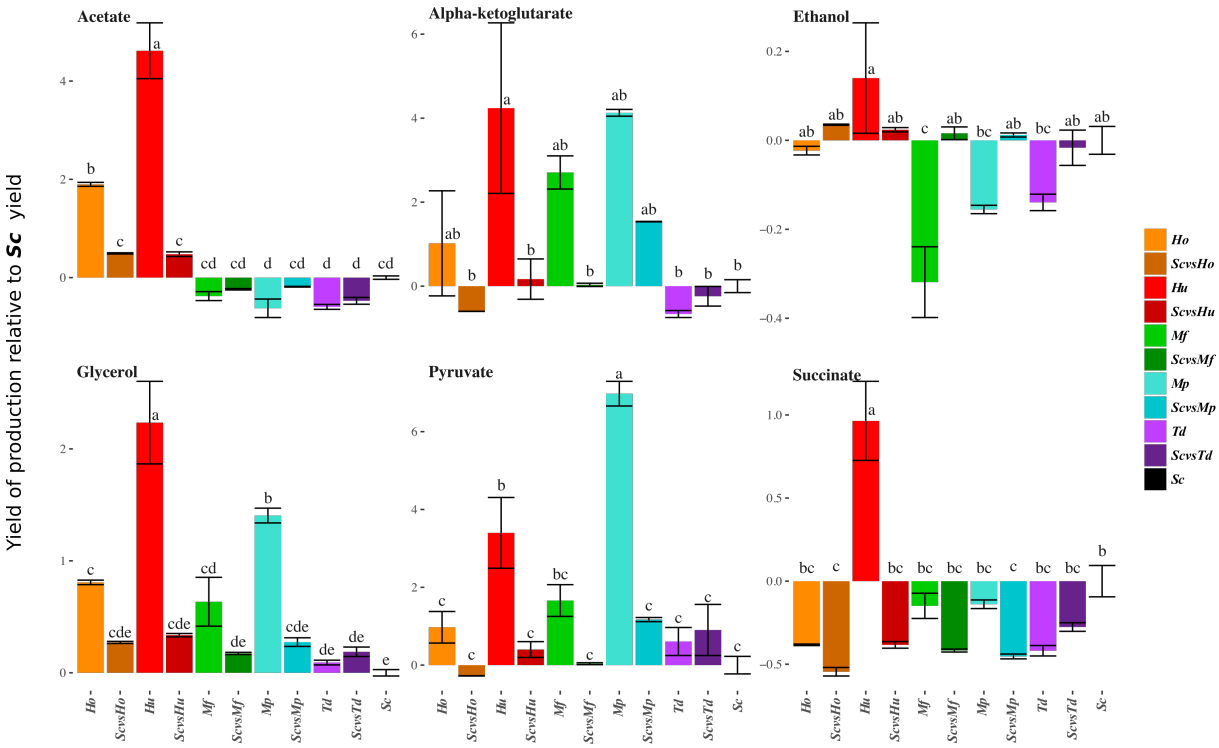
527 **Consumption of sugars and nitrogen assimilable sources (NAS) for each type of**
528 **fermentation.** A) Final concentration of sugar (average ± standard error). B) Final
529 concentration of NAS (average ± standard error). C) Percentage of consumption of each NAS
530 in each type of fermentation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




536

537 **FIG4**

538 Yield (average ± standard error) of carbon metabolite production relative to the yield of
539 production of isolated *S. cerevisiae* cultures for acetate, alpha-ketoglutarate, ethanol, glycerol,
540 pyruvate and succinate and each type of fermentation. ***Sc, Mp, Mf, Ho, Hu, Td, ScvsMp,***
541 ***ScvsMf, ScvsHo, ScvsHu, ScvsTd*** correspond respectively to cultures with: isolated *S.*
542 *cerevisiae*, isolated *M. pulcherrima*, isolated *M. fructicola*, isolated *H. opuntiae*, isolated
543 *H.uvarum*, isolated *T. delbrueckii*, *M. pulcherrima* mixed with *S. cerevisiae*, *M. fructicola*
544 mixed with *S. cerevisiae*, *H. opuntiae* mixed with *S. cerevisiae*, *H. uvarum* mixed with *S.*
545 *cerevisiae* and *T. delbrueckii* mixed with *S. cerevisiae*.



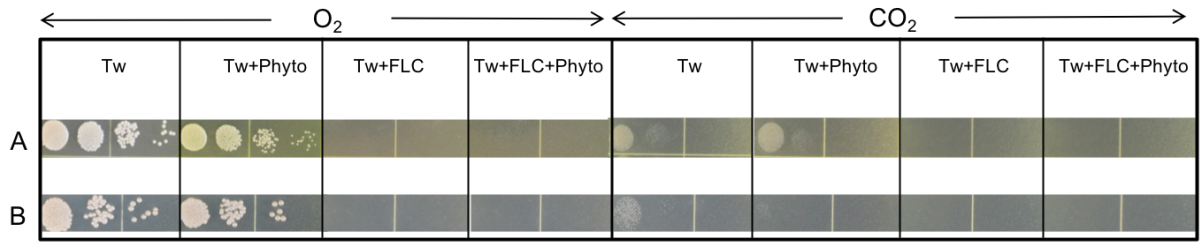

546

547 # Supplementary informations

548 **FIG S1**

549 Serial tenfold dilutions of two *Metschnikovia pulcherrima* strains (A and B) spotted onto
550 various synthetic standard agar media (SM425, 425 mg/l assimilable nitrogen) with Tween 80,
551 (Tw, 0.06%), supplemented or not with phytosterol (Phyto, 20 mg/L), in the presence or not of

552 fluconazole (FLC, 256 µg/mL). Plates were incubated at 28°C for five days in air or in

553 anaerobiosis.

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