Investigations of the mechanisms of interactions between four non-conventional species with *Saccharomyces cerevisiae* in oenological conditions

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Keywords: winemaking fermentation, population dynamics, non-*Saccharomyces* species, 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 (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...
Torulaspora delbrueckii, Metschnikowia pulcherrima, Metschnikowia fructicola, Hanseniaspora opuntiae and Hanseniaspora uvarum. Species in the Metschnikowia genus ferment poorly in oenological conditions but can have interesting attributes: in conjunction with S. cerevisiae, a strain of M. pulcherrima could reduce ethanol concentrations (6,10), increase ‘citrus/grape fruit’ and ‘pear’ attributes (11), as well as allow the persistence of ‘smoky’ and ‘flowery’ characteristics (12). M. pulcherrima also has an amensalism effect on S. cerevisiae through iron depletion via the production of pulcherriminic acid (13). M. fructicola has been less studied and never in conjunction with S. cerevisiae although it presents the interesting ability to inhibit Botrytis growth (14). Last, the Hanseniaspora genus, studied in sequential or simultaneous fermentation with S. cerevisiae, has been shown to increase volatile compound production during winemaking (6). It notably increased the ‘tropical fruit’, ‘berry’, ‘floral’ and ‘nut aroma’ characters (15), that were linked to higher concentrations of acetate esters, esters of MCFAs, isoamyl alcohol, 2-phenylethanol and α-terpineol (16).

Despite these various studies, the composition and protocol of inoculation of these multi-strains starters are still very empirical and only based on the input/output balance, without considering the dynamics of the microbial populations or their interactions. This lack of knowledge about yeast-yeast interactions prevents implementing a rational design of multi-strain starters (17). To address this problem, we decided to focus our study on population dynamics and metabolites produced during oenological fermentations performed in isolated or mixed yeast cultures. Since our goal was not to obtain optimal mixes but to understand the mechanism of microbial interaction, we chose to compare the population dynamics and yields between isolated cultures of strains from five species (one S. cerevisiae and four NS) and four corresponding mixed cultures always including the S. cerevisiae strain as reference. We were thus able to identify key microbial interaction mechanisms that are further discussed.
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. CO₂ kinetics

We first investigated the influence of species and co-culture on the dynamics of CO₂ production (proportional to sugar consumption), which is a good indicator of the fermentation progress. Indeed, CO₂ 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 CO₂ production (Vmax, Fig 1a) and of the maximum CO₂ produced were estimated (CO₂max, Fig 1b). Vmax was highly dependent on the species (p.value < 0.001): S. cerevisiae cultures (Sc) displayed the highest value (VmaxSc = 0.99 ± 0.02 g.L⁻¹.h⁻¹), followed by both Hanseniaspora species (VmaxHo = 0.33 ± 0.04 g.L⁻¹.h⁻¹, and VmaxHo = 0.42 ± 0.02 g.L⁻¹.h⁻¹) and finally both Metschnikowia species (VmaxMp = 0.165 ± 0.02 g.L⁻¹.h⁻¹ and VmaxMf = 0.17 ± 0.04 g.L⁻¹.h⁻¹). The four mixed cultures had intermediate Vmax values between those of Sc and...
the highest $V_{\text{max}}$ of all non-sacc cultures (Fig 1a). Mixed cultures containing Metschnikowia species had significantly higher $V_{\text{max}}$ values than those containing Hanseniaspora species (Fig 1a). Although we did not monitor all cultures until the exhaustion of glucose and fructose, it was however possible to estimate the capacity of a given species to complete fermentation by estimating the amount of CO$_2$ produced during the first 300 hours. $S_{\text{c}}$ fermentations finished after around 220 hours with a $CO_{2\text{max}}^{\text{Sc}} = 88.2 \pm 2.2$ g.L$^{-1}$. We therefore can make the hypothesis that all cultures that produced more than 80g CO$_2$.L$^{-1}$ (90% of $S_{\text{c}}$ maximum) within 300 hours will be able to complete fermentation. Under this assumption, all mixed cultures, but no NS single strain cultures, would eventually complete fermentation. Among the latter cultures, both Hanseniaspora species had the highest $CO_{2\text{max}}$ ($Hu_{30} = 30 \pm 0.4$ g.L$^{-1}$, and $Ho_{46} = 46 \pm 0.6$ g.L$^{-1}$) followed by Metschnikowia species ($Mp_{22} = 22 \pm 0.4$ g.L$^{-1}$ and $Mf_{20} = 20 \pm 1$ g.L$^{-1}$).

FIG 1

Maximum rate of CO$_2$ production, $V_{\text{max}}$ (A) and total CO$_2$ produced (B) in function of the single or mixed strain species driving each fermentation. Values corresponds to average ± standard deviation. The small letters indicate the statistical groups from a Tukey analysis.
2.2. Population Kinetics

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

Global monitoring of the kinetics of the total living population (left), and sub-population in the mixed cultures (right) across fermentation. Each population was detected by flow cytometry as indicated in the Material and Methods section. 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 single strain cultures of ‘non-Saccharomyces’ strains and dark ones to the corresponding culture in competition with 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 CO₂ 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
consumed glucose (S1 Fig). Sugar consumption was higher in mixed cultures than in single NS species cultures (Fig 3a). However, it was still lower than in *Sc* species cultures, also with a preference for glucose. This indicates the major impact of *S. cerevisiae* on sugar consumption (consistent with the CO$_2$ production observed), compared to the other NS species studied.

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

**FIG3**

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

In parallel with nutrient 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 \textit{Sc} in single strain culture (Fig 4).

**FIG 4**

Yield of carbon metabolite production relative to the yield of production of single \textit{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 \textit{Mf} fermentations had a relative yield significantly inferior (-32\%) to 0 (0 being \textit{Sc} yield). For glycerol, all isolated NS fermentations had a greater yield than \textit{Sc} and mixed fermentations were intermediate between (but not significantly different from) the corresponding single strain cultures. For acetate, only \textit{Hanseniaspora} strains 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 (S2 Fig). The case of glycerol was more interesting. Indeed, even if the average sugar consumption was lower in ScvsHu and ScvsMp mixed cultures than in single strain Sc culture, the total production of glycerol was significantly higher than that of the corresponding single strain cultures (GlycerolScvsMp = 6.1±0.1 g.L⁻¹, GlycerolSc = 5.3±0.4 g.L⁻¹, GlycerolMp = 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 single strain cultures (S1 Table).

3. Discussion

This study presents one of the first works focusing on the population dynamics and kinetics of yeast-yeast interactions between two species (S.C. and NS) during the alcoholic fermentative process (18), but dynamics are distinct according to the M. pulcherrima and H. uvarum strains tested in this study. Firstly, all strains grew in single strain and mixed strain cultures. Metschnikowia single strain cultures present lower Vmax values than Hanseniaspora
single strain cultures, while mixed cultures containing Metschnikowia species had significantly higher Vmax values than those containing Hanseniaspora species. It appears that the better growth behavior of Hanseniaspora strains limited more S. cerevisiae’s growth than Metschnikowia in mixed culture. The growth characteristics of the competing strains directly impact the mixed fermentation characteristics.

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

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

These overproductions of glycerol open the way for potentially more interesting results. Our results point out that inoculation conditions in mixed cultures must be considered according
to fermentation aims and strains characteristics. 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 mixed cultures.

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

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

Interestingly, even though S. cerevisiae, M. pulcherrima and M. fructicola mortality rates
were low in single strain cultures, the corresponding mixed cultures (ScvsMp and ScvsMf)
presented 30% mortality (after 200 hours of fermentation). Moreover, this mortality seemed to
impact both species differently. In the mixed ScvsMp culture, only S. cerevisiae cells eventually
died, whereas in *ScvsMf* both species were negatively affected. The survival of *M. pulcherrima* cells compared to *S. cerevisiae* cells could be explained by the production of pulcherriminic acid by *M. pulcherrima* (21). Indeed, pulcherriminic acid is known to deplete iron from the medium, which has a lethal effect on *S. cerevisiae* cells (13,22,23). In *ScvsMf* cultures, the mortality observed in both species suggests a more complex mechanism of interaction (although it is not clear whether *M. fructicola* also produces pulcherriminic acid; Kurtzman and Droby, 2001). To explain these results, we could hypothesize the conjunction of two different mechanisms of interaction. It could be that *M. fructicola* synthesized a metabolite (pulcherriminic acid?) impacting the viability of *S. cerevisiae* cells (through iron depletion?), with *M. fructicola* cells dying thereafter for another reason such as sensitivity to ethanol. Indeed, the production of ethanol was almost four times higher in mixed cultures than in single strain *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*.

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 or dual transcriptomics will be needed to answer this question (24–26).
To discuss more generally the mechanisms of interactions of *Metschnikowia* and *Hanseniaspora* cultures mixed 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), except for 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 were overall not different from *S. cerevisiae* single strain cultures. This result is in agreement with the good adaptation of *S. cerevisiae* to winemaking conditions (4–6,27) but could be different with a different set of strains. This result is important in the context of ecological engineering. In fact, our results confirmed that *S. cerevisiae* has a much better fitness than the NS strains studied in this paper. Therefore, if we want mixed culture behavior to deviate from that of *S. cerevisiae* single strain culture, it must be ensured that NS cells dominate the culture as soon as possible. To achieve this, two conceivable options are currently tested: either to reduce the proportion of *S. cerevisiae* at time *t*₀ or to successively inoculate (16) first the NS species, and only then the *S. cerevisiae* strain. These two options could be equivalent depending on the type of interaction(s) that occurs. If strain behaviors in single strain or mixed cultures are identical, then all interactions are mediated by the medium through the competition for resources and the production of constitutive toxins such as ethanol (producing a toxin only in mixed fermentation would be a behavior change) and could be qualified as “indirect”.

Mathematical models could be designed from data on isolated cultures to predict the impact of indirect interactions in mixed cultures. This would allow simulating numerous mixes of species with various initial conditions and identify optimal strategies depending on one or several given criteria. Using these approaches could limit the number of necessary tests, 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 \textit{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): \textit{Saccharomyces cerevisiae} (Sc), \textit{Metschnikowia pulcherrima} (Mp), \textit{M. fructicola} (Mf), \textit{Hanseniapora uvarum} (Hu) and \textit{H. opuntiae} (Ho). The \textit{S. cerevisiae} strain is a haploid strain from EC1118 labelled with GFP (59A-GFP, Marsit and Dequin, 2015). The \textit{Hanseniapora uvarum} (CLIB 3221) \textit{H. opuntiae} (CLIB 3093) and \textit{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 \textit{Metschnikowia fructicola} strain was from the Lallemand collection.

For each strain, 3 replicates of single strain cultures were performed (except for \textit{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 \textit{S. cerevisiae} cells was set at 10%. In this text, fermentations were referred
to be by the species that performed them, i.e. single strain cultures were referred to as: Sc, Mp, Mf, Hu and Ho and mixed strain cultures as ScvsMp, ScvsMf, ScvsHu and ScvsHo.

4.2. Medium

Initial cultures (12 h, in 50 ml YPD medium, 28 °C) were used to inoculate fermentation media at a total density of $10^6$ cells/mL; therefore, for mixed culture the S. cerevisiae cells density was $0.1 \times 10^6$/mL and the NS cells density was $0.9 \times 10^6$/mL. Fermentations were carried out in a synthetic medium (SM) mimicking standard grape juice (28). The SM used in this study contained 200 g/L of sugar (100 g glucose and 100 g fructose per liter) and 200 mg/L of assimilable nitrogen (as a mix 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 pH of the medium was adjusted to 3.3 with 10M NaOH. The SM medium was first saturated with bubbling air for 40 minutes, then it was supplemented with 5 mg/L phytosterols (85451, Sigma Aldrich) solubilized in Tween 80 to fulfill the lipid requirements (sterols and fatty acids) of yeast cells during anaerobic growth.

4.3. Measurements

Fermentation took place in 1.1-liter fermentors equipped with fermentation locks to maintain anaerobiosis, at 20 °C, with continuous magnetic stirring (500 rpm) during approximately 300h. CO$_2$ release was followed by automatic measurements of fermentor weight loss every 20 min. The amount of CO$_2$ released allowed us to monitor the progress of the fermentation and evaluate the maximum of released CO$_2$ ($CO2_{max}$) as well as the maximum rate of CO$_2$ released ($V_{max}$). Samples were harvested after 6h, 12h and 24h, then every 12h during the first week and every 24h during the second week of fermentation. For each sample, the population density cells were determined using a BD Accuri™ C6 Plus flow
cytometer as described in (30). Viability was determined using propidium iodide staining and BD Accuri™ C6 Plus flow cytometer adapted from (31). Proportions of S. cerevisiae in mixed-culture was established thanks to the green fluorescence produced by the S. cerevisiae 59A-GFP, the forward and side scatters from the BD Accuri™ C6 Plus flow cytometer and machine learning using the caret package in R (32). From these population densities (without taking into account viability), we fitted a growth population model (with the growthcurver package in R, (33), and determined the carrying capacity (K) and maximum growth rate (mu) for each fermentation.

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

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

4.4. Statistical Analysis

The experimental work was performed in 5 different blocks. Each block was composed of three replicates of NS fermentations (for example Hu), three replicates of the corresponding mixed fermentations with S. cerevisiae (for example ScvsHu) and one or two fermentations of
single strain *S. cerevisiae* cells (*Sc*). The block effect was evaluated on the parameters of the *Sc* fermentation. For most studied parameters, the block effect was not significant. For those parameters where a block effect was observed (*mu* and *K*), a statistical correction for block effect did not modify our results. Therefore, for simplification purposes, we compared all fermentations without any correction for the block effect parameter. For each measured parameter, an ANOVA was performed to evaluate the type of fermentation (*Sc, Mp, Mf, Hu, Ho, ScvsTd, ScvsMp, ScvsMf, ScvsHu* and *ScvsHo*) effect and then a Tukey t-test was performed to determine statistical groups and two-by-two statistical differences. All statistical analyses were performed using R (36) and Rstudio (37). All data, analysis and figures scripts can be found in this github address: https://github.com/tnidelet/Git-Harle-et-al-2019.

**Acknowledgments.**

We thank the CIRM, the Lallemand company, Jean-Luc Le grap, 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 advices.

**References**


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.
**S3 FIG**

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

| Sample  | μ     | K prop Vmax MaxCO2 Sugar NAS Ethanol Glycerol Succinate Acetate Pyruvate Alpha |
|---------|-------|----------------------|----------------------|----------------------|----------------------|----------------------|----------------------|----------------------|
| Sc      | 0.15±0.02bc | 1.6±0.2bc   | NA       | 0.99±0.03g8.8±2.2a  | 0.16±0.2a   | 10.4±8.6a  | 95.2±8.4a  | 5.3±0.4a  | 1.05±0.2a  | 0.63±0.06a  | 0.12±0.08bc  | 0.08±0.04bc  |
| Ho      | 0.19±0.03bc | 1.6±0.1bc   | NA       | 0.42±0.02g46.4±0.6d | 92.3±1.4b  | 122.2±3.5b | 50.1±0.2b  | 5.2±0.0f  | 0.35±0.00b  | 0.99±0.02b  | 0.13±0.05bc  | 0.09±0.1f   |
| Sc vs Ho| 0.01±0.0005 | 2.3±0.1f   | 0.83±0.02g0.57±0.01f | 82.7±0.6f   | 23.3±1.3f  | 6.4±2.4f  | 87.2±0.8f  | 6.0±0.0f  | 0.42±0.04f  | 0.84±0.01f  | **0.08±0.00f** | 0.03±0.05f   |
| Hu      | 0.6±0.18c  | 1.4±0.18c   | NA       | 0.34±0.04g30.7±0.5c | 134.3±11.2c | 173.3±24.8c | 34.5±1.1c  | 5.5±0.2c  | 0.66±0.03c  | 1.15±0.03c  | 0.17±0.04ac  | 0.14±0.09ac  |
| Sc vs Hu| 0.24±0.01b | 1.7±0.1b    | 0.76±0.01g0.55±0.04g | 82.1±1.5c  | 16.5±6.5b  | 4.6±4.2c  | 89.5±3.8c  | 6.6±0.3c  | 0.60±0.06b  | 0.86±0.07bc | 0.16±0.04bc  | 0.09±0.06bc  |
| Mf      | 0.17±0.02bc | 0.8±0.05f   | NA       | 0.17±0.04g20.6±1.2f | 135.8±9.7c | 256.6±2.9c | 20.6±0.3c  | 2.8±0.1f  | 0.29±0.01b  | 0.13±0.01f  | 0.10±0.00f   | 0.08±0.01f   |
| Sc vs Mf| 0.09±0.01bc | 1.3±0.03bc   | 0.96±0.02g0.76±0.03g | 86.8±0.7bc | 9.9±2.1bc  | 1.8±0.1c  | 92.1±3.2c  | 6.0±0.1bc | 0.58±0.02bc | 0.46±0.02c  | 0.12±0.01bc  | 0.08±0.01bc  |
| Mp      | 0.18±0.03bc | 0.6±0.01f   | NA       | 0.16±0.02g21.8±0.4f | 142±3.7c   | 305.5±5.9c | 23.4±1.8c  | 3.7±0.2d  | 0.26±0.03c  | 0.07±0.06f  | 0.29±0.04c   | 0.13±0.01bc  |
| Sc vs Mp| 0.09±0.00f  | 1.3±0.01f   | 0.50±0.02g0.68±0.04g | 83.3±2.2bc | 28.8±6.0b  | 2.1±0.2f  | 86.5±3.4f  | 6.1±1.1bc | 0.52±0.04bc | 0.46±0.02f  | 0.24±0.01bc  | 0.19±0.01f   |

Mu: h-1; K: 10⁸ 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.