Investigations of the mechanisms of interactions between four non-conventional species with *Saccharomyces cerevisiae* 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, namely competition, predation, commensalism and mutualism. Traditional wine fermentation is a complex microbial process performed by *Saccharomyces* and non-*Saccharomyces* (NS) 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 NS yeast species (*Metschnikowia pulcherrima, M. fructicola, Hanseniaspora opuntiae and H. uvarum*). In each case, we studied population dynamics, resource consumed and metabolites produced from central carbon metabolism. This phenotyping of competition kinetics allowed us to confirm the main mechanisms of interaction between strains of four NS 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 specific cell populations in mixed cultures 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. In particular the originality of this paper is for the first times to highlight the joint-effect of different species population dynamics on glycerol production and also to discuss on the putative role of lipid uptake on the limitation of some non-conventional species growth although interaction processes.

1. 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 . 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 (1,2). Most often, these "starters" are only composed of a single *Saccharomyces cerevisiae* (S. c.) 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 (3–6). 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 (3,4,6,7).

Indeed, there are numerous experiments and even industrial products making use of such mixed starters to improve wine's organoleptic qualities (4,8,9). The non-*Saccharomyces* (NS) strains used in these experiments are very diverse, with more than 23 different species including

71 *Torulaspora delbrueckii*, *Metschnikowia pulcherrima*, *Metschnikowia fructicola*,
72 *Hanseniaspora opuntiae* and *Hanseniaspora uvarum*. *S*pecies in the *Metschnikowia* genus
73 ferment poorly in oenological conditions but can have interesting attributes: in conjunction with
74 *S. cerevisiae*, a strain of *M. pulcherrima* could reduce ethanol concentrations (6,10), increase
75 'citrus/grape fruit' and 'pear' attributes (11), as well as allow the persistence of 'smocky' and
76 'flowery' characteristics (12). *M. pulcherrima* also has an amensalism effect on *S. cerevisiae*
77 through iron depletion via the production of pulcherriminic acid (13). *M. fructicola* has been
78 less studied and never in conjunction with *S. cerevisiae* although it presents the interesting
79 ability to inhibit *Botrytis* growth (14). 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 (6). It notably increased the 'tropical fruit', 'berry', 'floral' and
82 'nut aroma' characters (15), that were linked to higher concentrations of acetate esters, esters
83 of MCFAs, isoamyl alcohol, 2-phenylethanol and α-terpineol (16).

84 Despite these various studies, the composition and protocol of inoculation of these multi-
85 strains starters are still very empirical and only based on the input/output balance, without
86 considering the dynamics of the microbial populations or their interactions. This lack of
87 knowledge about yeast-yeast interactions prevents implementing a rational design of multi-
88 strain starters (17). To address this problem, we decided to focus our study on population
89 dynamics and metabolites produced during oenological fermentations performed in isolated or
90 mixed yeast cultures. Since our goal was not to obtain optimal mixes but to understand the
91 mechanism of microbial interaction, we chose to compare the population dynamics and yields
92 between isolated cultures of strains from five species (one *S. cerevisiae* and four NS) and four
93 corresponding mixed cultures always including the *S. cerevisiae* strain as reference. We were
94 thus able to identify key microbial interaction mechanisms that are further discussed.
95

2. Results

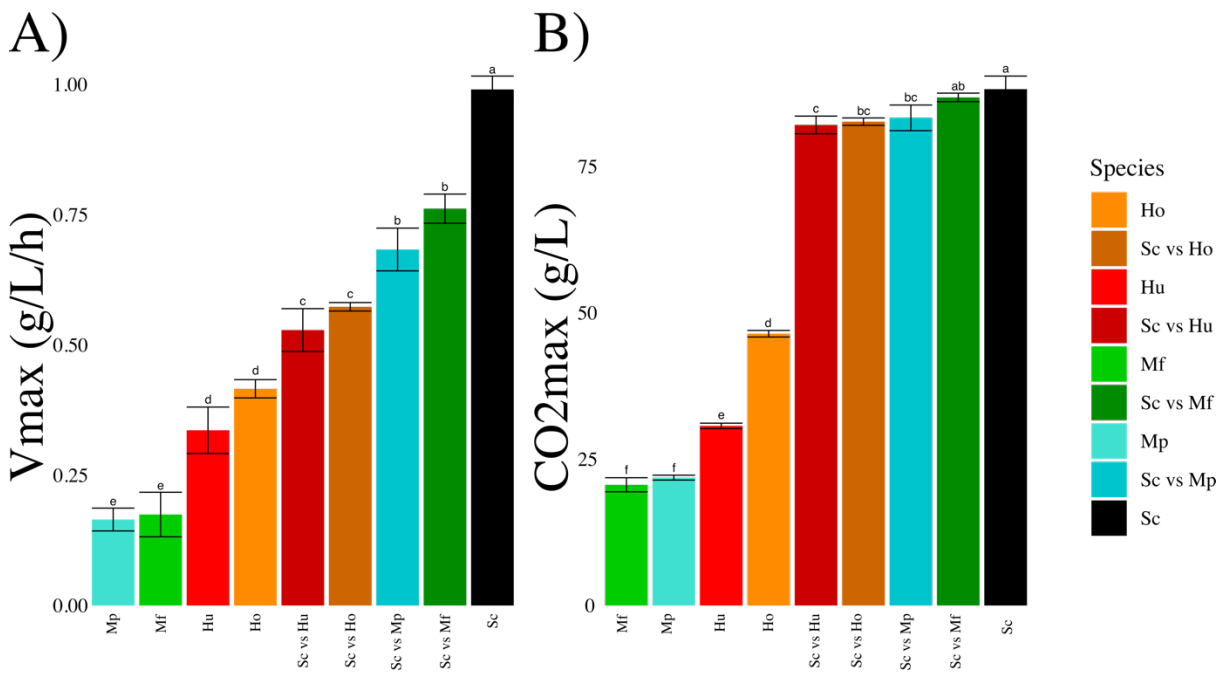
In this work, we compared in winemaking conditions the performance of single cell cultures of five different strains from five yeast species (*Saccharomyces cerevisiae*, *Metschnikowia pulcherrima*, *Metschnikowia fructicola*, *Hanseniaspora opuntiae* and *Hanseniaspora uvarum*) and mixed co-cultures combining each of the four NS species with one GFP-labelled *S. cerevisiae* strain representing 10% of the initial inoculate. We choose to stop the monitoring of fermentation at a given time, even if the sugar supply was not completely exhausted. Thus, for all fermentation with the *S. cerevisiae* reference strain, sugars were exhausted after around 200-220 h while in fermentations with single NS 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 single strain and mixed strain cultures, we evaluated the intensity of yeast-yeast interactions and/or their consequences on ecosystem service production.

2.1. 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) and of the maximum CO_2 produced were estimated (***CO_2max***, Fig 1b). ***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 $\boldsymbol{Vmax_{Mf}}$ = 0.17 ± 0.04 $g.L^{-1}.h^{-1}$). The four mixed cultures had intermediate ***Vmax*** values between those of ***Sc*** and

121 the highest **Vmax** of all *non-sacc* cultures (Fig 1a). Mixed cultures containing *Metschnikowia*
122 species had significantly higher ***Vmax*** values than those containing *Hanseniaspora* species (Fig
123 1a). Although we did not monitor all cultures until the exhaustion of glucose and fructose, it
124 was however possible to estimate the capacity of a given species to complete fermentation by
125 estimating the amount of CO_2 produced during the first 300 hours. ***Sc*** fermentations finished
126 after around 220 hours with a $\boldsymbol{CO2max_{Sc}}$ = 88.2 ± 2.2 g.L^{-1}. We therefore can make the
127 hypothesis that all cultures that produced more than 80g CO_2.L^{-1} (90% of ***Sc*** maximum) within
128 300 hours will be able to complete fermentation. Under this assumption, all mixed cultures, but
129 no NS single strain cultures, would eventually complete fermentation. Among the latter
130 cultures, both *Hanseniaspora* species had the highest $\boldsymbol{CO_2max}$ (***Hu*** 30 ± 0.4 g.L^{-1}, and ***Ho*** 46
131 ± 0.6 g.L^{-1}) followed by *Metschnikowia* species (***Mp*** 22 ± 0.4 g.L^{-1} and ***Mf*** 20 ± 1 g.L^{-1}).
132
133 **FIG 1**
134 **Maximum rate of CO_2 production, *Vmax* (A) and total CO_2 produced (B) in function of**
135 **the single or mixed strain species driving each fermentation.** Values corresponds to average
136 ± standard deviation. The small letters indicate the statistical groups from a Tukey analysis.



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138

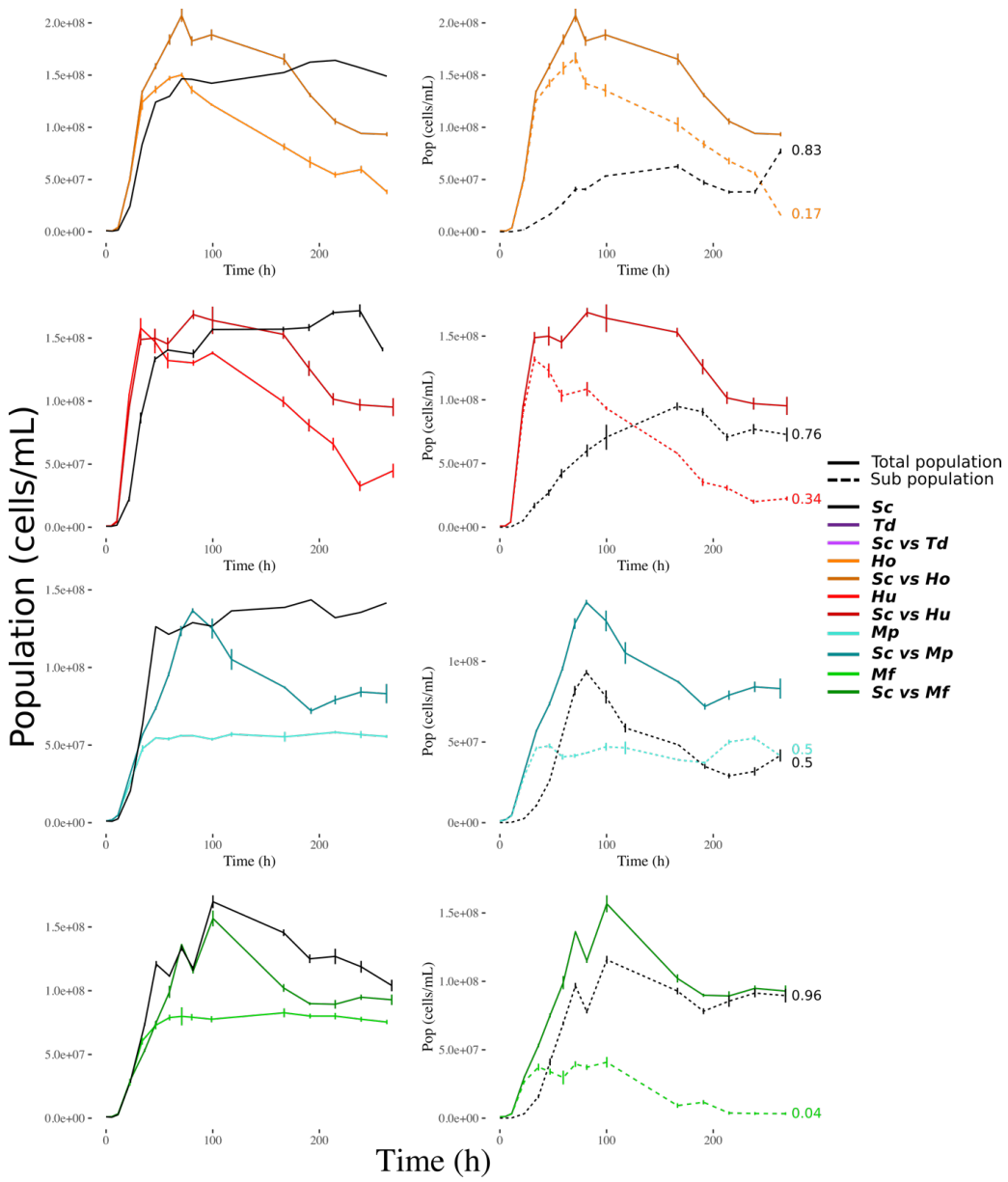
139 **2.2. Population Kinetics**

140 We also looked at population dynamics in each culture (Fig 2) and determined the
141 maximum growth rate of the population ($\boldsymbol{\mu}$), the maximum population size, also termed carrying
142 capacity ($\boldsymbol{K}$) and the relative abundance (by cytometry) of each species after 300 hours of mixed
143 culture, corresponding in our case to the end of the monitoring period (S1 Table). Fermentations
144 with *S. cerevisiae* alone went through an exponential growth rate ($\boldsymbol{\mu_{Sc}}$ = 0.15 ± 0.02 h^{-1}) and
145 reached a maximum population of around 1.5*10^8cells.mL^{-1} ($\boldsymbol{K_{Sc}}$ = 1.55 ± 0.15 10^8cells.mL^{-1})
146 that remained constant until the end of the fermentation. Fermentations with either
147 *Hanseniaspora* species alone had a growth dynamic like ***Sc*** at the beginning of the fermentation
148 but a higher growth rate ($\boldsymbol{\mu_{Ho}}$ = 0.19 ± 0.03 h^{-1}, $\boldsymbol{\mu_{Hu}}$ = 0.62 ± 0.18 h^{-1}). On the opposite, their
149 stationary phase was quite different from that of ***Sc*** and characterized by a higher cell mortality
150 with a population drop of about 70% by the end of the process. Fermentations performed by
151 *Metschnikowia* species in single strain culture had growth dynamics mostly similar to ***Sc***
152 fermentations: a similar growth rate ($\boldsymbol{\mu_{mp}}$ = 0.18 ± 0.03 h^{-1}, $\boldsymbol{\mu_{Mf}}$ = 0.17 ± 0.2 h^{-1}), no mortality
153 during the stationary phase but a much reduced maximum population ($\boldsymbol{K_{Mp}}$ = 0.57 ± 0.01
154 10^6cells.mL^{-1}, $\boldsymbol{K_{Mf}}$ = 0.8 ± 0.05 10^6cells.mL^{-1}). In most cases, mixed cultures displayed an
155 intermediate pattern between the two corresponding single strain cultures (Fig 2). However,
156 mixed or single strain cultures with *Metschnikowia* displayed different cell mortality rates
157 during the stationary phase: in the case of ***ScvsMp*** fermentations, only the *S. cerevisiae*
158 population decreased significantly during the stationary phase, while in ***ScvsMf*** fermentations,
159 both subpopulations significantly decreased. As a measure of fitness, we also followed the
160 variations of *S. cerevisiae* frequency along the fermentation. In all mixed cultures, *S. cerevisiae*
161 was found dominant (frequency > 50%) in the end, increasing significantly during fermentation
162 from 10% initially to frequencies varying between 50% (***ScvsMp***) and 96% (***ScvsMf***) (Fig 2).

163

164 **FIG 2**

165 **Global monitoring of the kinetics of the total living population (left), and sub-population**
166 **in the mixed cultures (right) across fermentation**. Each population was detected by flow
167 cytometry as indicated in the Material and Methods section. Each point represents a sample
168 (average ± standard error). Full lines are for total population and dashed lines for the two sub
169 populations in mixed cultures. At the end of dashed lines, the final proportion of both sub-
170 populations in mixed cultures is indicated. The light colors represent single straind cultures of
171 'non-*Saccharomyces*' strains and dark ones to the corresponding culture in competition with
172 *S. cerevisiae*. The single strand cultures of *S. cerevisiae* are represented in black.



2.3. Sugar and nitrogen assimilable source consumption

We then looked at the final concentration of resources: sugars (fructose and glucose) and nitrogen assimilable source (NAS) i.e. ammonium and amino-acids (Fig 3). In ***Sc*** fermentations, less than 0.1% of the initial concentration of both sugars remained (Fig 3a). As seen in the paragraph concerning CO_2 production, NS species in single strain cultures did not complete fermentation in the 300h period and left respectively 45% of sugars for ***Ho***, 67% for ***Hu***, 68% for ***Mf*** and 71% for ***Mp***. Furthermore, all species except *H. opuntiae* preferentially

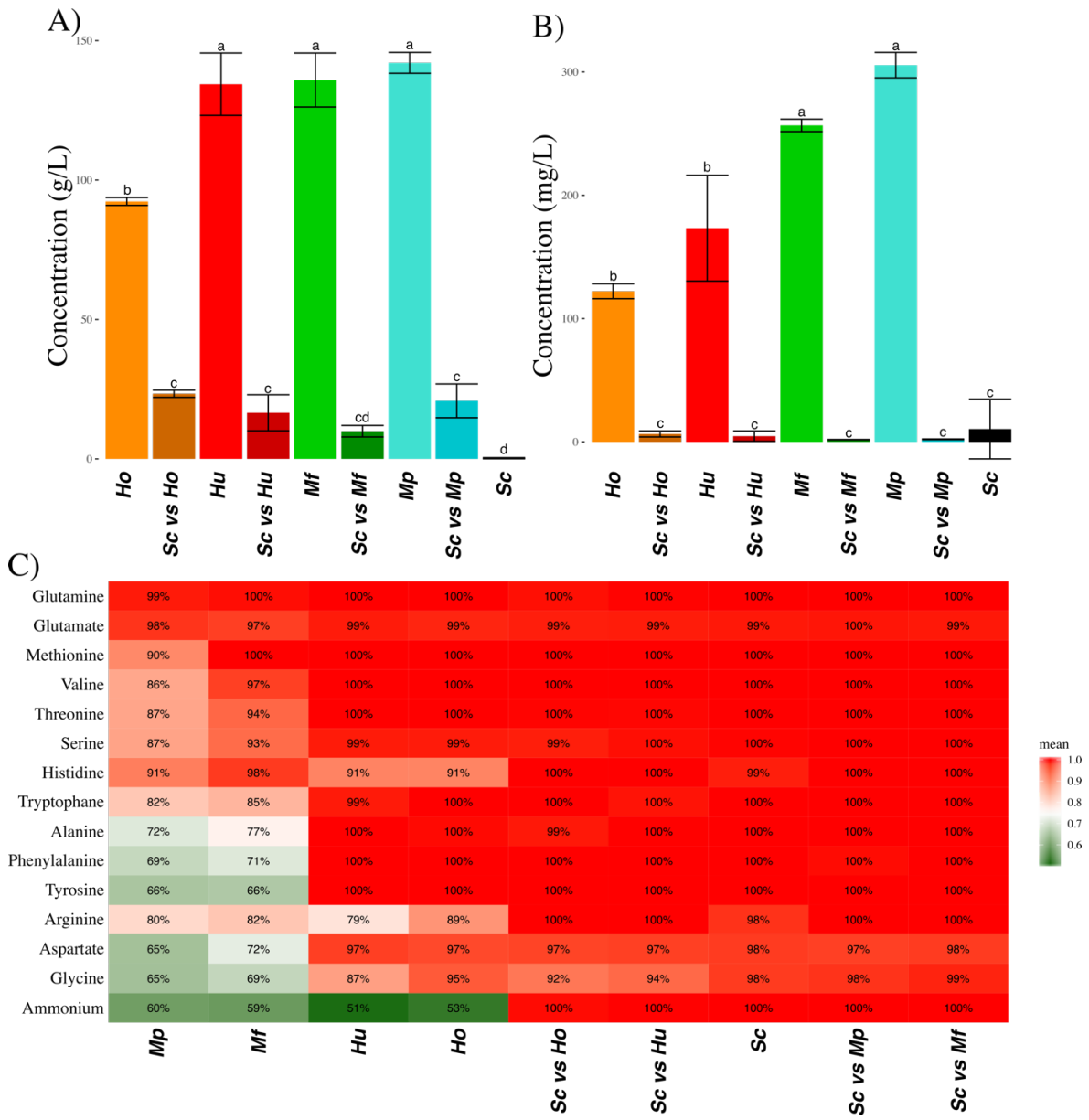
182 consumed glucose (S1 Fig). Sugar consumption was higher in mixed cultures than in single NS
183 species cultures (Fig 3a). However, it was still lower than in ***Sc*** species cultures, also with a
184 preference for glucose. This indicates the major impact of *S. cerevisiae* on sugar consumption
185 (consistent with the CO_2 production observed), compared to the other NS species studied.

186 The consumption of NAS displayed the same pattern (Fig 3b). NAS were almost entirely
187 consumed both in ***Sc*** single strain cultures and in all co-cultures, whereas in NS single strain
188 cultures the fraction of NAS consumed varied between 84% and 94%. However, the preference
189 for different nitrogen sources varied with each NS species (Fig 3c). Both *Hanseniaspora*
190 species had similar behaviors, consuming only half of the available ammonium, 90% of
191 histidine and 89% or 79% of arginine (Fig 3c). *Metschnikowia* species presented a similar
192 pattern. It was possible to classify these NS species preferences for the various NAS. The
193 resulting ranking by order of preference was glutamine, methionine, glutamate, valine,
194 threonine, serine, tryptophan, alanine, histidine, arginine, aspartate, glycine and, surprisingly
195 last, ammonium.

196

197 **FIG3**

198 **Consumption of sugars and nitrogen assimilable sources (NAS) for each type of**
199 **fermentation.** A) Final concentration of sugar (average ± standard deviation). B) Final
200 concentration of NAS (average ± standard deviation). C) Percentage of consumption of each
201 NAS in each type of fermentation represented as a color gradient from green (<75 %) to red (>
202 75%).



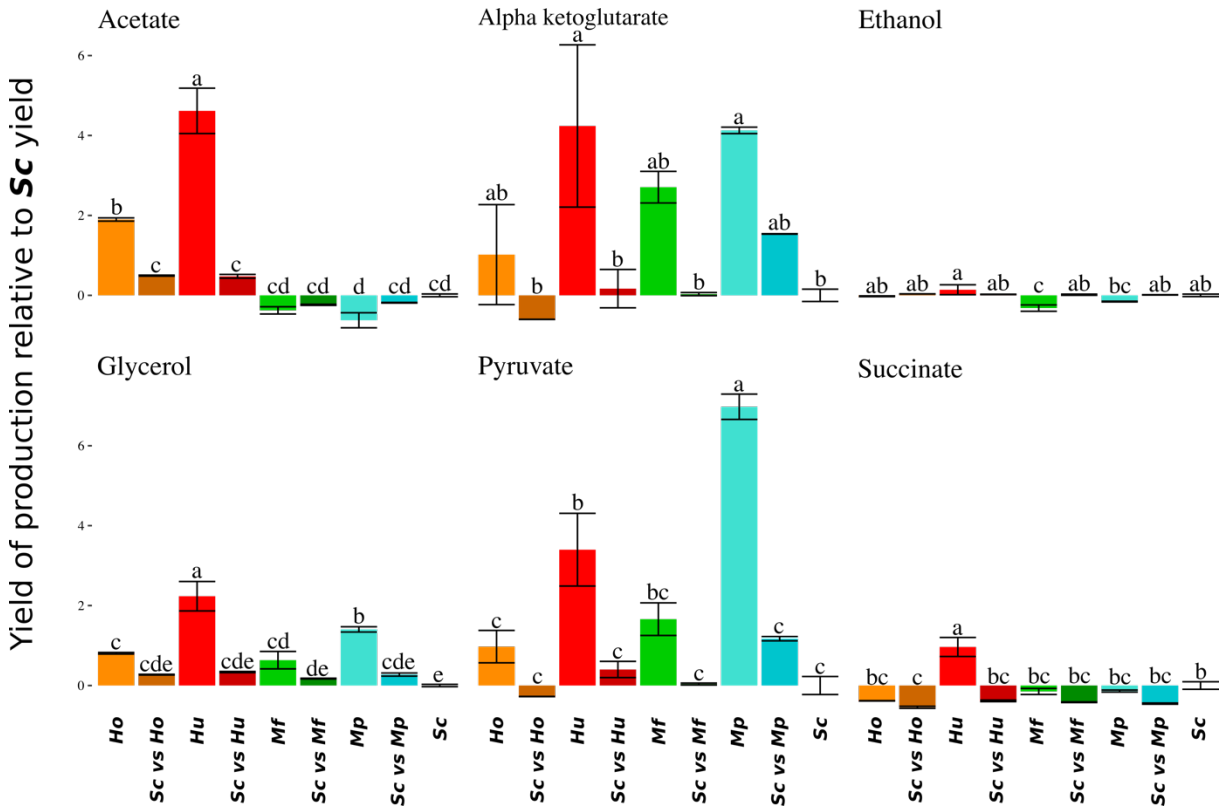
2.4. Metabolite production

In parallel with must resources consumption monitoring, we also investigated the production of metabolites from Central Carbon Metabolism (CCM): ethanol, glycerol, succinate, pyruvate, acetate and alpha-ketoglutarate (Fig 4). These measurements of metabolite production were taken after 300 hours when sugars consumptions were quite different from one culture to another depending on their dynamics. To allow figures comparison, we computed the

production yield (total production / sugar consumption) for each culture and, from these data, we then estimated this yield relatively to that of ***Sc*** in single strain culture (Fig 4).

FIG 4

Yield of carbon metabolite production relative to the yield of production of single *S. cerevisiae*. Average Yield are given with standard deviations for acetate, alpha-ketoglutarate, ethanol, glycerol, pyruvate and succinate and each type of fermentation.



In the case of ethanol, only ***Mf*** fermentations had a relative yield significantly inferior (-32%) to 0 (0 being ***Sc*** yield). For glycerol, all isolated NS fermentations had a greater yield than ***Sc*** and mixed fermentations were intermediate between (but not significantly different from) the corresponding single strain cultures. For acetate, only *Hanseniaspora* strains displayed a higher yield (Fig 4).

225 Finally, all mixed cultures seemed to have a lower succinate yield than both
226 corresponding isolated cultures (but not significantly after correction for multiple tests).
227
228 For each fermentation, the total production of metabolites resulted from the combination
229 of species yields, total sugar consumption and respective population dynamics during
230 fermentation. Therefore, differences observed in the total productions of mixed cultures were
231 the consequences of additive or subtractive effects observed for these 3 components.
232 Considering ethanol, its total production was directly linked to the consumption of resources
233 and all mixed cultures were equivalent to ***Sc*** fermentations (S2 Fig). The case of glycerol was
234 more interesting. Indeed, even if the average sugar consumption was lower in ***ScvsHu*** and
235 ***ScvsMp*** mixed cultures than in single strain ***Sc*** culture, the total production of glycerol was
236 significantly higher than that of the corresponding single strain cultures ($Glycerol_{SCvsMp}$ =
237 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
238 positive combination of the greater glycerol yield by *Hanseniaspora* and *Metschnikowia* and
239 their population dynamics. For all other metabolites, the total production of mixed cultures was
240 not significantly different from the corresponding single strain cultures (S1 Table).
241
242

243 3. Discussion

244 This study presents one of the first works focusing on the population dynamics and
245 kinetics of yeast-yeast interactions between two species (S.C. and NS) during the alcoholic
246 fermentative process (18), but dynamics are distinct according to the *M. pulcherrima* and *H.*
247 *uvarum* strains tested in this study. Firstly, all strains grew in single strain and mixed strain
248 cultures. *Metschnikowia* single strain cultures present lower Vmax values than *Hanseniaspora*

249 single strain cultures, while mixed cultures containing *Metschnikowia* species had significantly
250 higher Vmax values than those containing *Hanseniaspora* species. It appears that the better
251 growth behavior of *Hanseniaspora* strains limited more *S. cerevisiae*'s growth than
252 *Metschnikowia* in mixed culture. The growth characteristics of the competing strains directly
253 impact the mixed fermentation characteristics.

254 Mixed population dynamics were characterized by similar growth rate (reaching a
255 maximum population like *S. cerevisiae* single strain culture), followed by a long phase with
256 decreasing mortality. NS viability dropped to 30% in accordance with Moreira et al., 2008 and
257 Tristezza et al., 2016. Their NAS consumption was also similar, with half of the available
258 ammonium not being consumed. Even their yield and total production of CCM metabolites
259 were very similar. For almost all these characteristics, mixed cultures presented intermediate
260 phenotypes compared to the corresponding single strain cultures.

261 However, there is a remarkable exception considering the total production of glycerol
262 that is superior in mixed cultures whereas their sugar consumption was inferior (Figure 4). This
263 point is characteristic of a transgressive interaction (often referred to as over-yielding), i.e. a
264 situation in which the ecosystem performance is higher than that of the best-yielding species
265 present (when cultivated alone). This glycerol overproduction in mixed cultures has already
266 been observed in previous works (16,19) but is not systematic depending on the strains and
267 experimental conditions (20). In the present study, the glycerol and sugar consumption
268 observed in mixed cultures is for the first time explained by the joint effect of population
269 dynamics (*S. cerevisiae* slowly dominating the population), resource consumptions (the **NS**
270 fermentation leaving ⅔ of sugars) and the glycerol yields of NS strains that were two to three
271 times higher that of *S. cerevisiae*.

272 These overproductions of glycerol open the way for potentially more interesting results.
273 Our results point out that inoculation conditions in mixed cultures must be considered according

274 to fermentation aims and strains characteristics. Exploiting this specific yield and population
275 dynamics, for example by inoculating the S. *cerevisiae* strain at lower relative frequencies
276 (0.01, 0.001, 0.0001), more important transgressive interactions (such as lower ethanol
277 production) could be achieved in mixed cultures.

278 Meanwhile NS fermentations present similarities, it was possible to group the NS strains
279 performance according to their genus (see PCA in figures 5). While *H. uvarum* and *H. opuntiae*
280 both presented similar phenotypes, *M. pulcherrima* and *M. fructicola* presented a more distinct
281 phenotype. Regarding *Metschnikowia* species, ***Mp*** and ***Mf*** single strain cultures were
282 characterized by a short growth phase reaching a small carrying capacity and followed by a
283 long stationary phase, maintaining viability at a high level.

284 Nevertheless, despite *Metschnikowia* high viability, the resources measured (sugars and
285 nitrogen) were not entirely consumed. The reason why the cells stopped growing remains
286 unclear. A possible explanation could be linked to resources that have not been considered in
287 the present study. For instance, the third most important resource to be considered during
288 fermentation, even using synthetic medium, is lipid content, available as phytosterols and fatty
289 acids. In fact, for all yeast species, yeast lipid synthesis requires oxygen and is therefore
290 impossible in anaerobic conditions. With the progress of fermentation and ensuing oxygen
291 limitation yeast should import lipids to survive. If not, it stops to multiply. It is the lack of lipid
292 uptake under anaerobiosis that we can hypothesized for some *Metschnikowia* species. In fact,
293 we have shown that some of them were unable to import lipids from the medium, depending
294 instead on their own (internal) lipid synthesis for their growth (see Fig S1).

295 Interestingly, even though *S. cerevisiae*, *M. pulcherrima* and *M. fructicola* mortality rates
296 were low in single strain cultures, the corresponding mixed cultures (***ScvsMp*** and ***ScvsMf***)
297 presented 30% mortality (after 200 hours of fermentation). Moreover, this mortality seemed to
298 impact both species differently. In the mixed ***ScvsMp*** culture, only *S. cerevisiae* cells eventually

299 died, whereas in ***ScvsMf*** both species were negatively affected. The survival of *M. pulcherrima*
300 cells compared to *S. cerevisiae* cells could be explained by the production of pulcherriminic
301 acid by *M. pulcherrima* (21). Indeed, pulcherriminic acid is known to deplete iron from the
302 medium, which has a lethal effect on *S. cerevisiae* cells (13,22,23). In ***ScvsMf*** cultures, the
303 mortality observed in both species suggests a more complex mechanism of interaction
304 (although it is not clear whether *M. fructicola* also produces pulcherriminic acid; Kurtzman and
305 Droby, 2001). To explain these results, we could hypothesize the conjunction of two different
306 mechanisms of interaction. It could be that *M. fructicola* synthesized a metabolite
307 (pulcherriminic acid?) impacting the viability of *S. cerevisiae* cells (through iron depletion?),
308 with *M. fructicola* cells dying thereafter for another reason such as sensitivity to ethanol.
309 Indeed, the production of ethanol was almost four times higher in mixed cultures than in single
310 strain *Metschnikowia* fermentations. Under such hypothesis, the reason why no loss of viability
311 was observed for *M. pulcherrima* in mixed culture with *S. cerevisiae* could probably be related
312 to a better tolerance of *M. pulcherrima* to ethanol stress compared to *M. fructicola*.
313
314 For all *Metschnikowia* and *Hanseniaspora* mixed cultures, we only discussed
315 transgressive interactions when mixed cultures over-produced (or under-produced) a given
316 metabolite. Indeed, it was very difficult to identify interactions when the productions of mixed
317 fermentations were within the range of isolated cultures productions. As the relative frequency
318 of both strains in mixed cultures evolved during fermentation, it was difficult to link the final
319 mix to the contributions of each species. It was even more difficult to assess whether these
320 contributions combined additively or with interaction. New statistical developments or dual
321 transcriptomics will be needed to answer this question (24–26).
322

323 To discuss more generally the mechanisms of interactions of *Metschnikowia* and
324 *Hanseniaspora* cultures mixed with *S. cerevisiae*, we could not evidence any major antagonistic
325 phenomena. For almost all assays, mixed cultures performance stood always between that of
326 the corresponding isolated cultures (Fig 1, 2 & 3), except for the total production of glycerol.
327 Moreover, despite the differences in yield and interactions between species, the rapid
328 dominance of *S. cerevisiae* (increasing from 10% to at least 50% during the fermentation)
329 resulted in mixed cultures that were overall not different from *S. cerevisiae* single strain
330 cultures. This result is in agreement with the good adaptation of *S. cerevisiae* to winemaking
331 conditions (4–6,27) but could be different with a different set of strains. This result is important
332 in the context of ecological engineering. In fact, our results confirmed that *S. cerevisiae* has a
333 much better fitness than the NS strains studied in this paper. Therefore, if we want mixed culture
334 behavior to deviate from that of *S. cerevisiae* single strain culture, it must be ensured that NS
335 cells dominate the culture as soon as possible. To achieve this, two conceivable options are
336 currently tested: either to reduce the proportion of *S. cerevisiae* at t_0 or to successively inoculate
337 (16) first the NS species , and only then the *S. cerevisiae* strain. These two options could be
338 equivalent depending on the type of interaction(s) that occurs. If strain behaviors in single
339 strain or mixed cultures are identical, then all interactions are mediated by the medium through
340 the competition for resources and the production of constitutive toxins such as ethanol
341 (producing a toxin only in mixed fermentation would be a behavior change) and could be
342 qualified as "indirect".
343 Mathematical models could be designed from data on isolated cultures to predict the
344 impact of indirect interactions in mixed cultures. This would allow simulating numerous mixes
345 of species with various initial conditions and identify optimal strategies depending on one or
346 several given criteria. Using these approaches could limit the number of necessary tests,
347 potentially saving a lot of time and money and opening the way to a more methodical ecological

engineering. The development of such mathematical models will only be possible thanks to a deep tracking of population dynamics to understand underlying mechanisms of growth and mortality. Obviously, it is also critical to validate this approach by i) first extending the number of species 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.).

4. Mat & Met

4.1. 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 strain, 3 replicates of single strain cultures were performed (except for *S. cerevisiae* that had a total of 8 replicates in different blocks). In addition, for each NS strain, 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

372 to by the species that performed them, i.e. single strain cultures were referred to as: ***Sc***, ***Mp***,
373 ***Mf***, ***Hu*** and ***Ho*** and mixed strain cultures as ***ScvsMp***, ***ScvsMf***, ***ScvsHu*** and ***ScvsHo***.
374
375 **4.2. Medium**
376 Initial cultures (12 h, in 50 ml YPD medium, 28 °C) were used to inoculate fermentation
377 media at a total density of 10^6 cells/mL; therefore, for mixed culture the *S. cerevisiae* cells
378 density was $0.1x10^6$ /mL and the NS cells density was $0.9x10^6$ /mL. Fermentations were carried
379 out in a synthetic medium (SM) mimicking standard grape juice (28). The SM used in this study
380 contained 200 g/L of sugar (100 g glucose and 100 g fructose per liter) and 200 mg/L of
381 assimilable nitrogen (as a mix of ammonium chloride and amino acids). The concentrations of
382 weak acids, salts and vitamins were identical to those described by Seguinot et al. (29). The pH
383 of the medium was adjusted to 3.3 with 10M NaOH. The SM medium was first saturated with
384 bubbling air for 40 minutes, then it was supplemented with 5 mg/L phytosterols (85451, Sigma
385 Aldrich) solubilized in Tween 80 to fulfill the lipid requirements (sterols and fatty acids) of
386 yeast cells during anaerobic growth.
387
388 **4.3. Measurements**
389 Fermentation took place in 1.1-liter fermentors equipped with fermentation locks to
390 maintain anaerobiosis, at 20 °C, with continuous magnetic stirring (500 rpm) during
391 approximately 300h. CO_2 release was followed by automatic measurements of fermentor
392 weight loss every 20 min. The amount of CO_2 released allowed us to monitor the progress of
393 the fermentation and evaluate the maximum of released CO_2 (***CO2max***) as well as the
394 maximum rate of CO_2 released (***Vmax***). Samples were harvested after 6h, 12h and 24h, then
395 every 12h during the first week and every 24h during the second week of fermentation. For
396 each sample, the population density cells were determined using a BD Accuri™ C6 Plus flow

397 cytometer as described in (30). Viability was determined using propidium iodide staining and
398 BD Accuri™ C6 Plus flow cytometer adapted from (31). Proportions of *S. cerevisiae* in mixed-
399 culture was established thanks to the green fluorescence produced by the *S. cerevisiae* 59A-
400 GFP, the forward and side scatters from the BD Accuri™ C6 Plus flow cytometer and machine
401 learning using the caret package in R (32). From these population densities (without taking into
402 account viability), we fitted a growth population model (with the growthcurver package in R,
403 (33), and determined the carrying capacity (***K***) and maximum growth rate (***mu***) for each
404 fermentation.

405 The final concentrations of carbon metabolites in the medium (acetate, succinate,
406 glycerol, alpha-ketoglutarate, pyruvate, ethanol, glucose and fructose) were determined with
407 high-pressure liquid chromatography (34). From these metabolite concentrations, we first
408 calculated the consumed sugar concentration as the difference between the final and the initial
409 concentration of either glucose or fructose. Then we calculated the yield of metabolite
410 production by dividing the final concentration by the corresponding consumed sugar
411 concentration. Finally, we compared these yields to the yield of *S. cerevisiae* single strain
412 cultures considered as reference.

413 Finally, the ammonium concentration after 100h of fermentation was determined
414 enzymatically with R-Biopharm (Darmstadt, Germany) and the free amino acid content of the
415 must was estimated through cation exchange chromatography with post-column ninhydrin
416 derivatization (35).

417

418 **4.4. Statistical Analysis**

419 The experimental work was performed in 5 different blocks. Each block was composed
420 of three replicates of NS fermentations (for example ***Hu***), three replicates of the corresponding
421 mixed fermentations with *S. cerevisiae* (for example ***ScvsHu***) and one or two fermentations of

422 single strain *S. cerevisiae* cells (***Sc***). The block effect was evaluated on the parameters of the
423 ***Sc*** fermentation. For most studied parameters, the block effect was not significant. For those
424 parameters where a block effect was observed (***mu*** and ***K***), a statistical correction for block
425 effect did not modify our results. Therefore, for simplification purposes, we compared all
426 fermentations without any correction for the block effect parameter. For each measured
427 parameter, an ANOVA was performed to evaluate the type of fermentation (***Sc***, ***Mp***, ***Mf***, ***Hu***,
428 ***Ho***, ***ScvsTd***, ***ScvsMp***, ***ScvsMf***, ***ScvsHu*** and ***ScvsHo***) effect and then a Tukey t-test was
429 performed to determine statistical groups and two-by-two statistical differences. All statistical
430 analyses were performed using R (36) and Rstudio (37). All data, analysis and figures scripts
431 can be found in this github address: https://github.com/tnidelet/Git-Harle-et-al-2019.
432

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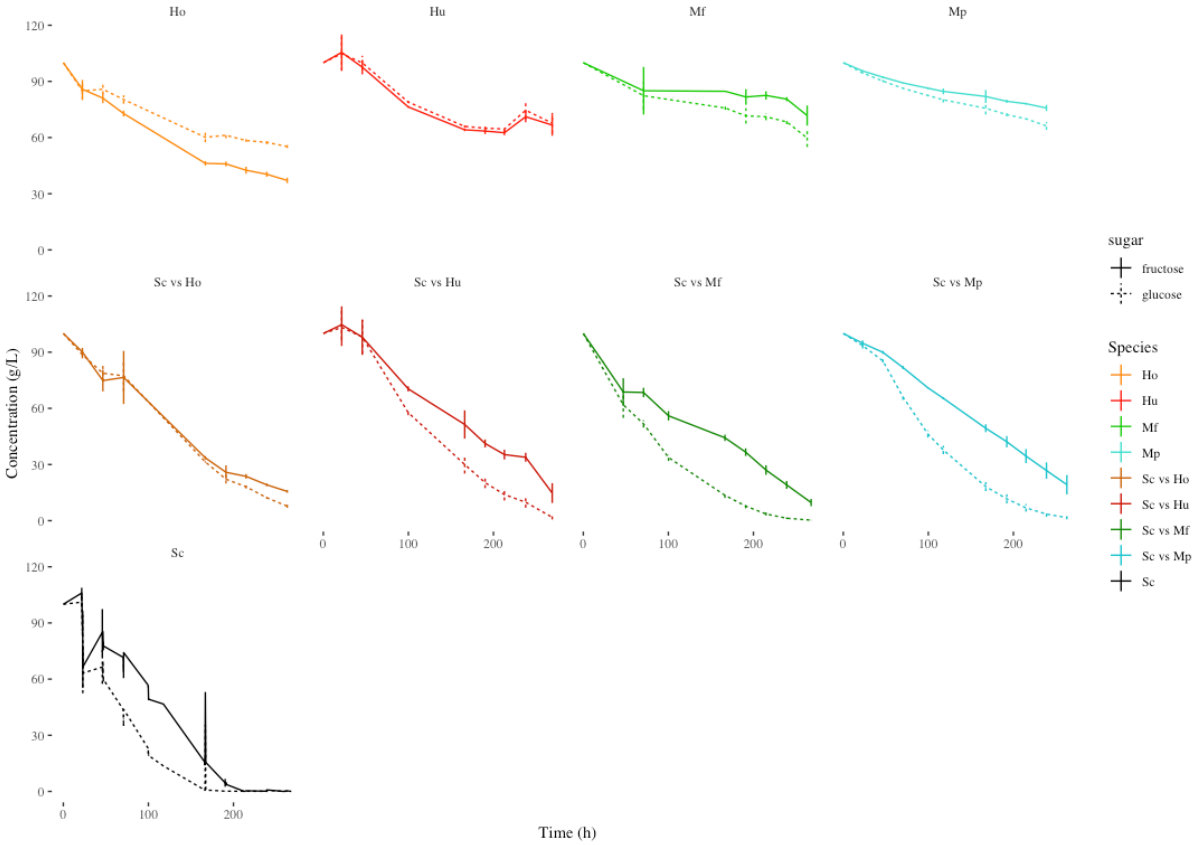
Supporting information

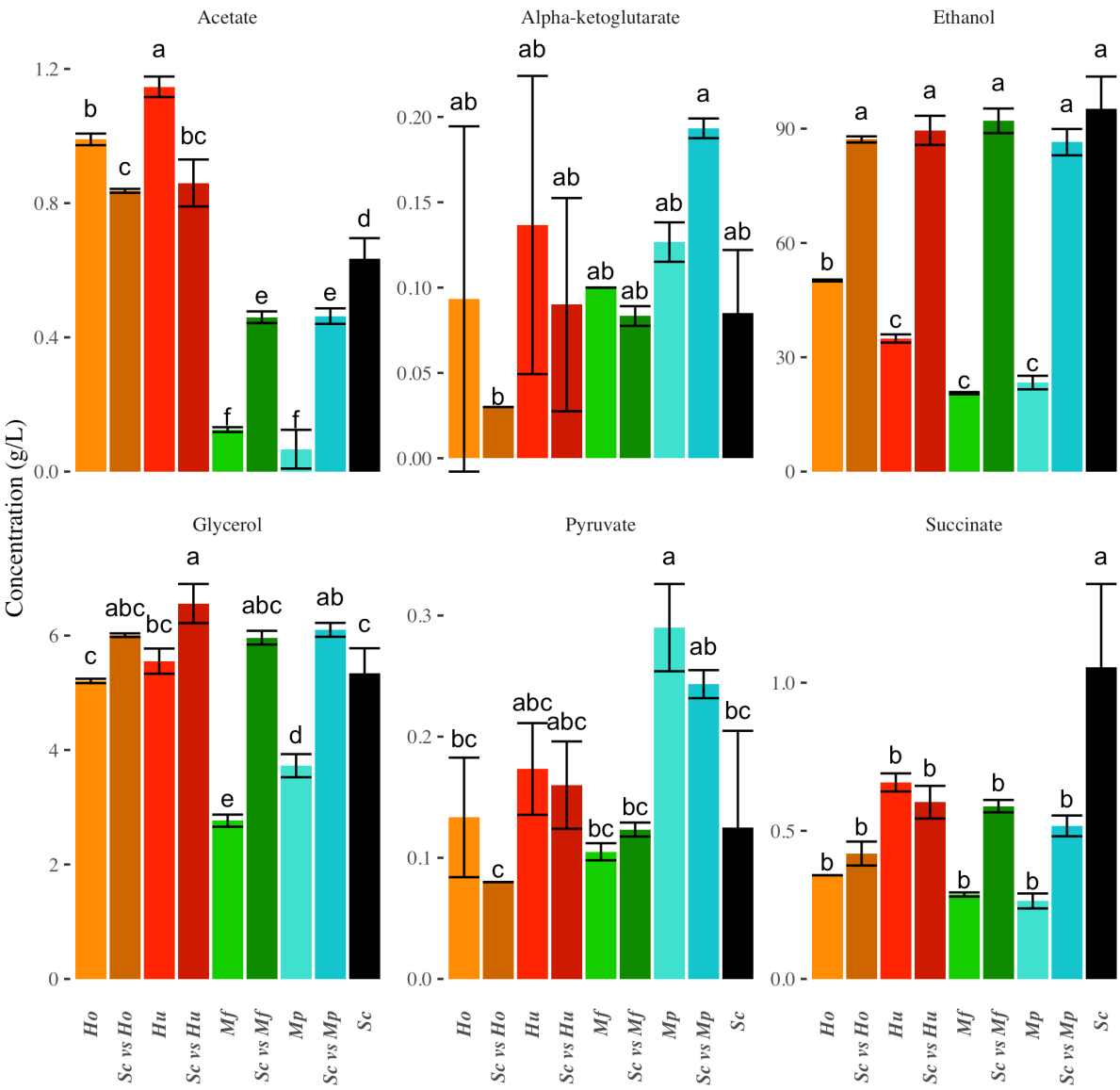
S1 FIG

Glucose and Fructose consumption kinetics in function of different strains. Each point represents a sample (average ± standard deviation).



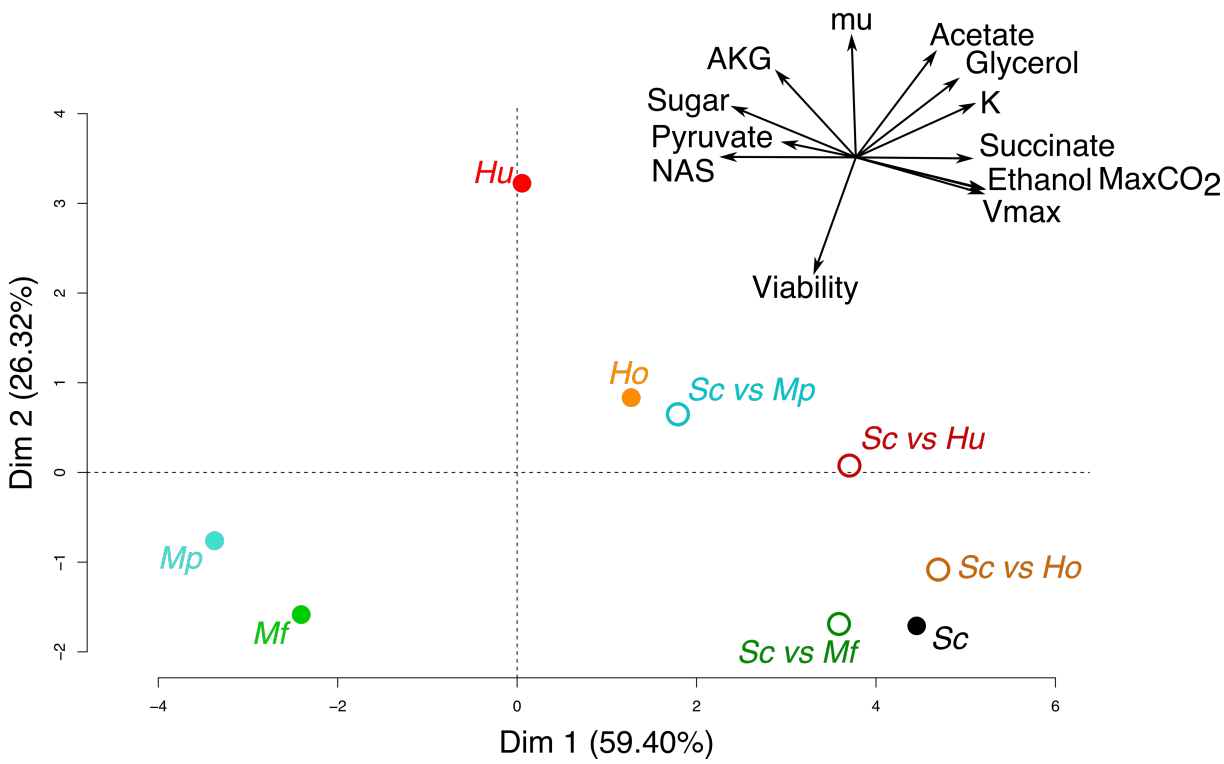
S2 FIG

Total production of carbon metabolite in function of the strains driving the fermentation. Average production are given with standard deviations for acetate, alpha-ketoglutarate, ethanol, glycerol, pyruvate and succinate.



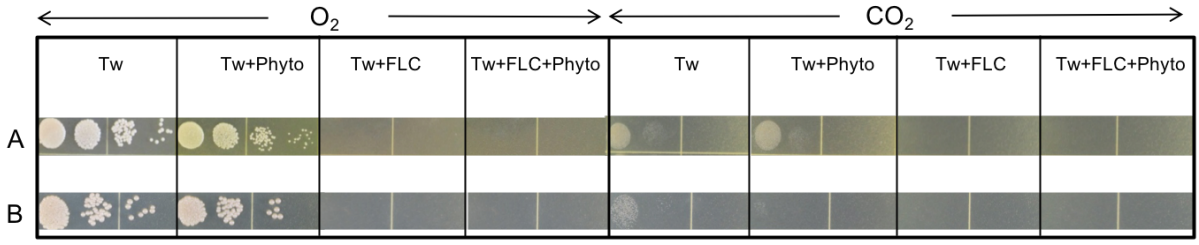
551
552
553 **S3 FIG**

554 **Principal component analysis of carbon metabolites and growth parameter of isolated**
555 **cultures.** The mixed cultures are a second time projected on the plan determiner by only
556 isolated cultures. In the top right is represented the circle of variables.
557



S4 FIG

Serial tenfold dilutions of two Metschnikovia pulcherrima strains (A and B) spotted onto various synthetic standard agar media (SM425, 425 mg/l assimilable nitrogen) with Tween 80, (Tw, 0.06%), supplemented or not with phytosterol (Phyto, 20 mg/L), in the presence or not of fluconazole (FLC, 256 µg/mL). Plates were incubated at 28°C for five days in air or in anaerobiosis.



S1 TABLE

Growth parameter values 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.