Kinetic analysis of yeast-yeast interactions in oenological conditions

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Abstract

Fermentation by microorganisms is a key step in the production of traditional food products such as bread, cheese, beer and wine. In these fermentative ecosystems, microorganisms interact in various ways, namely competition, predation, commensalism and mutualism. Traditional wine fermentation is a complex microbial process performed by Saccharomyces and non-Saccharomyces yeast species. To better understand the different interactions occurring
within wine fermentation, isolated yeast cultures were compared with mixed co-cultures of one reference strain of *S. cerevisiae* with one strain of four non-*Saccharomyces* yeast species (*Metschnikowia pulcherrima, M. fructicola, Hanseniaspora opuntiae* and *H. uvarum*). In each case, we studied population dynamics, resource consumption and the production of metabolites from central carbon metabolism. This deep phenotyping of competition kinetics allowed us to identify the main mechanisms of interaction between species. *S. cerevisiae* competed with *H. uvarum* and *H. opuntiae* for resources although both *Hanseniaspora* species were characterized by a strong mortality either in isolated or mixed fermentations. *M. pulcherrima* and *M. fructicola* displayed a negative interaction with the *S. cerevisiae* strain tested, with a decrease in viability in co-culture, probably due to iron depletion via the production of pulcherriminic acid. Overall, this work highlights the importance of measuring cell populations and their metabolite kinetics to understand yeast-yeast interactions. These results are a first step towards ecological engineering and the rational design of optimal multi-species starter consortia using modeling tools.

**Introduction**

In natural or anthropized environments, microbial species are part of an ecosystem and interact positively or negatively, forming a complex network. Until recently, process optimization in agriculture or food processing was mostly based on the selection of single strains. However, this paradigm is now being challenged and the scientific community is increasingly seeking to exploit and optimize consortia of several strains and/or species. Indeed, many studies have shown that more diverse anthropized environments have many advantages in terms of resilience, disease resistance or yield (Barot et al., 2017). Efforts are now being made to design
optimal consortia of various species and strains whose interactions will be exploited to
maximize given criteria such as fermentation quality, aromatic complexity or other organoleptic
characteristics.

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

Indeed, there are numerous experiments and even industrial products making use of such mixed
starters to improve wine’s organoleptic qualities (Jolly et al., 2014; P et al., 2008; Sadineni et
al., 2012). The non-Saccharomyces strains used in these experiments are very diverse, with
more than 23 different species (Jolly et al., 2014) including Torulaspora delbrueckii,
Metschnikowia pulcherrima, Metschnikowia fructicola, Hanseniaspora opuntiae and
Hanseniaspora uvarum. Species in the Metschnikowia genus ferment poorly in oenological
conditions but can have interesting attributes (Varela, 2016): in conjunction with S. cerevisiae,
M. pulcherrima can reduce ethanol concentrations (Contreras et al., 2014; Varela, 2016),
increase ‘citrus/grape fruit’ and ‘pear’ attributes (Belda et al., 2015), as well as allow the persistence of ‘smoky’ and ‘flowery’ characteristics (Elena et al., 2015). *M. pulcherrima* also has an amensalism effect on *S. cerevisiae* through iron depletion via the production of pulcherriminic acid (Oro et al., 2014). *M. fructicola* has been less studied and never in conjunction with *S. cerevisiae* although it presents the interesting ability to inhibit *Botrytis* growth (Kurtzman and Droby, 2001). Last, the *Hanseniaspora* genus, studied in sequential or simultaneous fermentation with *S. cerevisiae*, has been shown to increase volatile compound production during winemaking (Varela, 2016). It notably increased the ‘tropical fruit’, ‘berry’, ‘floral’ and ‘nut aroma’ characters (Hu et al., n.d.), that were linked to higher concentrations of acetate esters, esters of MCFAs, isoamyl alcohol, 2-phenylethanol and α-terpineol (Tristezza et al., 2016).

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 (Song et al., 2014). To address this problem, we decided to focus our study on population dynamics and metabolite production 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 non-*Saccharomyces*) 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.
Results

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

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

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 ($V_{max}$, Fig. 1a, Table. 1) and of the maximum CO₂ produced were estimated ($CO_{2max}$, Fig. 1b, Table. 1). $V_{max}$ was highly dependent on the species (p.value < 0.001): S. cerevisiae cultures (Sc) displayed the highest value ($V_{max}^{Sc} = 0.99 ± 0.02$ g.L$^{-1}$h$^{-1}$), followed by both Hanseniaspora species ($V_{max}^{Hu} = 0.33 ± 0.04$ g.L$^{-1}$h$^{-1}$, and $V_{max}^{Ho} = 0.42 ± 0.02$ g.L$^{-1}$h$^{-1}$) and finally both Metschnikowia species ($V_{max}^{Mp} = 0.165 ± 0.02$ g.L$^{-1}$h$^{-1}$ and $V_{max}^{Mf}$...
The four mixed cultures had intermediate \( V_{max} \) values between those of \( Sc \) and the highest \( V_{max} \) of all non-sacc cultures (Fig. 1a). Mixed cultures containing Metschnikowia species had significantly higher \( V_{max} \) 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. \( Sc \) fermentations finished after around 220 hours with a \( CO_{2\text{max}}^{Sc} = 88.2 \pm 2.2 \text{ g.L}^{-1} \). We therefore can make the hypothesis that all cultures that produced more than 80g CO\(_2\).L\(^{-1}\) (90% of \( Sc \) maximum) within 300 hours will be able to complete fermentation. Under this assumption, all mixed cultures, but not isolated non-\( Saccharomyces \) cultures, would eventually complete fermentation. Among the latter cultures, both Hanseniaspora species had the highest \( CO_{2\text{max}} \) (\( Hu \) 30 ± 0.4 g.L\(^{-1}\), and \( Ho \) 46 ± 0.6 g.L\(^{-1}\)) followed by Metschnikowia species (\( Mp \) 22 ± 0.4 g.L\(^{-1}\) and \( Mf \) 20 ± 1 g.L\(^{-1}\)).

### 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 of each species after 300 hours of mixed culture, corresponding in our case to the end of the monitoring period (Table. 1). Fermentations with \( S. \text{ cerevisiae} \) 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\(^8\)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 similar to \( Sc \) 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 isolation had growth dynamics mostly similar to Sc fermentations: a similar growth rate ($\mu_{mp} = 0.18 \pm 0.03$ h$^{-1}$, $\mu_{Mf} = 0.17 \pm 0.2$ h$^{-1}$), no mortality during the stationary phase but a much reduced maximum population ($K_{mp} = 0.57 \pm 0.01$ 10$^6$cells.mL$^{-1}$, $K_{Mf} = 0.8 \pm 0.05$ 10$^6$cells.mL$^{-1}$). In most cases, mixed cultures displayed an intermediate pattern between the two corresponding isolated cultures (Fig. 2). However, mixed or isolated 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) (Table 1).

Sugar and NAS consumption

We then looked at the final concentration of resources: sugars (fructose and glucose) and NAS (sum of all assimilable nitrogen sources, i.e. ammonium and amino-acids) (Fig. 3, Table 1). 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, non-Saccharomyces species in monocultures 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 (see supplementary figure 1). Sugar consumption was higher in mixed cultures than in single non-Saccharomyces species cultures (Table 1). 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 species studied.
The consumption of nitrogen assimilable sources (NAS, amino-acids and ammonium) displayed the same pattern (Fig. 3b). NAS were almost entirely consumed both in \textit{Sc} isolated cultures and in all co-cultures, whereas in non-\textit{Saccharomyces} isolated cultures the fraction of NAS consumed varied between 84% and 94%. However, the preference for different nitrogen sources varied with each species (Fig. 3c). Both \textit{Hanseniaspora} species had similar behaviors, consuming only half of the available ammonium, 90% of histidine and 89% or 79% of arginine (Fig. 3c). \textit{Metschnikowia} species presented a similar pattern. It was possible to classify these non-\textit{Saccharomyces} 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.

**Metabolite production**

In parallel with must resources consumption monitoring, we also investigated the production of metabolites from CCM (Central Carbon Metabolism): ethanol, glycerol, succinate, pyruvate, acetate and alpha-ketoglutarate (Table 1). 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 isolated culture (Fig. 4).

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 non-\textit{Saccharomyces} fermentations had a greater yield than \textit{Sc} and mixed fermentations were intermediate between (but not significantly different from) the corresponding isolated 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 \textit{Sc} fermentations (Table 1, supplementary figure S2). The case of glycerol was more interesting. Indeed, even if the average sugar consumption was lower in \textit{ScvsHu} and \textit{ScvsMp} mixed cultures than in isolated \textit{Sc} culture, the total production of glycerol was significantly higher than that of the corresponding isolated cultures (Glycerol\textsubscript{ScvsMp} = 6.1\pm0.1 \text{ g.L}^{-1}, Glycerol\textsubscript{Sc} = 5.3\pm0.4 \text{ g.L}^{-1}, Glycerol\textsubscript{Mp} = 3.7\pm0.2 \text{ g.L}^{-1}). This resulted from the positive combination of the greater glycerol yield by \textit{Hanseniaspora} and \textit{Metschnikowia} and their population dynamics. For all other metabolites, the total production of mixed cultures was not significantly different from the corresponding isolated cultures (Table 1).

Discussion

This study presents one of the first works focusing on the population dynamics and kinetics of yeast-yeast interactions between two species during the alcoholic fermentative process. By contrast, to our knowledge, most of the previous studies were concerned only with input-output balances (García et al., 2016; Varela, 2016). Firstly, it was possible to group the non-
Saccharomyces strains performance according to their genus (see PCA in supplementary figures S3); H. uvarum and H. opuntiae both presented similar phenotypes. Their population dynamics were characterized by a similarly high growth rate (reaching a maximum population similar to S. cerevisiae), followed by a long mortality phase where viability dropped to 30% (Moreira et al., 2008; 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, Hanseniaspora mixed cultures presented intermediate phenotypes compared to the corresponding isolated cultures. The only exception was the total production of glycerol that seemed to be superior in mixed cultures whereas their sugar consumption was inferior; this 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 (Ciani and Ferraro, 1996; Tristezza et al., 2016) but is not systematic depending on the strains and experimental conditions (Moreira et al., 2008). In the present study, the glycerol overproduction and sugar consumption observed in mixed cultures could be explained by the joint effect of population dynamics (S. cerevisiae slowly dominating the population), resource consumptions (the Ho and Hu fermentation leaving ⅔ of sugars) and the glycerol yields of Hanseniaspora strains that were two to three times higher that of S. cerevisiae.

Regarding Hanseniaspora species, Mp and Mf isolated cultures were characterized by a short growth phase reaching a small carrying capacity and followed by a long stationary phase with no mortality. Nevertheless, despite this very 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 unmonitored resources. For instance, the
third most important resource in the synthetic medium was lipids, available as phytosterols.
Concurrently, it has been shown that some Metschnikowia species were unable to import lipids
from the medium, depending instead on their own lipid synthesis (see Fig S1). For all yeast
species, lipid synthesis requires oxygen and is therefore impossible in anaerobic conditions.
From these data, one can hypothesize that Metschnikowia cells were able to produce their own
lipids in the aerobic conditions prevailing at the beginning of the fermentation. Then, with the
progress of fermentation and ensuing oxygen limitation, Metschnikowia cells became unable to
synthetize the necessary lipids and stopped multiplying.
Interestingly, even though S. cerevisiae, M. pulcherrima and M. fructicola mortality rates were
low in isolated cultures, the corresponding mixed cultures (ScvsMp and ScvsMf) presented 30%
mortality. 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 (MacDonald, 1965).
Indeed, pulcherriminic acid is known to deplete iron from the medium, which has a lethal effect
on S. cerevisiae cells (Oro et al., 2014; Sipiczki, 2006; Türkel and C, 2009). 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
isolated Metschnikowia fermentations. Under such hypothesis, the reason why no loss of
viability was observed for *M. pulcherrima* in mixed culture with *S. cerevisiae* could probably be related to a better tolerance of *M. pulcherrima* to ethanol stress compared to *M. fructicola*. As in *Hanseniaspora*, we also observed an overproduction of glycerol in *Metschnikowia* mixed cultures. This also could be explained by the same conjunction of yield, sugar consumption and population dynamics. These overproductions of glycerol open the way for potentially more interesting results. In fact, one can hypothesize that, by exploiting this specific yield and population dynamics, for example by inoculating the *S. cerevisiae* strain at lower relative frequencies (0.01, 0.001, 0.0001), more important transgressive interactions (such as lower ethanol production) could be achieved in such mixed cultures.

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

If we look more generally at *Metschnikowia* and *Hanseniaspora* mixed cultures with *S. cerevisiae*, we could not evidence any major antagonistic phenomena. For almost all assays, mixed cultures performance stood always between that of the corresponding isolated cultures (Fig 1, 2 & 3), with the exception of the total production of glycerol. Moreover, despite the differences in yield and interactions between species, the rapid dominance of *S. cerevisiae* (increasing from 10% to at least 50% during the fermentation) resulted in mixed cultures that...
were overall not different from *S. cerevisiae* pure cultures. This result is in agreement with the good adaptation of *S. cerevisiae* to winemaking conditions (Garcia et al., 2016; Jolly et al., 2014; Pinto et al., 2015; Varela, 2016) 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 non-*Saccharomyces* strains studied in this paper. Therefore, if we want mixed culture behavior to deviate from that of *S. cerevisiae* monoculture, it must be ensured that non-*Saccharomyces* 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 t₀ or to successively inoculate (Tristezza et al., 2016) first the non-*Saccharomyces* 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 isolated 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”. Indeed, all the interactions observed in the present study could be explained by these types of interactions.

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 *S. cerevisiae*, ii) investigating intra-specific variability and strain-strain
interactions between species, iii) investigating the impact of the environment of culture (temperature, grape variety, etc.).

Mat & Met

Strains

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

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

Medium
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 non-*Saccharomyces* cells density was $0.9 \times 10^6$ /mL. Fermentations were carried out in a synthetic medium (SM) mimicking standard grape juice (Bely et al., 1990). 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. (Seguinot et al., 2017). The pH of the medium was adjusted to 3.3 with 10M NaOH. The SM medium was first saturated with bubbling air during 40 minutes, then it was supplemented with 5 mg/L phytosterols (85451, Sigma Aldrich) to fulfill the lipid requirements of yeast cells during anaerobic growth.

**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, viability and proportion of *S. cerevisiae* cells were determined using a BD Accuri™ C6 Plus flow cytometer as described in Delobel et al., (2012. From these population densities (without taking into account viability), we fitted a growth population model (with the growthcurver package in R, Sprouffske and Wagner, 2016), 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 (Camarasa et al., 2011). 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 isolated 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 (Crépin et al., 2012).

**Statistical Analysis**

The experimental work was performed in 5 different blocks. Each block was composed of three replicates of non-Saccharomyces fermentations (for example Hu), three replicates of the corresponding mixed fermentations with S. cerevisiae (for example ScvsTu) and one or two fermentations of isolated 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 (“R Core Team (2016). R: A language and environment for...
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### Tables

#### Table 1

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

|       | µ         | K   | prop | Vmax | MaxCO2 | Sugar | NAS | Ethanol | Glycerol | Succinate | Acetate | Pyruvate | Alpha |
|-------|-----------|-----|------|------|--------|-------|-----|---------|----------|-----------|---------|----------|-------|
| µ     | 0.15±0.02bc | 1.6±0.2med | NA | 0.99±0.03* | 88.2±12.2a | 0.2±0.2d | 10.4±1.6f | 95.2±8.4a | 5.3±0.4c | 1.05±0.28a | 0.63±0.06d | 0.12±0.08bc | 0.08±0.04ab |
| Ho    | 0.19±0.03bc | 1.6±0.1e  | NA | 0.4±0.02e | 46.4±0.6a | 92.3±1.4b | 122.2±3.5a | 50.1±0.23b | 5.2±0.06a | 0.35±0.00b | 0.99±0.072a | 0.13±0.05bc | 0.09±0.14ab |
| ScvsHo| 0.11±0.00bc | 2.3±1.1b  | NA | 0.9±0.02* | 30.7±0.5a | 134.3±1.1c | 173.3±23.8a | 34.9±11.1b | 5.5±0.2bc | 5.2±0.07bc | 11.5±0.03ab | 0.17±0.04bc | 0.14±0.09ab |
| Hu    | 0.62±0.18b  | 1.4±0.1de | NA | 0.34±0.04d | 10.5±0.5b | 134.3±11.2c | 173.3±23.8a | 34.9±11.1b | 5.5±0.2bc | 5.2±0.07bc | 11.5±0.03ab | 0.17±0.04bc | 0.14±0.09ab |
| ScvsHu| 0.24±0.01c  | 1.7±0.1d  | NA | 0.76±0.01d | 6.5±0.5c | 89.5±13.8e | 6.6±0.3c | 0.6±0.06a | 0.86±0.07a | 0.16±0.04bc | 0.09±0.06ab |
| Mu    | 0.17±0.02bc | 0.8±0.05bc | NA | 0.17±0.04c | 20.6±1.2b | 135.8±9.7e | 256.6±12.9a | 20.6±1.0c | 2.8±0.1a | 0.29±0.001b | 0.13±0.01bc | 0.10±0.01ab | 0.10±0.00ab |
| ScvsMu| 0.09±0.01bc | 1.3±0.03e  | NA | 0.96±0.02a | 86.8±11.7a | 9.9±0.1bc | 1.8±0.1a | 92.1±3.2b | 6.0±0.1ab | 0.58±0.02b | 0.46±0.02a | 0.12±0.01bc | 0.08±0.01ab |
| Mu    | 0.18±0.03bc | 0.6±0.01f  | NA | 0.16±0.02c | 81.8±0.4f | 142±3.7a | 305.5±5.9a | 23.4±1.8c | 3.7±0.2bc | 0.28±0.03bc | 0.07±0.06c | 0.29±0.04c | 0.13±0.01ab |
| ScvsMu| 0.09±0.00bc | 1.3±0.01c  | 0.5±0.02e | 0.68±0.04c | 83.3±12.2m | 20.8±6.0c | 2.1±0.2a | 86.5±3.4c | 6.1±0.1a | 0.52±0.04bc | 0.46±0.02a | 0.24±0.01bc | 0.19±0.01c |

**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.
FIGURES

FIG 1

Representation of a) the maximum rate of CO₂ production (\(V_{\text{max}}\)) and b) the total CO₂ produced in function of the species driving each fermentation (average ± standard deviation). The small letters indicate the statistical groups from a Tukey analysis.
FIG2

Global monitoring of the kinetics of the total living population (left), and sub-population in the mixed cultures (right) across fermentation. Each point represents a sample (average ± standard error). Full lines are for total population and dashed lines for the two sub-populations in mixed cultures. At the end of dashed lines, the final proportion of both sub-populations in mixed cultures is indicated. The light colors represent pure cultures of ‘non-Saccharomyces’
strains and dark ones to the corresponding culture in competition with *S. cerevisiae*. The isolated cultures of *S. cerevisiae* are represented in black. *Sc, Mp, Mf, Ho, Hu, ScvsMp*, *ScvsMf, ScvsHo* and *ScvsHu* correspond respectively to cultures with: isolated *S. cerevisiae*, isolated *M. pulcherrima*, isolated *M. fructicola*, isolated *H. opuntiae*, isolated *H. uvarum*, *M. pulcherrima* mixed with *S. cerevisiae*, *M. fructicola* mixed with *S. cerevisiae*, *H. opuntiae* mixed with *S. cerevisiae* and *H. uvarum* mixed with *S. cerevisiae*.
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%). Sc, Mp, Mf, Ho, Hu, ScvsMp, ScvsMf, ScvsHo and ScvsHu correspond respectively to cultures with: isolated S. cerevisiae, isolated M. pulcherrima, isolated M. fructicola, isolated H. opuntiae, isolated H. uvarum, M. pulcherrima mixed with S. cerevisiae, M. fructicola mixed with S. cerevisiae, H. opuntiae mixed with S. cerevisiae and H. uvarum mixed with S. cerevisiae.

![Graph showing yield of metabolite production relative to S. cerevisiae yield](https://example.com/graph.png)

**FIG4**

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