Design of a new model yeast consortium for ecological studies of enological fermentation

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ABSTRACT

Wine fermentation involves complex microbial communities of non-*Saccharomyces* yeast species besides the well-known *Saccharomyces cerevisiae*. While extensive research has enhanced our understanding of *S. cerevisiae*, the development of multi-species fermentation starters has led to increased interest in yeast interactions and the role of microbial diversity in winemaking. Consequently, molecular methods have emerged to identify the different species at different stages of the winemaking process. Model microbial communities or consortia, which provide simplified systems resembling natural microbial diversity, offer opportunities to investigate population dynamics and understand the role of community diversity in ecosystem performance.

Here, this work aims to design a yeast consortium reflecting the diversity of wine yeasts and to develop a method for accurately tracking their population dynamics during fermentation. We developed and characterized a six-species consortium, with *S. cerevisiae*, *Hanseniaspora uvarum*, *Starmerella bacillaris*, *Metschnikowia pulcherrima*, *Lachancea thermotolerans* and *Torulaspora delbrueckii*. By tagging each yeast species with distinct fluorescent markers, the study enables real-time monitoring of individual species within the consortium using flow cytometry. We have carried out a complete analysis of this consortium, studying the evolution of populations over time and examining factors such as metabolite production and fermentation kinetics.

In addition, the yeast consortium was used to test the diversity-function relationship as a proof of concept. We sought to determine the impact of the initial evenness on communities' performances subjected to osmotic stress. To this end, ten randomly designed consortia with varying initial species proportions were followed in enological fermentation with 200 and 280 g/L of initial sugars. Although no demonstrable effect of the initial evenness was shown, the usefulness of this consortium has been demonstrated. Moreover, the initial proportion of certain species on population dynamics and metabolite production were observed.

The consortium presented in this work and available to the scientific community can contribute to future work trying to decipher multispecies dynamics and exploring the role of yeast diversity in wine fermentation.

Keywords: Synthetic ecology, non-*Saccharomyces*, wine fermentation, diversity, flow cytometry

Introduction

Fermented foods and beverages are consumed worldwide, and their production relies on diversified microbial communities that include numerous bacteria and yeasts (Furukawa et al., 2013; Wolfe & Dutton, 2015; Tamang et al., 2016). Wine alcoholic fermentation involves different yeast species from the *Saccharomyces, Hanseniaspora, Pichia, Metschnikowia, Starmerella, Torulaspora, Lachancea*, or *Rhodotorula* genera (Barata et al., 2012; Drumonde-Neves et al., 2021). Research has mainly been focused on optimizing the process with *Saccharomyces cerevisiae*, as it is the main species carrying out the alcoholic fermentation. Thus extensive knowledge has been provided on *S. cerevisiae* growth, and its influence on wine aroma as well as the genetic basis of its suitability for fermentation (Lambrechts & Pretorius, 2000; Sablayrolles, 2009; Peter et al., 2018). However, the development of multispecies starters, with one or two non-*Saccharomyces* strains alongside *S. cerevisiae* (Roudil et al., 2020), has spurred research on interactions between yeast species and on the role of wine microbial diversity in the process (Ivey et al., 2013; Ciani & Comitini, 2015; Bordet et al., 2020).

Consequently, an increasing number of studies on wine microbial ecology, ranging from investigation on the factors influencing yeast and bacterial diversity in grape must, such as climate and vine management (Bokulich et al., 2014; Grangeteau et al., 2017; Bagheri et al., 2018), to the mechanisms of interactions involved between the different species (Bordet et al., 2020). Studies on population dynamics have highlighted mechanisms of competition for nutrient, secretion of toxic compounds or involvement of cellular contact (Holm Hansen et al., 2001; Medina et al., 2012; Kemsawasd et al., 2015). Molecular mechanisms and phenotypic changes caused by interactions have also been studied through transcriptomic analysis or description of physical interactions of cell wall proteins (Brückner et al., 2020; Mencher et al., 2021; Conacher et al., 2022). In addition, interactions and population dynamics of yeasts can be modulated by environmental conditions, such as the different stress factors they encounter during winemaking. Indeed, across the alcoholic fermentation yeasts are exposed to harsh conditions, encompassing low pH, low oxygen availability, or high sugar content (Heard & Fleet, 1988; Bauer & Pretorius, 2000; Varela et al., 2021). The varied environmental conditions and complex microbiota involved in fermentation makes it difficult to draw conclusion.

Model microbial communities allow working on simplified systems that mimic the diversity found in natural communities (Roy et al., 2014; Blasche et al., 2017). These consortia include species found in natural communities, and are used in controlled environments to foster knowledge on ecological drivers of population dynamics as well as decipher the role of community diversity in its functioning (Wolfe et al., 2014; Venturelli et al., 2018; Piccardi et al., 2019; Niccum et al., 2020). Several methods can be used to evaluate the evolution of population in such consortia, such as differential culture media, quantitative PCR or metabarcoding. Cytometry has recently proven useful to monitor population dynamics in multispecies communities (Rigottier-Gois et al., 2003; Kylilis et al., 2018) and model wine yeast pairwise cocultures and consortia (Longin et al., 2017; Conacher et al., 2020).

The present study aimed to develop a novel six-species consortium representative of wine yeast diversity as well as the method to discriminate its different species population. This consortium consisted of one strain of six different species, modified with a specific fluorescent tag, at different initial abundance. We also verified our ability to accurately measure species proportions in complex mixtures with mock communities and finally used this novel model consortium to test the diversity-function relationship as a proof of concept.

Material and methods

Strains and media

In this study, six wine yeasts species were used: *Hanseniaspora uvarum, Lachancea thermotolerans, Metschnikowia pulcherrima, Saccharomyces cerevisiae, Starmerella bacillaris*, and *Torulaspora delbrueckii*. Details of the strain are available in Table 1. Strains were kept at -80°C in yeast peptone dextrose YPD (1% yeast extract, 2% bactopeptone, 2% glucose) supplemented with 20% of glycerol before being streaked on YPD agar and incubated at 28°C. *Escherichia coli* DH5 bacteria (New England Biolabs), used for plasmid constructions, were grown on LB medium (1% tryptone, 0,5% yeast extract,

0,5% NaCl) supplemented with 100 µg/mL of ampicillin antibiotic (Sigma A9817). For solid media preparation, agar was added to the media at 2 %.

Table 1: List of yeast strains used in this study.

Species	Strain name	Code	Genotype*	Provider/Reference
S. cerevisiae	MTF 4798		*TDH3-mCherry-kanMX*	This study
S. cerevisiae	CLIB 3930	Sc	*TDH3-mCherry-kanMX ENO2-GFP-natMX4*	This study
H. uvarum	CLIB 3118			CIRM Levures
H. uvarum	CLIB 3929	Hu	*TDH3-BFP2-kanMX*	This study
L. thermotolerans	MTF 5257			SPO lab
L. thermotolerans	CLIB 3932	Lt	*KLTH0G15730-mCherry-natMX4*	This study
M. pulcherrima	CLIB 3329	Mp		CIRM Levures
S. bacillaris	CLIB 3147			CIRM Levures
S. bacillaris	CLIB 3933	Sb	*TDH3-eGFP-hphMX6*	This study
T. delbrueckii	CLIB 3152			CIRM Levures
T. delbrueckii	CLIB 3931	Td	*TDEL0E04750-mCitrine-natMX4*	This study

*Due to lack of information as of writing time, *TDH3* was used for non-*Saccharomyces* to indicate the closest homologuous orthologues to *S. cerevisiae* S288C *TDH3* gene sequence when no gene code was available.

Fluorescent strains construction

Plasmid construcion

The following plasmids were constructed by Gibson assembly (Gibson et al., 2009): pFA6a-link-yEmCitrine-NATMX, pFA6a-link-yomCherry-NATMX, pFA6-TDH3.1kb.Td-mCitrine-NATMX, pFA6-Hu1kb-BFP2-KAN. Gibson assembly were done using the NEB Builder HiFi DNA Assembly Master Mix (New England Biolabs) and transformed into *E. coli* DH5 (New England Biolabs) following the manufacturer instructions. pFA6 plasmid backbone, antibiotic resistance cassettes (*KanMX, hphMX*) and fluorescent protein genes (EGFP, mCherry, mCitrine, mTagBFP2) were obtained from plasmids ordered from AddGene (#44900, #44899, #44645, #44903, **Supplementary Table 1**, Lee et al., 2013; Sheff and Thorn, 2004). Primers used for the amplification of fragments used for the assembly are listed in **Supplementary Table 2**. Where necessary, homologous sequences of approximately 1kb were amplified from the target species (*T. delbruecki* CLIB3069, *H. uvarum* CLIB3221, *S. bacillaris* CLIB3147). All plasmids were checked by enzymatic digestion (New England Biolabs). Plasmid DNA was extracted from 3 mL of overnight *E. coli* LB (with 100 µg/mL of ampicillin) culture with the NucleoSpin Plasmid extraction kit (Macherey Nagel, Düren, Germany) according to manufacturer instructions.

Yeast genetic modification

Genes coding for fluorescent proteins were integrated into the genome in fusion of *TDH3* gene (or its orthologue in non-*Saccharomyces*). Cassettes used for transformation were amplified from plasmid matrices listed in **Supplementary Table 1** with a high fidelity enzyme, either the KAPA HiFi kit (Cape Town, South Africa) or Phusion High-Fidelity DNA polymerase (Thermo Fisher Scientific, Vilnius, Lithuania), using primers specific to each species, as listed in **Supplementary Table 3**. PCR amplification products were purified with the PCR clean-up Kit (Machery Nagel, Düren, Germany, REF 740609). In addition, *S. cerevisiae* MTF4798 was tagged with a second fluorescent protein, in fusion of *ENO2*, another gene involved in glycolysis. *TDH3* has a strong promoter and is expressed continuously during fermentation in *S. cerevisiae*. Similarly, *ENO2* is also expressed throughout *S. cerevisiae* fermentation, even though to a lesser extent (Puig & Pérez-Ortín, 2000; Peng et al., 2015). Yeast transformation was carried out by electroporation, as described by Pourcelot et al. (2023). Transformants were selected on YPD agar supplemented with the appropriate antibiotic: 800 µg/mL of hygromycin B for *S. bacillaris*, 100 µg/mL of nourseothricin for *S. cerevisiae*, *T. delbrueckii*, and *L. thermotolerans*, and 200 µg/mL of geneticin for *H. uvarum* and *S. cerevisiae*. Integration at the locus was verified with two independent PCR (**Supplementary Table 4**). To check that transformation did not modify cells behavior, growth of WT strains and transformants were compared by microplate assay (**Supplementary Figure 1**).

Fermentations

Synthetic must

Synthetic grape must was prepared according to Bely et al., (1990) with 425 mg/L of yeast assimilable nitrogen (as a mixture of amino-acids and ammonium), supplemented with 5 mg/L of phytosterols (Beta-sitosterol, Sigma, St-Louis, US, MO) and a final pH of 3.3. Total sugar concentration was either 200 g/L (S200, with 100 g/L of glucose and 100 g/L of fructose), or 280 g/L (S280, with 140 g/L of glucose and 140 g/L of fructose). Synthetic must was pasteurized at 100°C for 15 min before use.

Cell inoculation

Cell cultures were prepared with a first propagation in 5 mL YPD for 18 hours at 28°C, and a second propagation of 1 mL in 50 mL of synthetic must S200 for 24 hours at 28°C with agitation. Cell concentration of each pre-culture was measured by flow cytometry before inoculation. Appropriate cell culture volume was used to start fermentation with a final cell concentration of 10^6 cells/mL. In the case of consortia, the volume of different species cultures were pooled to obtain the desired initial abundance (**Table 2**). Cell suspensions were centrifuged at 4415 g for 5 min and washed in physiological water (9 g/L of sodium chloride, Sigma), and suspended in 5 mL of synthetic must for inoculation (either S200 or S280).

Scale of fermentation

Fermentations were carried out either in 1 L or 250 mL scale fermentation. One liter fermentation were run in 1.2 L fermenters (200mL headspace) with automated weighing every 20 min to evaluate CO_2 weight loss. Fermenters filled with 1 L of synthetic grape must were aerated for 40 min before inoculation. An internally developed software was used to measure in real time the rate of CO_2 production (g/L/h) and fermentations were stopped when it was lower than < 0.02 g/L/h, or after 312 hours if fermentation had not finished yet. At 0, 4, 6, 12, 24, 48, 72, 168 hours of fermentation and when fermentation stopped or after 312 hours, 6 mL were sampled for population and metabolite analysis.

Two-hundred and fifty mL fermentations were run in 300 mL fermenters with manual weighing twice to thrice a day. Fermenters were aerated for 20 min before inoculation. At the start and end of fermentation, 5 mL were sampled for both population and metabolite analysis. At 21, 45, 120, 168, 210, 290 h, 1.5 mL were sampled for population analysis only.

Inoculation and consortia composition

Precultures of each strain were done by inoculating 5 mL of YPD with colony grown on a YPD agar plate and incubated for 18 hours at 28°C. One milliliter of the preculture was then propagated in 50 mL in synthetic must S200 in 125 mL Erlenmeyer flasks and incubated for 24 hours at 24 °C with shaking (250 rpm). Volume of cell culture of each species was calculated after measuring cell concentration of each strain culture by flow cytometry. Consortia were made by mixing the adequate volume of each species in individual 50 mL Falcon tubes for each fermenter. The cell mixes were centrifuged for 5 min at 4415 g and the cell pellet was rinsed in physiological water once (NaCL at 9 g/L) then thoroughly ressuspended in 5 mL of synthetic must (S200 or S280 accordingly). Fermenters were inoculated at a total 10^6 cell/mL rate with this final cell suspension.

Composition of the different consortia used in this study can be found in **Table 2**. The 6-species consortia (Co) had the following initial proportions of the six species were: 35% *H. uvarum* (3.5×10^5 cells/mL), 25% *M. pulcherrima* (2.5×10^5 cells/mL), 20% *S. bacillaris* (2×10^5 cells/mL), 10% *S. cerevisiae (*1×10^5 cells/mL), 5% *T. delbrueckii* (0.5×10^5 cells/mL), 5% *L. thermotolerans* (0.5×10^5 cells/mL). The consortia used for testing the diversity-functionnality relationship included eleven consortia with the five fluorescent species (noted C02 to C12). These consortia had varying initial relative abundance of the five different species (except for *S. cerevisiae* which had an initial abundance of 5% in C02 to C11, and 15 % in C12) encompassing different levels of evenness (**Table 2**). Diversity was evaluated with the Shannon index with the following formula: $-\sum^n p_i \ln(p_i)$ where p_i is the relative abundance of species i, and n the number of species. C12 composition is adapted from the 6-species consortium but without *M. pulcherrima*. Two consortia consisting of equal initial abundance for all species, with *S. cerevisiae* (C07) or without (C13), as well as a control monoculture with *S. cerevisiae* alone (noted C01) were also included.

Table 2: Composition (in %) and diversity index of the different consortia tested in this study.

Consortium	Sc	Hu	Sb	Lt	Td	Mp	Shannon index
6 species							
Co	10	35	20	5	5	25	
5 species							
C01	100	0	0	0	0		0.000
C02	5	75	5	10	5		0.895
C03	5	5	15	5	70		0.983
C04	5	25	60	5	5		1.102
C05	5	5	50	35	5		1.163
C06	5	30	5	10	50		1.237
C07	5	23.75	23.75	23.75	23.75		1.515
C08	5	25	50	15	5		1.277
C09	5	5	45	20	25		1.327
C10	5	45	25	10	15		1.370
C11	5	25	30	30	10		1.449
C12	15	40	25	10	10		1.458
C13	0	25	25	25	25		1.386

Cell numbering by flow cytometry

Mock communities

Cell number of each species was monitored by flow cytometry with the Attune NxT™ Thermofisher® Flow Cytometer (Life Technologies, Singapore). Each population tagged with one or two fluorescent protein was detected with a specific set of channels described in **Table 3**. To validate the method, accuracy of the numbering of the 6 species was made with mock communities. The mock communities were constructed by incubating single species cultures overnight in synthetic must at 28°C with agitation. Subsequently, one mL of culture was centrifuged at 4415 g for 5 min and the resulting cell pellet was resuspended in 1 mL of PBS (130 mM NaCl, 2.6 mM KCl, 7 mM Na_2HPO_4, 1.2 mM KH_2PO_4, pH 7.4; Sigma). Cell suspensions at a concentration of 10^6 cells/mL were prepared in PBS and combined to create mock communities in 96-well microplates (200 µL of final volume). In total, 30 communities were tested, each included one species with theoretical abundance of either 1, 5, 10, 50 or 90% while the five other species were in equal proportions. Cell numbering was performed both immediately after preparation of the mock communities in the microplate and after 2 hours at room temperature.

Table 3: Description of the set of channels (with dichroic filters details) used for the detection of the different populations considered in this study. Voltages were set using fluorescent and wild type cells to ensure a proper signal of both. The fluorescence phenotype of each strain is indicated with + (fluorescent) and – (non-fluorescent) on the corresponding channel. Viability was measured using Propidium Iodide (PI).

Population	Fluorochrome	Channel (laser and filter wavelengths in nm)	Voltage	Fluorescence
Dead cells	PI	BL3 (488 - 695-40)	340	+
Live cells	PI	BL3 (488 - 695-40)	340	-
S. cerevisiae	mCherry + GFP	YL2 (561 - 600DLP - 620/15)	320	+
		BL1 (488 - 495DLP-530/30)	260	+
L. thermotolerans	mCherry	YL2 (561 - 600DLP-620/15)	320	+
		BL1 (488 - 495DLP-530/30)	260	-
S. bacillaris	mCitrine	BL1 (488 - 495DLP-530/30)	260	+
		VL2 (405 - 495DLP-512/25)	400	-
T. delbrueckii	EGFP	BL1 (488 - 495DLP-530/30)	260	+
		VL2 (405 - 495DLP-512/25)	400	-
H. uvarum	mTagBFP2	YL2 (561 - 600DLP-620/15)	320	-
		BL1 (488 - 495DLP-530/30)	260	-
		VL1 (405 - 417LP-440/50)	340	+

M. pulcherrima	YL2 (561 - 600DLP-620/15)	320	-
	BL1 (488 - 495DLP-530/30)	260	-
	VL1 (405 - 417LP-440/50)	340	-

Population dynamics during fermentation

At the different sampling timepoints, 200 µL of sample were ressuspended in 200 µL of PBS after centrifugation in 96-wells microplate (V-bottom) at 4415g for 5 min. Samples were diluted in PBS before reading so that the cell concentration was approximately 1 to 5.10^5cells/mL. Total cell count was measured with the FSC and SSC channels (488 nm laser, 488/10 filter). To restore the fluorescence, cells suspended in PBS were kept at room temperature for 2 hours. This incubation step likely enables the maturation of fluorescent proteins which is oxygen-dependent (Hansen et al., 2001; Zimmer, 2002), while oxygen is rapidly depleted during fermentation (Bardi et al., 1999; Moenne et al., 2014). Moreover, we observed a decrease in fluorescence of eGFP under the control of the *ENO2* promoter in *S. cerevisiae* near the end of fermentation, reducing the accuracy of measurement after 144 hours of fermentation (up to 5% of inaccurate events at 144 hours). This was corrected with an incubation step in YPD for 1 hour (Breslow et al., 2008) followed with an incubation step of 1h in PBS. These steps did not impair total cell count or viability. Viability was assessed after staining cells with 1 µg/mL propidium iodide (PI, stored at 4°C protected from light; Calbiochem). Cell number and percentage were obtained from gatings done on the Attune NxT software.

Metabolites analysis

For the metabolite analysis, 5 mL of samples were centrifuged 5 min at 4415 g at 4°C to get rid of cells. Supernatant aliquots were kept at -18°C before being diluted with 2.5 mM sulfuric acid (H_2SO_4, Merck, Darmstadt, Germany) at 1:6, and centrifuged again at 16 000 g for 5 min. Diluted samples were kept at -18°C until analysis. Extracellular metabolite and sugar concentrations (glucose, fructose, ethanol, glycerol, acetic acid, lactic acid, pyruvic acid, and α-ketoglutaric acid) were determined by high-performance liquid chromatography (HPLC), following the method described in Deroite et al., (2018). Analyses were run on the HPLC (HPLC 1260 Infinity, Agilent Technologies, Santa Clara, California, USA) using a Rezex ROA ion exclusion column (Phenomenex, Torrance, CA, USA) at 60°C, with a flow rate of 0.6 mL/min of 2.5 mM H_2SO_4. Concentrations of acetic acid, lactic acid and α-ketoglutaric acid have been measured with a UVmeter at 210 nm and other compounds with a refractive index detector and chromatograms were analyzed on OPEN LAB 2X software. Fermentation samples were analyzed with technical duplicates and data analysis were carried out on the mean of both replicates.

Data analysis

Mock communities were analyzed by comparing theoretical cell concentration to the observed cell concentration, as measured by cytometry.

All fermentations were performed in biological triplicates, on three different runs.

Fermentations of the six monocultures and the 6-species model consortium were compared based on 10 fermentation parameters. They included four CO_2 kinetics parameters - latency (time needed to reach 5 g/L of total produced CO_2), time of fermentation (tF, time when CO_2 production rate < 0.02 g/L/h), maximum CO_2 production rate (Vmax) - and six metabolic parameters, namely the yields of ethanol, glycerol, and acetic, α-ketoglutaric, lactic and pyruvic acids. Metabolite yields were calculated with the formula:

(1) $$Ymetabolite = \frac{Final\ metabolite\ concentration\ (g/L)}{Initial\ sugar\ concentration\ (g/L) - Final\ sugar\ concentration\ (g/L)}$$

Fermentations of the 5-species consortia were compared with the six metabolites yieds and CO_2 yield. Differences in yields between the S200 and S280 conditions were visualized by calculating the difference between both conditions with the following formula:

(2) $$\Delta_{S280,S200} = \frac{Y_{S280} - Y_{S200}}{Y_{S200}}$$

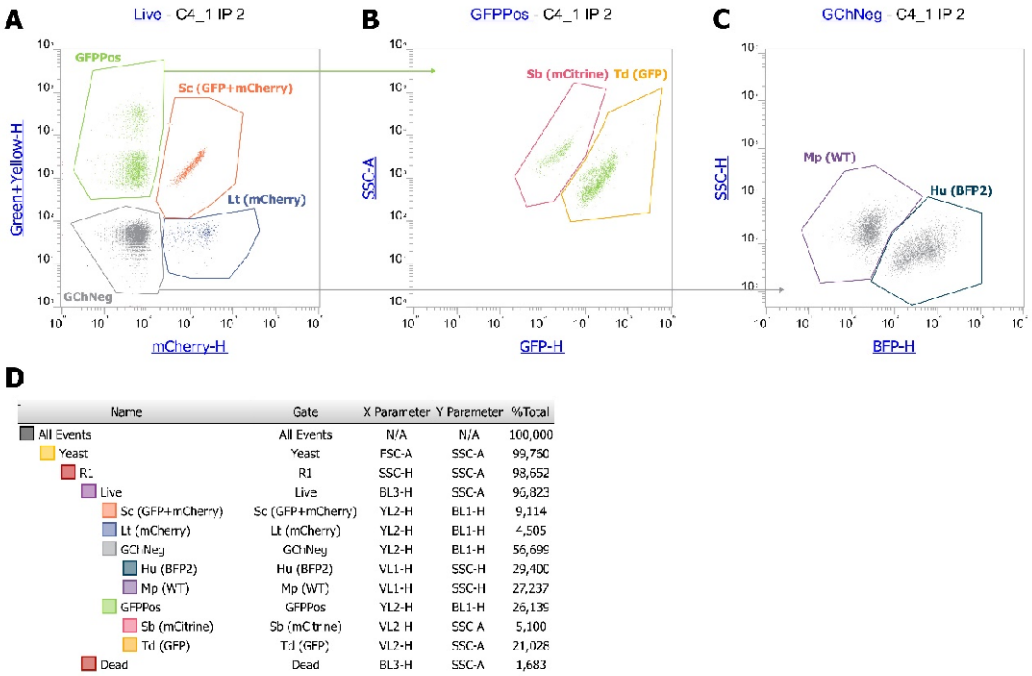
Data were analyzed with R studio software (version 4.3.1, Core Team, 2023). Mock communities' data were analyzed with the 'ggpmisc' package (Aphalo, 2023). ANOVA were performed to compare the different monocultures and 6-species consortium parameters, followed by a post hoc Tukey test when significant using the `rstatix` package (Kassambara, 2023). For the 5-species consortia, comparison between both sugar conditions were done with t-test for consortia C01 to C13, on the following parameters: residual sugars, yield in CO_2, and end yields in ethanol, glycerol, and acetic, lactic, α-ketoglutaric and pyruvic acids

Results

Construction of consortium and cytometry numbering optimization

The model community that we used in this study was constituted of 6 different species at different initial abundance: *Saccharomyces cerevisiae* (10% of total population), *Hanseniaspora uvarum* (35%), *Metschnikowia pulcherrima* (25%), *Starmerella bacillaris* (20%), *Lachancea thermotolerans* (5%), and *Torulaspora delbrueckii* (5%). The species and their proportions were determined based on a literature analysis of 18 articles, and the species initial abundance was determined from the average of the initial abundance found in a subselection of 11 articles (Combina et al., 2005; Hierro et al., 2006; Di Maro et al., 2007; Zott et al., 2008; Ocón et al., 2010; Maturano et al., 2015; Ghosh et al., 2015; Bagheri et al., 2017b; Simonin et al., 2018; Mateus et al., 2020; González-Alonso et al., 2021). These articles needed to include at least one sampling without inoculation and without enrichment steps, and the identification had to be at the species level. They encompassed different world regions and winemaking practices (grape variety, conventional and organic, etc). Despite the clear influence of these factors on yeast diversity, we found little relationship between the regions and practices with the presence of specific species, which is in line with the conclusions of a recent meta-analysis (Drumonde-Neves et al., 2021). Among the most frequent species, we decided to select only one species per genera with the assumption that it might increase phenotypic diversity and the overall usefulness of our model consortium. Thus, the six following species were selected: S. *cerevisiae*, *H. uvarum*, *M. pulcherrima*, *S. bacillaris*, *T. delbrueckii* and *L. thermotolerans* (**Supplementary Figure 2A**). Then, the initial abundance for each species was determined from their natural initial abundance in must and early stages of fermentation (**Supplementary Figure 2B**).

The consortium was designed to track individually six species (*H. uvarum*, *L. thermotolerans*, *M. pulcherrima*, *S. bacillaris*, *S. cerevisiae*, *Torulaspora delbrueckii*) during fermentation. To this end, our strategy was to label the different species with different fluorescent proteins. The fluorescent proteins were selected based on their excitation and emission spectra to minimize overlaps. Genes of the fluorescent proteins were successfully integrated by homologous recombination in fusion of the *TDH3* gene into the genomes of *H. uvarum* (mTagBFP2), *S. bacillaris* (eGFP), *L. thermotolerans* (mCherry) and *T. delbrueckii* (mCitrine). *S. cerevisiae* was tagged with mCherry fused with *TDH3* and eGFP with *ENO2* (Table 1). For *M. pulcherrima*, the WT strain was used since we did not manage to integrate genes at the locus even when using long homology sequences, which is in accordance with previous studies (Gordon et al., 2019; Moreno-Beltrán et al., 2021). As shown on **Figure 1**, which displays derived dot plots and gates used in this study, the different transformants could be well discriminated. *L. thermotolerans* and *S. cerevisiae* are distinguished by the mCherry fluorescence and *S. cerevisiae* by the additional GFP fluorescence. *T. delbrueckii* (eGFP-tagged) cells could be separated from *S. bacillaris* (mCitrine-tagged) using the violet laser, not needing any specific optical configuration, as reported for YFP (Yellow Fluorescent Protein) (Marcus & Raulet, 2013). Initially, the T-Sapphire fluorescent protein was also selected, but its signal overlapped with the mTagBFP2 signal and was not used further.



Name	Gate	X Parameter	Y Parameter	%Total
All Events	All Events	N/A	N/A	100,000
Yeast	Yeast	FSC-A	SSC-A	99,750
R1	R1	SSC-H	SSC-A	98,652
Live	Live	BL3-H	SSC-A	96,823
Sc (GFP+mCherry)	Sc (GFP+mCherry)	YL2-H	BL1-H	9,114
Lt (mCherry)	Lt (mCherry)	YL2-H	BL1-H	4,505
GChNeg	GChNeg	YL2-H	BL1-H	56,699
Hu (BFP2)	Hu (BFP2)	VL1-H	SSC-H	29,400
Mp (WT)	Mp (WT)	VL1-H	SSC-H	27,237
GFPPos	GFPPos	YL2-H	BL1-H	26,139
Sb (mCitrine)	Sb (mCitrine)	VL2-H	SSC-A	5,100
Td (GFP)	Td (GFP)	VL2-H	SSC-A	21,028
Dead	Dead	BL3-H	SSC-A	1,683

Figure 1: Cytometry set up with an example of sample at inoculation. **A:** YL2 (600DLP-620/15)/BL1 (495DLP-530/30) dot plot is used to separate GFP positive and mCherry positive cells. **B**: VL2 (495DLP-512/25)/SSC dot plot derived from the GFPPos gate on A, is used to separate mCitrine (-) from eGFP signal (+). **C:** VL1 (417LP-440/50)/SSC dot plot derived from the GFPNeg gate on A, is used to separate non-fluorescent cells from BFP positive cells. **D**: Hierarchy of gating and percentage of each population. % Total indicates the % of the total population of events in the corresponding gate.

In addition, accurate numbering of the different yeast populations was verified by comparing measured to theoretical cell concentration in mock communities (**Figure 2**). Mock communities are constructed with known composition and used to validate microbial quantification methods, with metagenomics or cytometry, since they (Tourlousse et al., 2021; van de Velde et al., 2022; Rué et al., 2023). In this study, 6-species yeast mock communities were constructed from overnight cultures in S200 and included one species with theoretical abundance of either 1, 5, 10, 50 or 90% while the five other species were in equal proportions. In total, 30 communities were tested in microplates, with a final cell concentration of 10^6 cells/mL, and the concentration of each species was measured by flow cytometry. When species concentrations were numbered immediately after the mock assembly, the observed and theoretical cell concentrations were well correlated (R^2 ranging between 0.8 and 0.99 , **Figure 2**A). However, during fermentation, a loss of cell fluorescence was observed after 24 hours, especially for the mCherry-tagged cells. To restore fluorescence, samples needed to stay at least 2 hours in PBS at room temperature. This step was also tested on the mock community, and except for *M. pulcherrima* that was

under-estimated after the 2 hours (R^2_{Mp} = 0.73, **Figure 2**B), the other species were still accurately numbered ($R^2 \geq 0.90$).

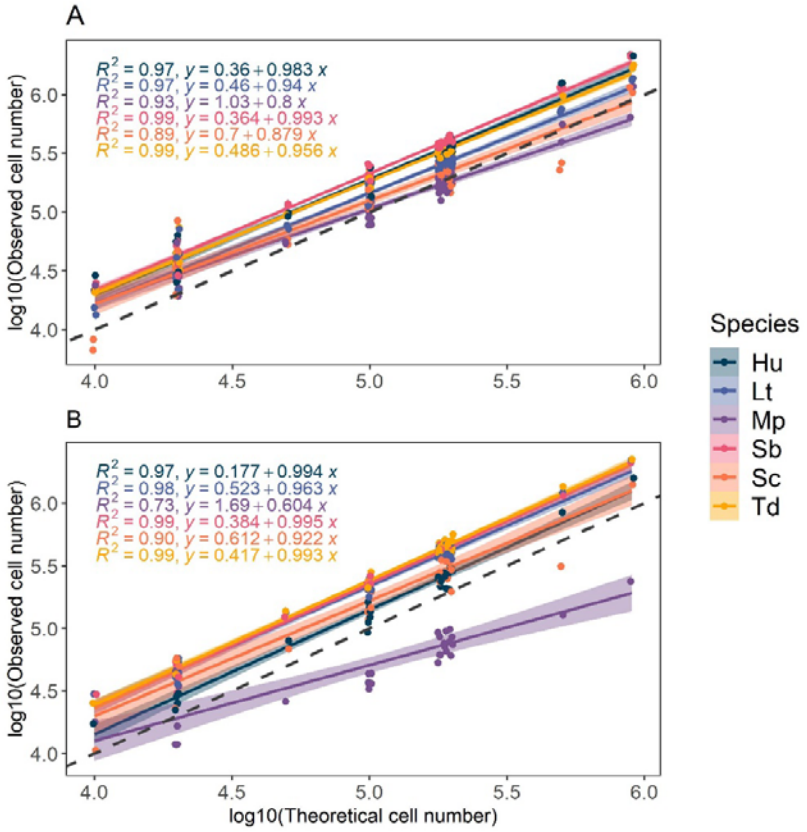


Figure 2: Comparison of observed population and theoretical population in 30 mock communities for immediate measure (**A**) and after 2 hours in PBS (**B**). Correlation factor and linear equation of observed population in function of theoretical populations are indicated for each species. Dashed line: log10(Observed cell number) = log10(Theoretical cell number). Hu: *H. uvarum*, Lt: *L. thermotolerans*, Mp: *M. pulcherrima*, Sb: *S. bacillaris*, Sc: *S. cerevisiae*, Td: *T. delbrueckii*.

Characterization of the 6-species model consortium

Population dynamics in the consortium and monocultures.

Monocultures and the 6-species model consortium (Co) were characterized with fermentation in synthetic grape must (S200) in 1 L-scale fermentation. All fermenters were inoculated with a total cell concentration of 10^6 cells/mL and initial abundance of the six species in the 6-species consortium (Co) were 35% *H. uvarum*, 25% *M. pulcherrima*, 20% *S. bacillaris*, 10% *S. cerevisiae*, 5% *T. delbrueckii*, 5% *L. thermotolerans*. The dynamic of species abundance in the consortium are shown in **Figure 3** and population dynamics of monocultures in **Supplementary Figure 3**. In the consortium, the early fermentation phase was characterized by a rapid growth of *H. uvarum* that reached 65% of the total population at 12 h, with a maximum population of 6.7 log of cells/mL (**Figure 3**, **Supplementary Table 5**). On the contrary, *S. bacillaris* and *M. pulcherrima* abundance dropped under 5% after 12 hours, even though they were inoculated at 20 and 25%, respectively. *L. thermotolerans*, showed a little growth at 24h, reaching 6.3 log of cells/mL, (**Supplementary Table 5**), but quickly decreased after 48 hours. After 24 h, a drop of viability was observed (live cells < 75%) associated with the drop in abundance of NS species, while *S. cerevisiae* became dominant in the consortium and represented 41% of the total population and 58% of the live population (**Figure 3**). Maximum population (8 log) in the consortium was reached after 48 h, with almost only *S. cerevisiae* remaining alive(**Figure 3**).

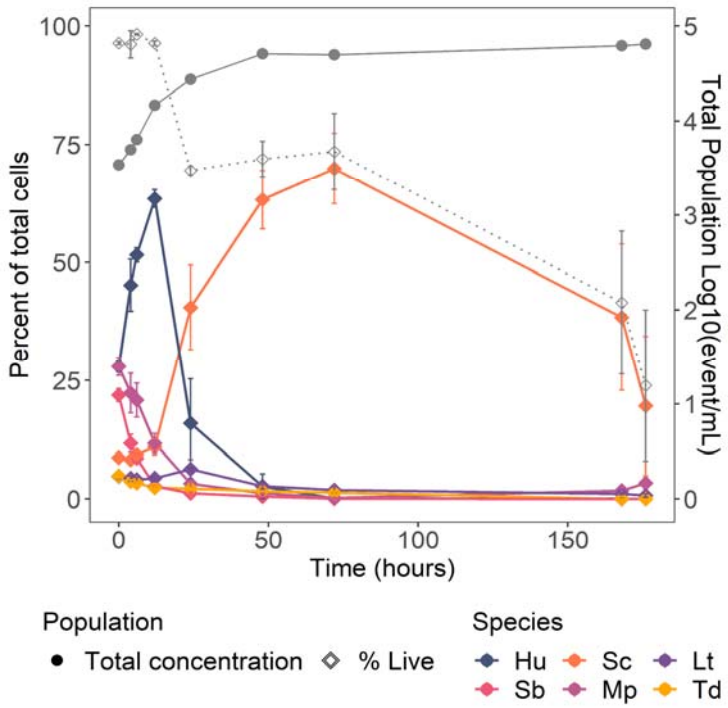


Figure 3: Individual growth of the different species in the 6-species consortium. The grey solid line corresponds to the cell concentration log10(cells/mL), the grey dotted line to the percent of live cell. Solid color lines describe the percent of the different species. Initial abundances were: 10 % *S. cerevisiae* (Sc), 35% *H. uvarum* (Hu), 25% *M. pulcherrima* (Mp), 20% *S. bacillaris* (Sb), 5% *L. thermotolerans* (Lt), and 5% *T. delbrueckii* (Td).

Fermentation of the 6-species consortium and monocultures

Fermentation capacity of the 6-species consortium and its member species alone was evaluated through CO_2 production (**Figure 4**). The consortium performance was similar to that of *S. cerevisiae* alone, with a slightly longer latency and fermentation time (latency = 25.3±1.9 h and tF = 158.5±6.1 h compared to 18.7±0.7 and 120.6±3.9 h respectively. The maximum CO_2 rate (Vmax) of consortium Co was significantly different from any monocultures. It reached 1.74 ± 0.0591 g/L/h (**Figure 4B**), which was between the maximum CO_2 rate of *S. cerevisiae* (2.16 ± 0.02 g/L/h), *T. delbrueckii* (1.11 ± 0.11 g/L/h) and *L. thermotolerans* (1.01 ± 0.06 g/L/h) alone. The consortium reached the same total CO_2 production as *S. cerevisiae* (93.08 ± 1.06 g), while *M. pulcherrima*, *H. uvarum*, and *S. bacillaris* alone showed the lowest CO_2 production (**Figure 4B**).

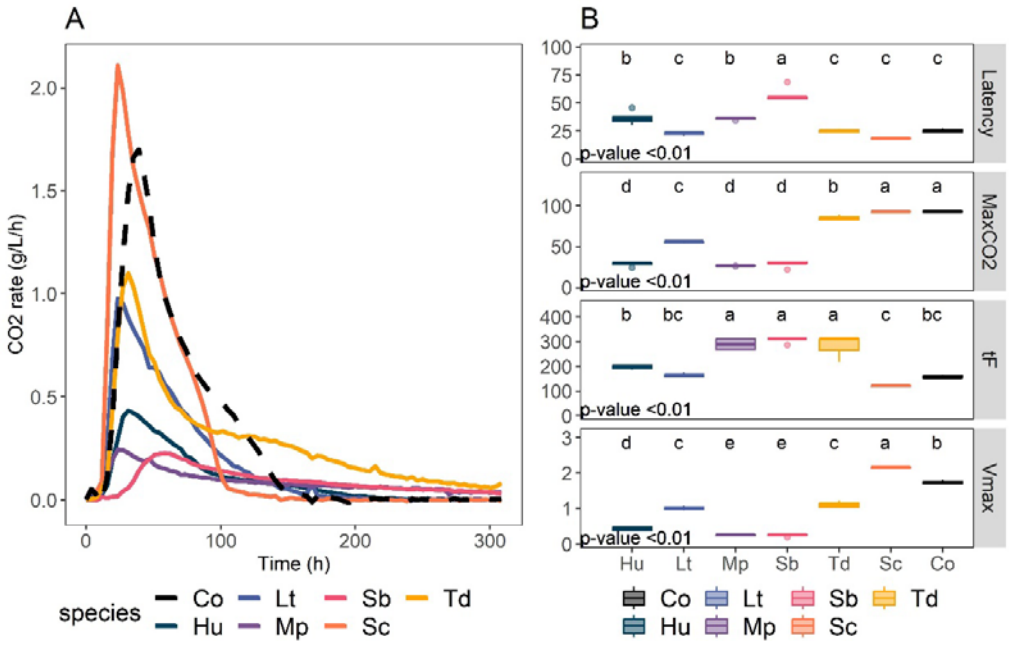


Figure 4: Fermentation kinetics of monocultures and consortium Co. **A**: CO_2 production rate kinetics of each monoculture (solid line) and consortium Co (dashed line). Smoothing with the Loess method was applied on the results of triplicates. **B**: Kinetics parameters obtained from CO_2 loss. MaxCO2 = maximum CO_2 produced (in g/L); tF = fermentation time (time to reach CO_2 production rate < 0.02 g/L/h), Vmax = maximum rate of CO_2 production, and Latency = time necessary for total CO_2 production to 5 g/L. ANOVA results are indicated by the p-value. Statistical groups determined with post-hoc Tukey tests are indicated with lowercase letters. Co: Consortium, Hu: *H. uvarum*, Lt: *L. thermotolerans*, Mp: *M. pulcherrima*, Sb: *S bacillaris*, Sc: *S. cerevisiae*, Td: *T. delbrueckii*.

In addition, changes in concentration of sugars and metabolites from the central carbon metabolism were evaluated. Once again, the 6-species consortium followed the trend of *S. cerevisiae* alone with a delay (**Figure 5**), showing the same end yields in ethanol (0.499 ± 0.001), acetic acid (0.002 ± 0.00), lactic acid (0.003 ± 0.00), and succinic acid (0.003 ± 0.00) as *S. cerevisiae* was alone at the end of fermentation (**Supplementary Figure 4**). Both the consortium and *S. cerevisiae* monoculture reached dryness (total residual sugars < 2g/L), and *T. delbrueckii* left 15.3 ± 5.28 of residual sugars. Other non-*Saccharomyces* left between 80 ± 3.32 g/L for *L. thermotolerans* and 137 g/L of sugars for *M. pulcherrima* and *H. uvarum*. Some difference can also be seen for the α-ketoglutaric acid release by the consortium, which seemed to be produced more slowly than *S. cerevisiae* monoculture (**Figure 5**) but reached a yield similar to monocultures of *S. cerevisiae, L. thermotolerans, T. delbrueckii* or *H. uvarum* ($Y_{\alpha\text{-keto, Co}}$ = 1.94e-04 ± 8.66e-06, **Supplementary Figure 4**). *M. pulcherrima* and *S. bacillaris* monocultures overproduced α-ketoglutaric acid in monoculture ($Y_{\alpha\text{-keto, Mp}}$ = 28.6e-04 ± 5.9e-04 g/g, $Y_{\alpha\text{-keto, Sb}}$ = 15.8e-04 ± 1.4e-04 g/g, **Supplementary Figure 4**). Similarly, *M. pulcherrima* and *S. bacillaris* had the two highest glycerol yields ($Y_{glycerol, Mp}$ = 0.068 ± 0.004 g/g, $Y_{glycerol, Sb}$ = 0.096 ± 0.002 g/g, **Supplementary Figure 4**). Despite that and their high initial abundance in the consortium (45% in total), their early disappearance likely prevented them from producing noticeable amounts of α-ketoglutaric acid and glycerol in the consortium. In the consortium, pyruvic acid was produced in the first 48 hours of fermentation (reaching 0.166 ± 0.005 g/L) then almost entirely consumed at the end (0.023 ± 0.001 g/L, **Supplementary Figure 4**). Monocultures of *S. cerevisiae, L. thermotolerans*, and *H. uvarum* showed a similar behavior, reaching respectively a maximum concentration of 0.230 ± 0.013 g/L at 48h, 0.050 ± 0.008 at 24h, 0.153 ± 0.007 at 48h (**Figure 5, Supplementary Figure 4**).

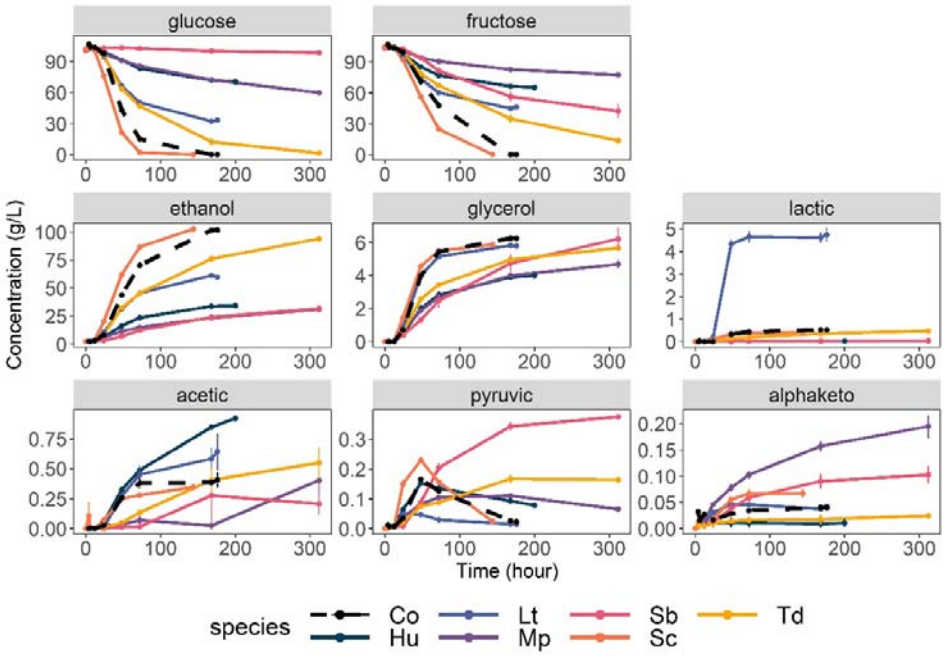


Figure 5: Kinetics of consumption and production of central carbon metabolites in monocultures and consortium. Error bars indicate the standard error. Co: Consortium, Hu: *H. uvarum*, Lt: *L. thermotolerans*, Mp: *M. pulcherrima*, Sb: *S. bacillaris*, Sc: *S. cerevisiae*, Td: *T. delbrueckii*.

Impact of consortium's diversity on osmotic stress response

The aforementionned consortium was developped as a tool to further study ecological drivers of the wine microbial community. As a first application, we focused on determining how the initial abundance of species would affect the fermentation process, especially when submitted to an osmotic stress. Twelve yeast consortia (numbered C02 to C13) with varying evenness of five species (excluding *M. pulcherrima* due to its underestimation), alongside a *S. cerevisiae* monoculture (C01) were tested. Fermentations were conducted in 250 mL-scale of synthetic grape must with two sugar concentration: 200 g/L (S200) and 280 g/L (S280). Furthermore the consortium C13 did not contain *Saccharomyces cerevisiae*.

In all consortia initially containing *S. cerevisiae*, this species largely dominated after 120 hours in both sugar conditions and constituted the only live cells, except in consortium C03 (**Figure 6**, **Supplementary Table 6)**. Unlike the others, in the C03 consortium there was 17% remaining of *T. delbrueckii* at 120h in condition S280, which led to a delayed dominance of *S. cerevisiae* that reached over 95% of total cells only after 168 hours instead of 120 hours in the S200 condition. *S. cerevisiae* monoculture was not much affected by the different sugar conditions and reached its maximum population of 8.12 log at 21 hours (**Supplementary Table 6**). On the other hand, observations revealed variations in the dynamics of non-*Saccharomyces* populations depending on the initial sugar concentration (**Figure 6**) with significant differences in population abundance between S200 and S280 found at 45 hours. In most consortia, *H. uvarum* reached a similar maximum proportion at 21 h for both sugar conditions but decreased more sharply at 45h in condition S280 (**Figure 6**). Similarly, *L. thermotolerans* tended to decrease more in condition S280, except in consortium C13 (without *S. cerevisiae*), while *T. delbrueckii* tended to maintain a higher proportion in condition S280 as compared to S200 (**Figure 6**). At 45 hours in consortium C03, the decrease in abundance between S200 and S280 of *L. thermotolerans* partially matched the increase in abundance of *T. delbrueckii* (6% less *L. thermotolerans* and 15% more *T. delbrueckii* in condition S280). The C03 consortium also showed a longer persistence of *T. delbrueckii* with S280 compared to the other consortia. Interestingly, in the *S. cerevisiae*-free consortium (C13), while both *L. thermotolerans* and *T. delbrueckii* were present at the end of fermentation, *L. thermotolerans* dominated the consortium after 21 hours of fermentation and tended to maintain higher abundance in S280. Regarding *S. bacillaris*, its

population decreased very quickly and its proportion was less than 5% after 21h in all consortia, for both sugar concentrations.

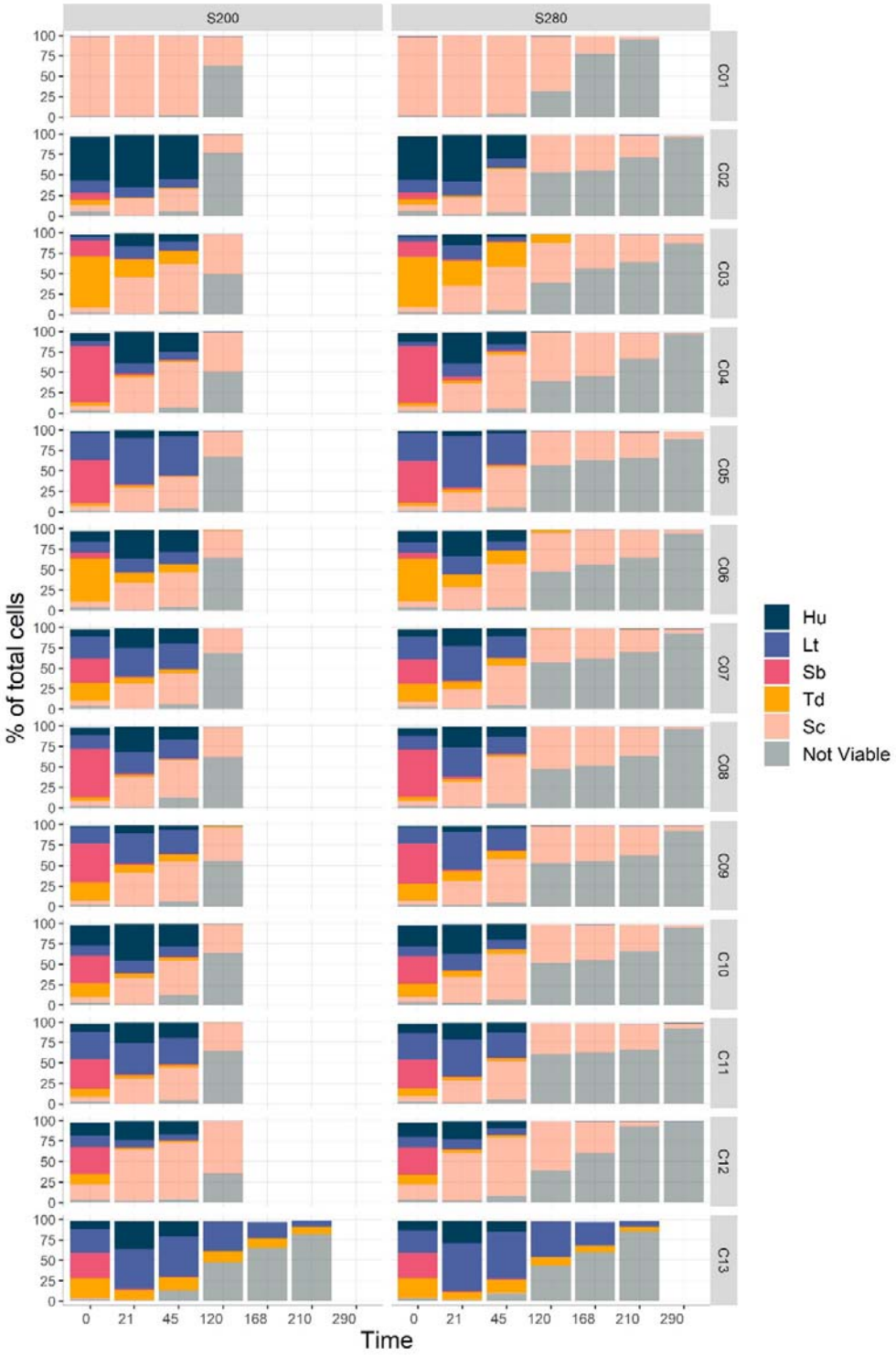


Figure 6: Population dynamics in consortia during fermentation with 200 and 280 g/L of sugars. C01 is a monoculture of *S. cerevisiae*. C02 to C11 initially contained 5% of *S. cerevisiae*. C12 initially contained 15% of *S. cerevisiae* and C13 did not contain *S. cerevisiae*. Hu: *H. uvarum*, Lt: *L. thermotolerans*, Sb: *S. bacillaris*, Td: *T. delbrueckii*, Sc: *S. cerevisiae*.

418 Fermentation performance was also assessed, through metabolite and CO_2 yields. The differences in
419 yield of the main metabolite from carbon metabolism between S280 and S200 are shown monocultures
420 in **Figure 7**. Positive values indicate higher yield in condition S280, while negative values indicate lower
421 yield in condition S280.
422 Higher initial sugar concentrations led *S. cerevisiae* to produce more acetic acid (increase by 75 % of
423 yield in acetic acid) coupled to a lower production in α-ketoglutaric acid (decrease by 60 % of yield in α-
424 ketoglutaric acid, orange diamond on **Figure 7**).
425 In addition, we also observed a lower yield in ethanol and lactic acid for *S. cerevisiae* monoculture
426 with high initial sugars, although very small for ethanol (decrease of 1.6 % for the ethanol yield and 25%
427 for the lactic acid yield). We did not observe differences for yield of pyruvic acid for both initial sugar
428 conditions in the *S. cerevisiae* monoculture. We next set out to compare the response to osmotic stress in
429 the consortia. The differences in metabolite yield between the two sugar conditions are shown in **Figure**
430 **7**. The different consortia exhibited a wide range of responses, especially regarding the production of
431 acetic and pyruvic acids. Contrary to *S. cerevisiae* monoculture, consortia tended to have lower pyruvic
432 acid yield in condition S280, but differences were significant only in consortia C07, C11 and C13.
433 Regarding acetic acid, its overproduction in condition S280 was greatly reduced in all consortia compared
434 to *S. cerevisiae* monoculture since the highest increase was of 31 % in consortium C02. In the consortium
435 inoculated with a higher proportion of *S. cerevisiae* (C12), 55 % more acetic acid was produced in
436 condition S280 than at S200. In addition, differences in yield of acetic acid in consortia were only
437 significant for consortia C02, C11 and C12. On the contrary, the yield in α-ketoglutaric acid remained
438 lower in condition S280 compared to S200. Differences ranged from 53% lower to 68% lower in consortia
439 C11 and C03 respectively. Only in consortium C02 was it not significantly different between both sugar
440 conditions. This could suggest a prevailing importance of *S. cerevisiae* concerning this metabolite since
441 the difference between both S280 and S200 was often close to that observed in *S. cerevisiae*
442 monocultures, as indicated by the orange diamonds (**Figure 7**). Regarding ethanol, consortia showed an
443 overall lower yield with S200 compared to *S. cerevisiae* monocultures (between 0.482 ± 0.001 in C05 and
444 0.494 ± 0.003 in C10. Surprisingly, even though glycerol is an essential osmolyte involved in osmotic
445 stress resistance, our results did not show significant differences in glycerol yields between both sugar
446 conditions (**Figure 7**). One possible explanation for this observation may be that the sugar difference
447 between both conditions was not enough to cause noticeable change in glycerol yield.

448 *Effect of initial composition on consortia behavior.*
449 Consortia C02 to C11 were designed to represent variable α diversity, through increasing evenness of
450 the initial relative abundance of non-*Saccharomyces* species (**Table 2**). Hence, the Shannon index of the
451 different consortia ranged from 0.895 in C02 to 1.449 in C11, except C07 that presents an equal
452 proportion of each NS species, and a 1,515 Shannon index. These 10 consortia also included the same
453 initial abundance of *S. cerevisiae* (5%), to avoid changes being related only to *S. cerevisiae*. We were
454 interested in knowing whether an increasing diversity in the yeast consortia would help maintain the
455 fermentation performance during the osmotic stress. However, we found no demonstrable effect of the
456 initial diversity on the different parameters related to fermentation performance, such as yield in CO_2,
457 yield in metabolites or residual sugars (**Supplementary Figure 5**). However, the initial composition still
458 influenced the behavior of the consortia, especially for the difference in acetic and α-ketoglutaric yields
459 between both sugar conditions (**Supplementary Figure 6**).

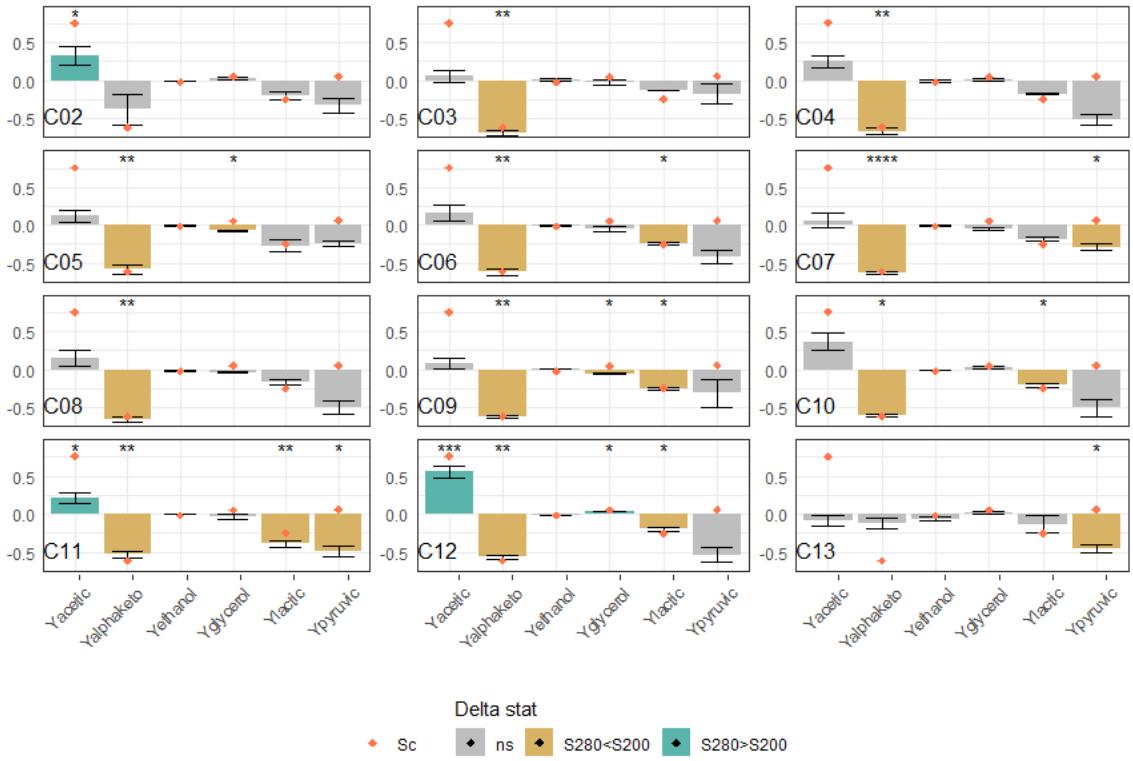


Figure 7: Metabolite yield changes in the different consortia (calculated by dividing the difference between measures for S280 and S200 by the measure for S200). Both sugar conditions were compared by T-test. Gray bars: p-value > 0.05; *, **, ***: p-value inferior to 0.05, 0.01, 0.0001 respectively (in blue when delta is positive, yellow when delta is negative). Orange diamonds indicate the values observed in the *S. cerevisiae* monoculture. C02 to C11 initially contained 5% of *S. cerevisiae*, C12 initially contained 15% of *S. cerevisiae* and C13 did not include *S. cerevisiae*.

Discussion

In the present study, we constructed a new model community of yeast species that can be numbered by flow cytometry thanks to the expression of different fluorescent protein. To validate the accuracy of this counting method, we employed mock communities. Then, we characterized the performances of the 6-species community by assessing various fermentation parameters. Finally, to further prove its usefulness, twelve consortia were tested in enological conditions with two differents sugar concentrations to evaluate the influence of varying initial eveness.

In the first part of this work, we tested whether the strategy of species quantification during fermentation was applicable and accurate. Five out of six species were successfully tagged with a set of four fluorescent proteins that can readily be discriminated with a flow cytometry equiped with the appropriate optical configuration. We then assembled 30 mock communities including *S. cerevisiae*, *H. uvarum*, *M. pulcherrima*, *S. bacillaris*, *L. thermotolerans*, and *T. delbrueckii* with abundance ranging from 1 to 90 %. In the range of concentrations tested here, our results indicate that the concentration of cells measured by cytometry highly correlates with the theroretical value). Hence, the quantification strategy could be validated on a consortium including 5 to 6 species. It should still be noted that the accuracy in numbering of *M. pulcherrima* decreased with longer handling time, which could lead to its understimation and should be taken into account when including this species.

During fermentation, species succession of the 6-species community described in this study reflected trends found in both natural communities and laboratory cocultures. For example, as observed in our data, several studies have reported a fast multiplication of *H. uvarum* in early fermentation, including

489 studies in cocultures and consortia (four species) grown in synthetic grape must (Lleixà et al., 2016; Harlé
490 et al., 2020), but also at industrial scale non-inoculated fermentations (Combina et al., 2005). However, in
491 industrial conditions, this increase of *H. uvarum* can be limited when treating must with SO_2, which is a
492 chemical commonly used as an antimicrobial and antioxidant agent, promoting *S. cerevisiae* (Grangeteau
493 et al., 2017). After 24 hours, *M. pulcherrima* and *S. bacillaris* were detected under 5 % in our consortium.
494 This early decline of *M. pulcherrima* in fermentation has been observed previously (Combina et al., 2005;
495 Chasseriaud et al., 2018), whereas several studies have found *S. bacillaris* able to multiply and maintain a
496 relatively high population in cocultures. For instance, in semi-industrial scale fermentations, *S. bacillaris*
497 could be found in mid fermentation. However, this was related to a later onset of *S. cerevisiae* (Ocón et
498 al., 2010). For other model consortia containing 8 species, tested in both synthetic and natural grape
499 must, *S. bacillaris* remained present up to the mid or end of fermentation (Bagheri et al., 2017).
500 Discrepancies on the presence of *S. bacillaris* during fermentation might be due to different media
501 composition, as was demonstrated with different initial sugar and nitrogen content (Lleixà et al., 2016).
502 Regarding *L. thermotolerans* and *T. delbrueckii*, both were detectable up to 72 hours in our
503 consortium despite their low inoculation ratio, showing a rather good persistence. In a four species
504 consortium, these two species were able to reach a sizable population despite the presence of *S.*
505 *cerevisiae.* However, their inoculation ratio was much higher (Conacher et al., 2020). Similarly, *L.*
506 *thermotolerans* was also found at the end of fermentation of Chenin Blanc grape must, in a consortium of
507 eight species (Bagheri et al., 2017). In natural communities, their maintenance is harder to evaluate since
508 they are present in only low abundance. However, they have been found mid-fermentations (Sternes et
509 al., 2017) and at the end of fermentation in some occasions (Díaz et al., 2013; Simonin et al., 2018).
510 As for *S. cerevisiae*, it was the main viable species after 24 hours, and likely caused the early drop in
511 viability observed between 12 and 24 hours. After 72 hours, it was the only viable species present in the
512 consortium. These results would be somewhat consistent with previous works on cocultures that found
513 that non-*Saccharomyces*, such as *L. thermotolerans*, *T. delbrueckii* or *M. pulcherrima*, populations tended
514 to decrease once *S. cerevisiae* reached a high cell number or a high cell number of live *S. cerevisiae* was
515 added (Nissen et al., 2003; Kapsopoulou et al., 2005; Comitini et al., 2011; Taillandier et al., 2014). This
516 phenomenon is also evidenced in sequential inoculation when non-*Saccharomyces* stop growing or even
517 die after the addition of *S. cerevisiae* (Benito et al., 2015; Binati et al., 2020; Seguinot et al., 2020).
518 In addition, our data presented species-related features, such as high glycerol and α-ketoglutaric acid
519 production for *M. pulcherrima* or *S. bacillaris* monocultures. These characteristics have previously been
520 described in monocultures in synthetic must (Englezos et al., 2018; Mbuyane et al., 2022) and in mixed
521 fermentaions (Comitini et al., 2011; Englezos et al., 2017; Binati et al., 2020; Seguinot et al., 2020).
522 Similarly, the great production of lactic acid observed here in *L. thermotolerans* monocultures is already
523 known, even though it varies greatly between strains and conditions (Benito et al., 2015; Vaquero et al.,
524 2020). Concerning the 6-species community, its metabolite production and fermentation kinetics seemed
525 mostly influenced by *S. cerevisiae* but was intermediate with non-*Saccharomyces*, which is commonly
526 observed in cocultures (Renault et al., 2013; Bagheri et al., 2017; Harlé et al., 2020). Our second
527 experiment also highlighted the influence of the different species on the fermentationa performance in
528 consortia leading to variation in the response to osmotic stress more or less influenced by the presence
529 of *S. cerevisiae*.
530 Taken together, our results confirm the applicability of the combined use of fluorescently tagged cells
531 and cytometry to follow several species individually in œnological fermentations. Indeed, this method
532 has been successfully applied in mixed fermentations of one non-*Saccharomyces* and *S. cerevisiae*
533 (Petitgonnet et al., 2019; Harlé et al., 2020), but rarely included more species (Conacher et al., 2020).
534 Other studies including more species in their consortium employ cultivation or molecular biology
535 methods to follow the microbial dynamics (Lleixà et al., 2016; Bagheri et al., 2017; Chasseriaud et al.,
536 2018). These methods allow enumeration in natural community without specific tagging of the different
537 species but involve heavier experiments and a longer time between sampling and results.
538
539 As a proof-of-concept, we employed the yeast community with only the 5 fluorescently tagged
540 species to investigate the effect of different initial eveness of consortia on their response to two sugar
541 concentrations (S200 and S280). Altogether, our results indicated a tendency of *T. delbrueckii* and *S.*
542 *cerevisiae* to be promoted in a complex community during a fermentation with high sugar content, while

H. uvarum and *L. thermotolerans* were negatively impacted. *T. delbrueckii* is described as relatively osmotolerant, to both salts and reducing sugars (Lages et al., 1999; Hernandez-Lopez et al., 2003), and has been found able to maintain a high population in natural grape musts with 250 to 360 g/L of sugars (Bely et al., 2008; Belda et al., 2015). Thus, the fact that it tended to be promoted at S280 in our study was to be expected. On the other hand, we found that *S. bacillaris* abundance decreased very quickly even in the S280 condition. This is somewhat surprising given this species is osmotolerant and could be expected to be promoted in high sugar must (Tofalo et al., 2012; Csoma et al., 2020). Its osmotolerant nature means it is often isolated in fermentations of high sugar must, such as icewine or botrytised wine that can usually contain at least 350 g/L of reducing sugar (Magyar & Soós, 2016). It can even dominate early and middle stages of such fermentations (Nisiotou et al., 2007; Magyar & Soós, 2016). Our inconsistent results might be due to the use of synthetic must with high nitrogen content that could limit the establishment of *S. bacillaris* in a community, in favor of faster growing species. Fewer data are available for *H. uvarum* or *L. thermotolerans* in conditions of high-sugar fermentation. In botrytised must, *H. uvarum* was shown to decrease more quickly than in healthy must, but other factors than the initial osmotic stress, such as presence of other species, may also explain these results since this observation stayed true even when there was no difference in initial sugar (Nisiotou et al., 2007; Contreras et al., 2015). Another interesting highlight from these data are the differences of population dynamics in the different consortia, which shows that initial abundance of the different species can influence their growth under different conditions. This could be explained by differences in response of the different species to the osmotic stress in the first hours, since different coping mechanisms seem to occur in *T. delbrueckii* and *S. cerevisiae* for example (Renault et al., 2009; Tondini et al., 2020).

In the osmotic stress condition, the most striking difference between consortia and *S. cerevisiae* monocultures was the overproduction acetic acid. *S. cerevisiae* overproduced acetic acid in the S280 condition, while the acetic acid overproduction was smaller in consortia and varied significantly between consortia. This may be partly due to the presence of non-*Saccharomyces* since several studies have reported a positive effect of mixed fermentation of *S. cerevisiae* with non-*Saccharomyces,* namely *S. bacillaris* and *M. pulcherrima,* in reducing the acetate yield (Rantsiou et al., 2012; Contreras et al., 2015; González-Royo et al., 2015; Mbuyane et al., 2018). Another explaination might also lie in the population changes between both sugar conditions. With S280, *H. uvarum* decreased more quickly while *T. delbrueckii* stayed longer. *T. delbrueckii* is known to produce low amount of acetic acid (Bely et al., 2008; Renault et al., 2009), while *H. uvarum* is associated with high production in acetic acid (Ciani & Maccarelli, 1998; Capece et al., 2005; Mendoza et al., 2019). Their difference in dynamics may therefore influence the overall response. However, the consortia and range of initial abundance tested here are too limited to allow us to properly disentangle the combined effect of each species on the production of metabolites in consortia in both sugar conditions.

Despite the effet of composition of the communities on microbial dynamics and metabolite production, we found no correlation to the initial eveness. The relatively low complexity of our environment may not be sufficient to generate a set of ecological sub-niches that are all the more covered as microbial diversity increases (Hooper et al., 2005; Shade et al., 2012). For example, Schmidt et al., (2020) observed differences in *S. cerevisiae* strains dynamics in natural must with 200 or 280 g/L of sugars, but no difference in synthetic must. In addition, the range of diversity tested in this study might be limited since it varied only based on initial abundance while the number of species (and therefore species richness) was constant. For example, Ruiz et al., (2023) showed a negative effect of an increasing richness in wine yeast communities. They observed that *S. cerevisiae* could not finish fermentation in some communities due to the higher probability of the presence of antagonistic species. Diversity effect has also been observed in microcosms, where fermentation performance was reduced in the highest and lowest diversity context, due to a lower establishment of *S. cerevisiae* (Boynton & Greig, 2016). Thus, further investigation on microbial diversity effect in winemaking might need an increased number of species. Model communities could also include bacterial species to follow both the alcooholic and malolactic fermentations.

Conclusion

In this study, we successfully developed a 6-species consortium of fluorescently tagged wine yeasts that allow accurate real time tracking of species subpopulations during fermentation. The consortium was also fully characterized in terms of population dynamics and phenotype, such as metabolite production and fermentation kinetics. The strains constructed in this work are available to the scientific community at the CIRM-levures. Such microbial consortium could be a valuable resource for applications in yeast ecology and support future research aimed at investigating the influence of stress factors relevant to the fermentation process, particularly when using multispecies starters. Factors such as oxygen availability, nitrogen levels, temperature and the use of SO_2 are critical parameters influence the presence of non-*Saccharomyces*, often with species-specific effects. Consequently, the trends observed within the 6- and 5-species communities and the our methodology could be applied and tested under a wider spectrum of environmental conditions. Ultimately, the insights gained from studying population dynamics might provide strategies to mitigate fermentations by managing the yeast population.

Acknowledgements

We are thankful to the CIRM Levures and C. Neuvéglise for providing the yeast strains, F. Macna for carrying out the HPLC analysis, D. Segond for his support with cytometry analysis.

Funding

This work was funded by a French doctoral scholarship provided by the University of Montpellier (GAIA doctoral school).

Conflict of interest disclosure

The authors declare that they comply with the PCI rule of having no financial conflicts of interest in relation to the content of the article. Thibault Nidelet is a recommender for PCI Microbiology.

Data, scripts, code, and supplementary information availability

All data, scripts and supplementary information are available online: XXXXDOI of the webpage hosting the data https://doi.org/10.5281/zenodo.11120746 ; Pourcelot et al., 2024.

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