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

Eléonore Pourcelot[1], Audrey Vigna[1], Thérèse Marlin[1], Virginie Galeote[1], Thibault Nidelet*[1]

[1] SPO, Univ Montpellier, INRAE, Institut Agro – Montpellier, France

*Corresponding author
Correspondence: thibault.nidelet@inrae.

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 *Rhodothorula* 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* DH5α 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	MTF4798		*TDH3-mCherry-kanMX*	This study
S. cerevisiae	CLIB3930	Sc	*TDH3-mCherry-kanMX ENO2-GFP-natMX4*	This study
H. uvarum	CLIB3118			CIRM-Levures
H. uvarum	CLIB3929	Hu	*TDH3-BFP2-kanMX*	This study
L. thermotolerans	MTF5257			SPO lab
L. thermotolerans	CLIB3932	Lt	*KLTH0G15730-mCherry-natMX4*	This study
M. pulcherrima	CLIB3329	Mp		CIRM-Levures
S. bacillaris	CLIB3147			CIRM-Levures
S. bacillaris	CLIB3933	Sb	*TDH3-eGFP-hphMX6*	This study
T. delbrueckii	CLIB3152			CIRM-Levures
T. delbrueckii	CLIB3931	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) $$\Delta_{S280,S200} = \frac{Y_{S280} - Y_{S200}}{Y_{S200}}$$

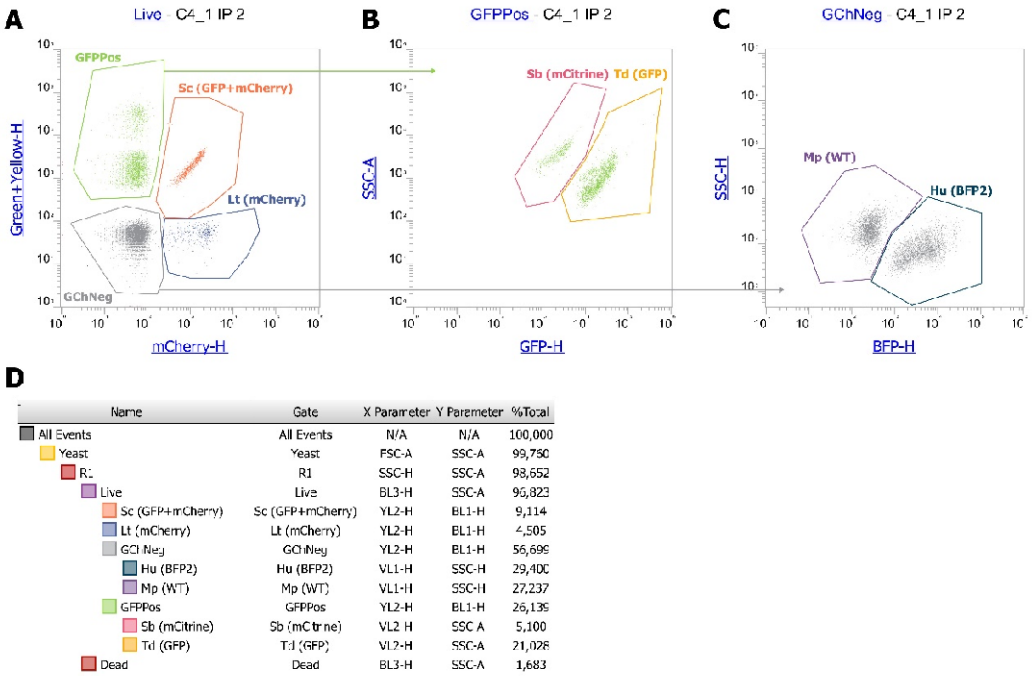
Data were analyzed with R studio software (version 4.3.1, Core Team, 2023). Mock communities' data were analyzed with the 'ggpmisc' package (Aphalo, 2023). ANOVA were performed to compare the different monocultures and 6-species consortium parameters, followed by a post hoc Tukey test when significant 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 ($R^2 \geq 0.90$).

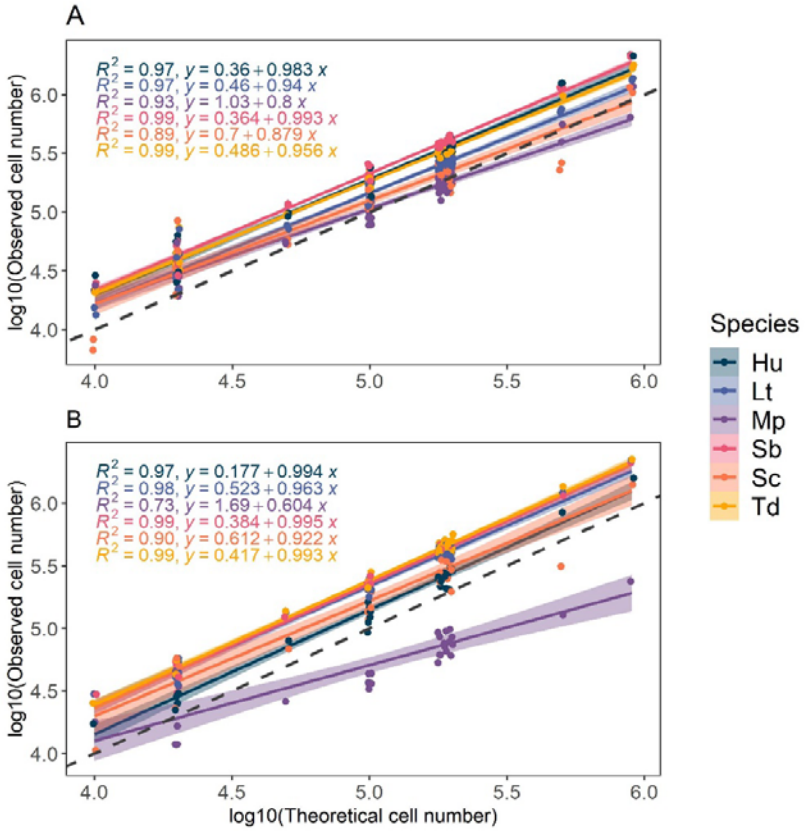


Figure 2: Comparison of observed population and theoretical population in 30 mock communities for immediate measure (**A**) and after 2 hours in PBS (**B**). Correlation factor and linear equation of observed population in function of theoretical populations are indicated for each species. Dashed line: 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**).

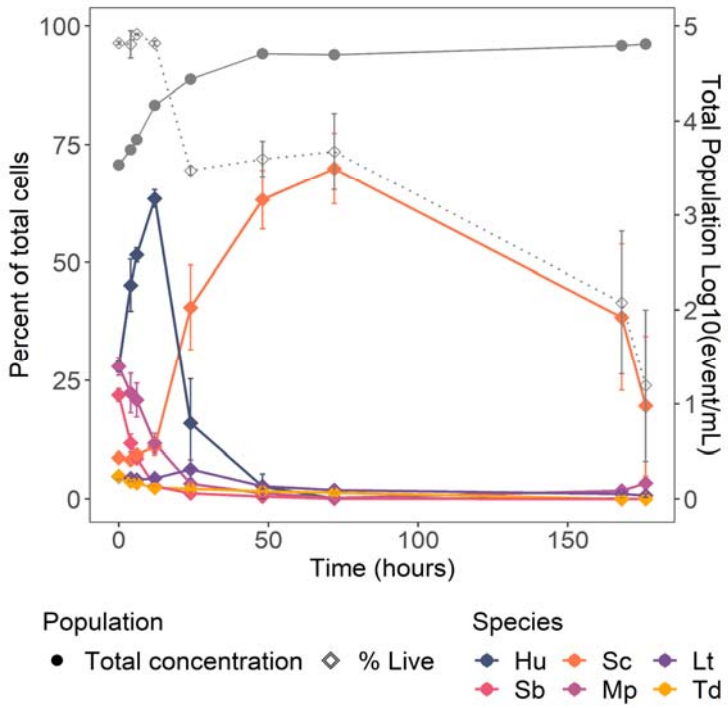


Figure 3: Individual growth of the different species in the 6-species consortium. The grey solid line corresponds to the cell concentration log10(cells/mL), the grey dotted line to the percent of live cell. Solid color lines describe the percent of the different species. Initial abundances were: 10 % *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**).

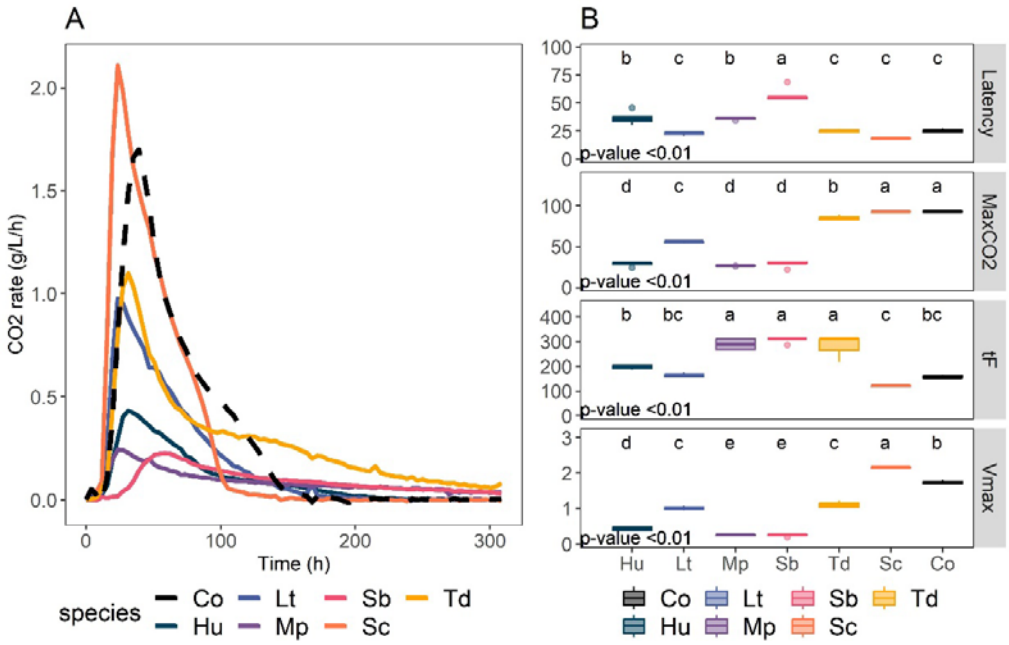


Figure 4: Fermentation kinetics of monocultures and consortium Co. **A**: CO_2 production rate kinetics of each monoculture (solid line) and consortium Co (dashed line). Smoothing with the Loess method was applied on the results of triplicates. **B**: Kinetics parameters obtained from CO_2 loss. MaxCO2 = 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**).

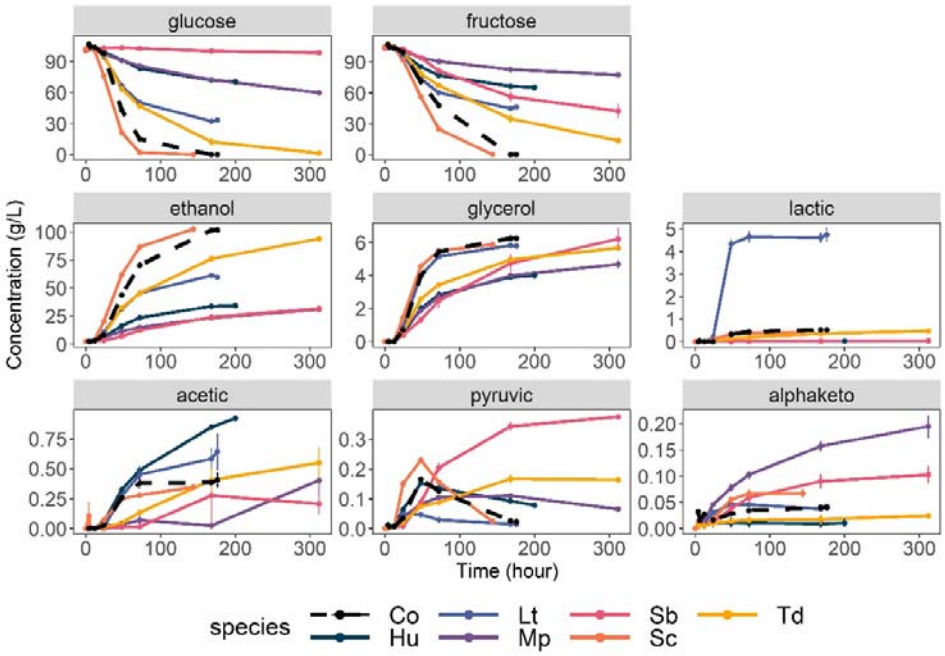


Figure 5: Kinetics of consumption and production of central carbon metabolites in monocultures and consortium. Error bars indicate the standard error. Co: Consortium, Hu: *H. uvarum*, Lt: *L. thermotolerans*, Mp: *M. pulcherrima*, Sb: *S. bacillaris*, Sc: *S. cerevisiae*, Td: *T. delbrueckii*.

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.

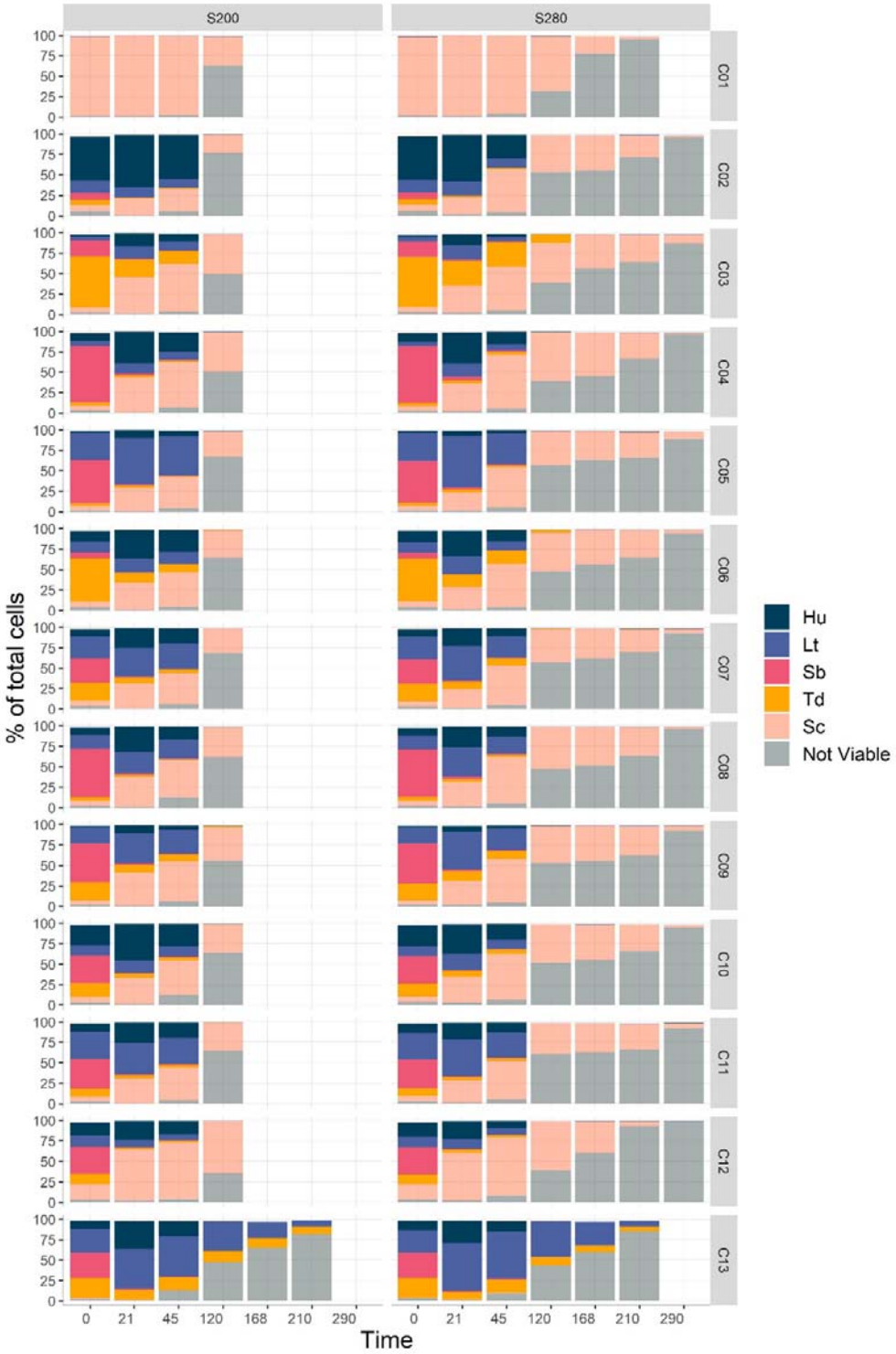


Figure 6: Population dynamics in consortia during fermentation with 200 and 280 g/L of sugars. C01 is a monoculture of *S. cerevisiae*. C02 to C11 initially contained 5% of *S. cerevisiae*. C12 initially contained 15% of *S. cerevisiae* and C13 did not contain *S. cerevisiae*. Hu: *H. uvarum*, Lt: *L.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

Fermentation performance was also assessed, through metabolite and CO_2 yields. The differences in yield of the main metabolite from carbon metabolism between S280 and S200 are shown monocultures in **Figure 7**. Positive values indicate higher yield in condition S280, while negative values indicate lower yield in condition S280.

Higher initial sugar concentrations led *S. cerevisiae* to produce more acetic acid (increase by 75 % of yield in acetic acid) coupled to a lower production in α-ketoglutaric acid (decrease by 60 % of yield in α-ketoglutaric acid, orange diamond on **Figure 7**).

In addition, we also observed a lower yield in ethanol and lactic acid for *S. cerevisiae* monoculture with high initial sugars, although very small for ethanol (decrease of 1.6 % for the ethanol yield and 25% for the lactic acid yield). We did not observe differences for yield of pyruvic acid for both initial sugar conditions in the *S. cerevisiae* monoculture. We next set out to compare the response to osmotic stress in the consortia. The differences in metabolite yield between the two sugar conditions are shown in **Figure 7**. The different consortia exhibited a wide range of responses, especially regarding the production of acetic and pyruvic acids. Contrary to *S. cerevisiae* monoculture, consortia tended to have lower pyruvic acid yield in condition S280, but differences were significant only in consortia C07, C11 and C13. Regarding acetic acid, its overproduction in condition S280 was greatly reduced in all consortia compared to *S. cerevisiae* monoculture since the highest increase was of 31 % in consortium C02. In the consortium inoculated with a higher proportion of *S. cerevisiae* (C12), 55 % more acetic acid was produced in condition S280 than at S200. In addition, differences in yield of acetic acid in consortia were only significant for consortia C02, C11 and C12. On the contrary, the yield in α-ketoglutaric acid remained lower in condition S280 compared to S200. Differences ranged from 53% lower to 68% lower in consortia C11 and C03 respectively. Only in consortium C02 was it not significantly different between both sugar conditions. This could suggest a prevailing importance of *S. cerevisiae* concerning this metabolite since the difference between both S280 and S200 was often close to that observed in *S. cerevisiae* monocultures, as indicated by the orange diamonds (**Figure 7**). Regarding ethanol, consortia showed an overall lower yield with S200 compared to *S. cerevisiae* monocultures (between 0.482 ± 0.001 in C05 and 0.494 ± 0.003 in C10. Surprisingly, even though glycerol is an essential osmolyte involved in osmotic stress resistance, our results did not show significant differences in glycerol yields between both sugar conditions (**Figure 7**). One possible explanation for this observation may be that the sugar difference between both conditions was not enough to cause noticeable change in glycerol yield.

Effect of initial composition on consortia behavior.

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

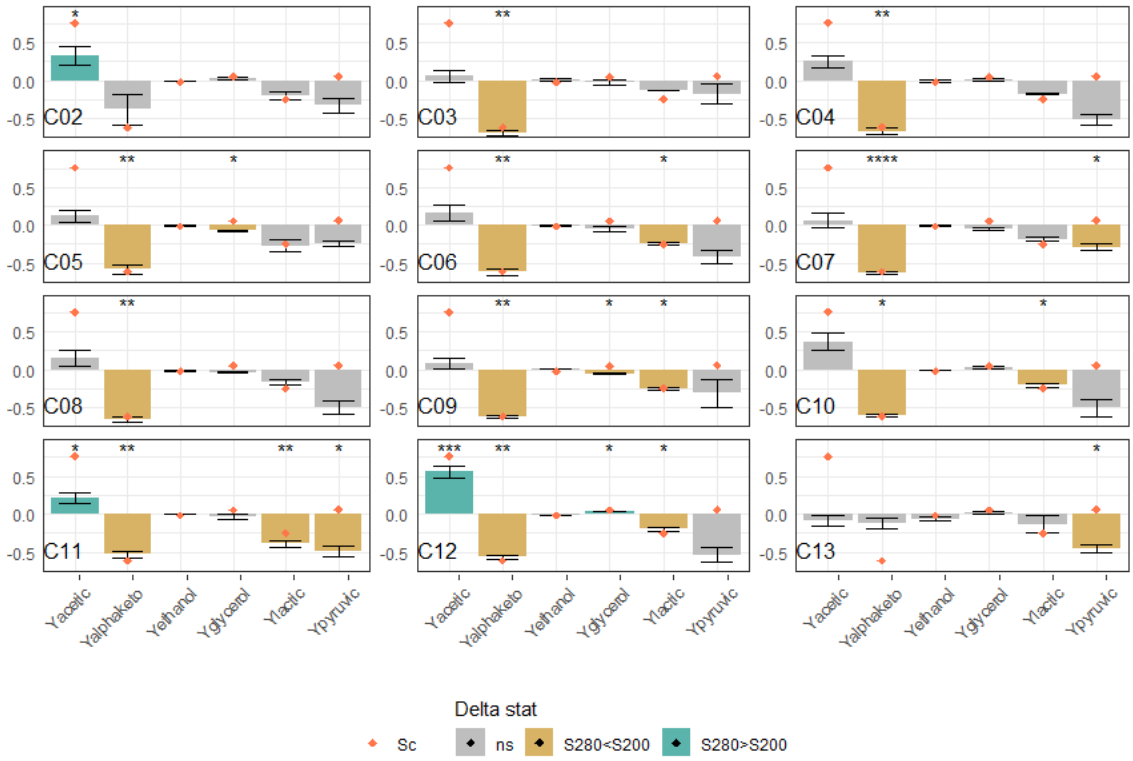


Figure 7: Metabolite yield changes in the different consortia (calculated by dividing the difference between measures for S280 and S200 by the measure for S200). Both sugar conditions were compared by T-test. Gray bars: p-value > 0.05; *, **, ***: p-value inferior to 0.05, 0.01, 0.0001 respectively (in 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

studies in cocultures and consortia (four species) grown in synthetic grape must (Lleixà et al., 2016; Harlé et al., 2020), but also at industrial scale non-inoculated fermentations (Combina et al., 2005). However, in industrial conditions, this increase of *H. uvarum* can be limited when treating must with SO_2, which is a chemical commonly used as an antimicrobial and antioxidant agent, promoting *S. cerevisiae* (Grangeteau et al., 2017). After 24 hours, *M. pulcherrima* and *S. bacillaris* were detected under 5 % in our consortium. This early decline of *M. pulcherrima* in fermentation has been observed previously (Combina et al., 2005; Chasseriaud et al., 2018), whereas several studies have found *S. bacillaris* able to multiply and maintain a relatively high population in cocultures. For instance, in semi-industrial scale fermentations, *S. bacillaris* could be found in mid fermentation. However, this was related to a later onset of *S. cerevisiae* (Ocón et al., 2010). For other model consortia containing 8 species, tested in both synthetic and natural grape must, *S. bacillaris* remained present up to the mid or end of fermentation (Bagheri et al., 2017). Discrepancies on the presence of *S. bacillaris* during fermentation might be due to different media composition, as was demonstrated with different initial sugar and nitrogen content (Lleixà et al., 2016).

Regarding *L. thermotolerans* and *T. delbrueckii*, both were detectable up to 72 hours in our consortium despite their low inoculation ratio, showing a rather good persistence. In a four species consortium, these two species were able to reach a sizable population despite the presence of *S. cerevisiae.* However, their inoculation ratio was much higher (Conacher et al., 2020). Similarly, *L. thermotolerans* was also found at the end of fermentation of Chenin Blanc grape must, in a consortium of eight species (Bagheri et al., 2017). In natural communities, their maintenance is harder to evaluate since they are present in only low abundance. However, they have been found mid-fermentations (Sternes et al., 2017) and at the end of fermentation in some occasions (Díaz et al., 2013; Simonin et al., 2018).

As for *S. cerevisiae*, it was the main viable species after 24 hours, and likely caused the early drop in viability observed between 12 and 24 hours. After 72 hours, it was the only viable species present in the consortium. These results would be somewhat consistent with previous works on cocultures that found that non-*Saccharomyces*, such as *L. thermotolerans*, *T. delbrueckii* or *M. pulcherrima*, populations tended to decrease once *S. cerevisiae* reached a high cell number or a high cell number of live *S. cerevisiae* was added (Nissen et al., 2003; Kapsopoulou et al., 2005; Comitini et al., 2011; Taillandier et al., 2014). This phenomenon is also evidenced in sequential inoculation when non-*Saccharomyces* stop growing or even die after the addition of *S. cerevisiae* (Benito et al., 2015; Binati et al., 2020; Seguinot et al., 2020).

In addition, our data presented species-related features, such as high glycerol and α-ketoglutaric acid production for *M. pulcherrima* or *S. bacillaris* monocultures. These characteristics have previously been described in monocultures in synthetic must (Englezos et al., 2018; Mbuyane et al., 2022) and in mixed fermentaions (Comitini et al., 2011; Englezos et al., 2017; Binati et al., 2020; Seguinot et al., 2020). Similarly, the great production of lactic acid observed here in *L. thermotolerans* monocultures is already known, even though it varies greatly between strains and conditions (Benito et al., 2015; Vaquero et al., 2020). Concerning the 6-species community, its metabolite production and fermentation kinetics seemed mostly influenced by *S. cerevisiae* but was intermediate with non-*Saccharomyces*, which is commonly observed in cocultures (Renault et al., 2013; Bagheri et al., 2017; Harlé et al., 2020). Our second experiment also highlighted the influence of the different species on the fermentationa performance in consortia leading to variation in the response to osmotic stress more or less influenced by the presence of *S. cerevisiae*.

Taken together, our results confirm the applicability of the combined use of fluorescently tagged cells and cytometry to follow several species individually in œnological fermentations. Indeed, this method has been successfully applied in mixed fermentations of one non-*Saccharomyces* and *S. cerevisiae* (Petitgonnet et al., 2019; Harlé et al., 2020), but rarely included more species (Conacher et al., 2020). Other studies including more species in their consortium employ cultivation or molecular biology methods to follow the microbial dynamics (Lleixà et al., 2016; Bagheri et al., 2017; Chasseriaud et al., 2018). These methods allow enumeration in natural community without specific tagging of the different species but involve heavier experiments and a longer time between sampling and results.

As a proof-of-concept, we employed the yeast community with only the 5 fluorescently tagged species to investigate the effect of different initial eveness of consortia on their response to two sugar concentrations (S200 and S280). Altogether, our results indicated a tendency of *T. delbrueckii* and *S. 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. Neuveglise 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.

References

Aphalo PJ (2023) *ggpmisc: Miscellaneous Extensions to "ggplot2."*

Bagheri B, Bauer FF, Setati ME (2017) The Impact of *Saccharomyces cerevisiae* on a Wine Yeast Consortium in Natural and Inoculated Fermentations. *Frontiers in microbiology*, **8**, 1988. https://doi.org/10.3389/fmicb.2017.01988

Bagheri B, Zambelli P, Vigentini I, Bauer FF, Setati ME (2018) Investigating the Effect of Selected Non-*Saccharomyces* Species on Wine Ecosystem Function and Major Volatiles. *Frontiers in bioengineering and biotechnology*, **6**, 169. https://doi.org/10.3389/fbioe.2018.00169

Barata A, Malfeito-Ferreira M, Loureiro V (2012) The microbial ecology of wine grape berries. *International Journal of Food Microbiology*, **153**, 243–59. https://doi.org/10.1016/j.ijfoodmicro.2011.11.025

Bardi L, Cocito C, Marzona M (1999) *Saccharomyces cerevisiae* cell fatty acid composition and release during fermentation without aeration and in absence of exogenous lipids. *International Journal of Food Microbiology*, **47**, 133–140. https://doi.org/10.1016/S0168-1605(98)00203-7

Bauer FF, Pretorius IS (2000) Yeast Stress Response and Fermentation Efficiency: How to Survive the Making of Wine - A Review. *South African Journal of Enology & Viticulture*, **21**. https://doi.org/10.21548/21-1-3557

Belda I, Navascués E, Marquina D, Santos A, Calderon F, Benito S (2015) Dynamic analysis of physiological properties of *Torulaspora delbrueckii* in wine fermentations and its incidence on wine quality. *Applied Microbiology and Biotechnology*, **99**, 1911–1922. https://doi.org/10.1007/s00253-014-6197-2

Bely M, Sablayrolles J-M, Barre P (1990) Automatic detection of assimilable nitrogen deficiencies during alcoholic fermentation in oenological conditions. *Journal of*

Fermentation and Bioengineering, **70**, 246–252. https://doi.org/10.1016/0922-338X(90)90057-4

Bely M, Stoeckle P, Masneuf-Pomarède I, Dubourdieu D (2008) Impact of mixed *Torulaspora delbrueckii–Saccharomyces cerevisiae* culture on high-sugar fermentation. *International Journal of Food Microbiology*, **122**, 312–320. https://doi.org/10.1016/j.ijfoodmicro.2007.12.023

Benito S, Hofmann T, Laier M, Lochbühler B, Schüttler A, Ebert K, Fritsch S, Röcker J, Rauhut D (2015) Effect on quality and composition of Riesling wines fermented by sequential inoculation with non-*Saccharomyces* and *Saccharomyces cerevisiae*. *European Food Research and Technology*, **241**, 707–717. https://doi.org/10.1007/s00217-015-2497-8

Binati RL, Lemos Junior WJF, Luzzini G, Slaghenaufi D, Ugliano M, Torriani S (2020) Contribution of non-*Saccharomyces* yeasts to wine volatile and sensory diversity: A study on *Lachancea thermotolerans, Metschnikowia* spp. and *Starmerella bacillaris* strains isolated in Italy. *International Journal of Food Microbiology*, **318**, 108470. https://doi.org/10.1016/j.ijfoodmicro.2019.108470

Blasche S, Kim Y, Oliveira AP, Patil KR (2017) Model microbial communities for ecosystems biology. *Current Opinion in Systems Biology*, **6**, 51–57. https://doi.org/10.1016/j.coisb.2017.09.002

Bokulich NA, Thorngate JH, Richardson PM, Mills DA (2014) Microbial biogeography of wine grapes is conditioned by cultivar, vintage, and climate. *Proceedings of the National Academy of Sciences of the United States of America*, **111**, 139–48. https://doi.org/10.1073/pnas.1317377110

Bordet F, Joran A, Klein G, Roullier-Gall C, Alexandre H (2020) Yeast-Yeast Interactions: Mechanisms, Methodologies and Impact on Composition. *Microorganisms*, **8**. https://doi.org/10.3390/microorganisms8040600

Boynton PJ, Greig D (2016) Species richness influences wine ecosystem function through a dominant species. *Fungal Ecology*, **22**, 61–72. https://doi.org/10.1016/j.funeco.2016.04.008

Breslow DK, Cameron DM, Collins SR, Schuldiner M, Stewart-Ornstein J, Newman HW, Braun S, Madhani HD, Krogan NJ, Weissman JS (2008) A comprehensive strategy enabling high-resolution functional analysis of the yeast genome. *Nature Methods*, **5**, 711–718. https://doi.org/10.1038/nmeth.1234

Brückner S, Schubert R, Kraushaar T, Hartmann R, Hoffmann D, Jelli E, Drescher K, Müller DJ, Oliver Essen L, Mösch H-U (2020) Kin discrimination in social yeast is mediated by cell surface receptors of the Flo11 adhesin family. *eLife*, **9**. https://doi.org/10.7554/eLife.55587

Capece A, Fiore C, Maraz A, Romano P (2005) Molecular and technological approaches to evaluate strain biodiversity in *Hanseniaspora uvarum* of wine origin. *Journal of Applied Microbiology*, **98**, 136–144. https://doi.org/10.1111/j.1365-2672.2004.02434.x

Chasseriaud L, Coulon J, Marullo P, Albertin W, Bely M (2018) New oenological practice to promote non-*Saccharomyces* species of interest: saturating grape juice with carbon dioxide. *Applied Microbiology and Biotechnology*, **102**, 3779–3791. https://doi.org/10.1007/s00253-018-8861-4

Ciani M, Comitini F (2015) Yeast interactions in multi-starter wine fermentation. *Current Opinion in Food Science*, **1**, 1–6. https://doi.org/10.1016/j.cofs.2014.07.001

Ciani M, Maccarelli F (1998) Oenological properties of non-*Saccharomyces* yeasts associated with wine-making. *World Journal of Microbiology and Biotechnology* , **14**, 199-203. https://doi.org/10.1023/A:1008825928354

Combina M, Elía A, Mercado L, Catania C, Ganga A, Martinez C (2005) Dynamics of indigenous yeast populations during spontaneous fermentation of wines from Mendoza,

Argentina. *International Journal of Food Microbiology*, **99**, 237–243. https://doi.org/10.1016/j.ijfoodmicro.2004.08.017

Comitini F, Gobbi M, Domizio P, Romani C, Lencioni L, Mannazzu I, Ciani M (2011) Selected non-*Saccharomyces* wine yeasts in controlled multistarter fermentations with Saccharomyces cerevisiae. *Food Microbiology*, **28**, 873–882. https://doi.org/10.1016/j.fm.2010.12.001

Conacher CG, Naidoo-Blassoples RK, Rossouw D, Bauer FF (2020) Real-time monitoring of population dynamics and physical interactions in a synthetic yeast ecosystem by use of multicolour flow cytometry. *Applied microbiology and biotechnology*, **104**, 5547–5562. https://doi.org/10.1007/s00253-020-10607-x

Conacher CG, Naidoo-Blassoples RK, Rossouw D, Bauer FF (2022) A Transcriptomic Analysis of Higher-Order Ecological Interactions in a Eukaryotic Model Microbial Ecosystem (SG Tringe, Ed,). *mSphere*, **7**, e00436-22. https://doi.org/10.1128/msphere.00436-22

Contreras A, Curtin C, Varela C (2015) Yeast population dynamics reveal a potential 'collaboration' between *Metschnikowia pulcherrima* and *Saccharomyces uvarum* for the production of reduced alcohol wines during Shiraz fermentation. *Applied Microbiology and Biotechnology*, **99**, 1885–1895. https://doi.org/10.1007/s00253-014-6193-6

Csoma H, Kállai Z, Antunovics Z, Czentye K, Sipiczki M (2020) Vinification without *Saccharomyces*: Interacting Osmotolerant and "Spoilage" Yeast Communities in Fermenting and Ageing Botrytised High-Sugar Wines (Tokaj Essence). *Microorganisms*, **9**, 19. https://doi.org/10.3390/microorganisms9010019

Deroite A, Legras J-L, Rigou P, Ortiz-Julien A, Dequin S (2018) Lipids modulate acetic acid and thiol final concentrations in wine during fermentation by *Saccharomyces cerevisiae* × *Saccharomyces kudriavzevii* hybrids. *AMB Express*, **8**, 130. https://doi.org/10.1186/s13568-018-0657-5

Di Maro E, Ercolini D, Coppola S (2007) Yeast dynamics during spontaneous wine fermentation of the Catalanesca grape. *International Journal of Food Microbiology*, **117**, 201–210. https://doi.org/10.1016/j.ijfoodmicro.2007.04.007

Díaz C, Molina AM, Nähring J, Fischer R (2013) Characterization and dynamic behavior of wild yeast during spontaneous wine fermentation in steel tanks and amphorae. *BioMed research international*, **2013**, 540465. https://doi.org/10.1155/2013/540465

Drumonde-Neves J, Fernandes T, Lima T, Pais C, Franco-Duarte R (2021) Learning from 80 years of studies: a comprehensive catalogue of non-*Saccharomyces* yeasts associated with viticulture and winemaking. *FEMS yeast research*, **21**, foab017. https://doi.org/10.1093/femsyr/foab017

Englezos V, Cocolin L, Rantsiou K, Ortiz-Julien A, Bloem A, Dequin S, Camarasa C (2018) Specific Phenotypic Traits of *Starmerella bacillaris* Related to Nitrogen Source Consumption and Central Carbon Metabolite Production during Wine Fermentation. *Applied and environmental microbiology*, **84**. https://doi.org/10.1128/AEM.00797-18

Englezos V, Giacosa S, Rantsiou K, Rolle L, Cocolin L (2017) *Starmerella bacillaris* in winemaking: opportunities and risks. *Current Opinion in Food Science*, **17**, 30–35. https://doi.org/10.1016/j.cofs.2017.08.007

Furukawa S, Watanabe T, Toyama H, Morinaga Y (2013) Significance of microbial symbiotic coexistence in traditional fermentation. *Journal of bioscience and bioengineering*, **116**, 533–9. https://doi.org/10.1016/j.jbiosc.2013.05.017

Ghosh S, Bagheri B, Morgan HH, Divol B, Setati ME (2015) Assessment of wine microbial diversity using ARISA and cultivation-based methods. *Annals of Microbiology*, **65**, 1833–1840. https://doi.org/10.1007/s13213-014-1021-x

González-Alonso I, Walker ME, Vallejo-Pascual M-E, Naharro-Carrasco G, Jiranek V (2021) Capturing yeast associated with grapes and spontaneous fermentations of the Negro Saurí minority variety from an experimental vineyard near León. *Scientific reports*, **11**, 3748. https://doi.org/10.1038/s41598-021-83123-1

González-Royo E, Pascual O, Kontoudakis N, Esteruelas M, Esteve-Zarzoso B, Mas A, Canals JM, Zamora F (2015) Oenological consequences of sequential inoculation with non-*Saccharomyces* yeasts (*Torulaspora delbrueckii* or *Metschnikowia pulcherrima*) and *Saccharomyces cerevisiae* in base wine for sparkling wine production. *European Food Research and Technology*, **240**, 999–1012. https://doi.org/10.1007/s00217-014-2404-8

Gordon ZB, Soltysiak MPM, Leichthammer C, Therrien JA, Meaney RS, Lauzon C, Adams M, Lee DK, Janakirama P, Lachance M-A, Karas BJ (2019) Development of a Transformation Method for *Metschnikowia borealis* and other CUG-Serine Yeasts. *Genes*, **10**. https://doi.org/10.3390/genes10020078

Grangeteau C, Roullier-Gall C, Rousseaux S, Gougeon RD, Schmitt-Kopplin P, Alexandre H, Guilloux-Benatier M (2017) Wine microbiology is driven by vineyard and winery anthropogenic factors. *Microbial biotechnology*, **10**, 354–370. https://doi.org/10.1111/1751-7915.12428

Hansen MC, Palmer RJ, Udsen C, White DC, Molin S (2001) Assessment of GFP fluorescence in cells of *Streptococcus gordonii* under conditions of low pH and low oxygen concentration. *Microbiology (Reading, England)*, **147**, 1383–1391. https://doi.org/10.1099/00221287-147-5-1383

Harlé O, Legrand J, Tesnière C, Pradal M, Mouret J-R, Nidelet T (2020) Investigations of the mechanisms of interactions between four non-conventional species with *Saccharomyces cerevisiae* in oenological conditions (Y Ohya, Ed,). *PLOS ONE*, **15**, e0233285. https://doi.org/10.1371/journal.pone.0233285

Heard GM, Fleet GH (1988) The effects of temperature and pH on the growth of yeast species during the fermentation of grape juice. *Journal of Applied Bacteriology*, **65**, 23–28. https://doi.org/10.1111/j.1365-2672.1988.tb04312.x

Hernandez-Lopez MJ, Prieto JA, Randez-Gil F (2003) Osmotolerance and leavening ability in sweet and frozen sweet dough. Comparative analysis between *Torulaspora delbrueckii* and *Saccharomyces cerevisiae* baker's yeast strains. *Antonie van Leeuwenhoek*, **84**, 125–134. https://doi.org/10.1023/a:1025413520192

Hierro N, González Á, Mas A, Guillamón JM (2006) Diversity and evolution of non-*Saccharomyces* yeast populations during wine fermentation: effect of grape ripeness and cold maceration. *FEMS Yeast Research*, **6**, 102–111. https://doi.org/10.1111/j.1567-1364.2005.00014.x

Holm Hansen E, Nissen P, Sommer P, Nielsen JC, Arneborg N (2001) The effect of oxygen on the survival of non-*Saccharomyces* yeasts during mixed culture fermentations of grape juice with *Saccharomyces cerevisiae*. *Journal of Applied Microbiology*, **91**, 541–547. https://doi.org/10.1046/j.1365-2672.2001.01426.x

Hooper D, Chapin F, Ewel J, Hector A, Inchausti P, Lavorel S, Lawton J, Lodge D, Loreau M, Naeem S, Schmid B, Setälä H, Symstad A, Vandermeer J, Wardle D (2005) effects of biodiversity on ecosystem functioning: a consensus of current knowledge. *Ecol Monogr*, **75**, 3–35. https://doi.org/10.1890/04-0922

Ivey M, Massel M, Phister TG (2013) Microbial interactions in food fermentations. *Annual review of food science and technology*, **4**, 141–62. https://doi.org/10.1146/annurev-food-022811-101219

Kapsopoulou K, Kapaklis A, Spyropoulos H (2005) Growth and Fermentation Characteristics of a Strain of the Wine Yeast *Kluyveromyces thermotolerans* Isolated in Greece. *World Journal of Microbiology and Biotechnology*, **21**, 1599–1602. https://doi.org/10.1007/s11274-005-8220-3

Kassambara A (2023) *rstatix: Pipe-Friendly Framework for Basic Statistical Tests*.

Kemsawasd V, Viana T, Ardö Y, Arneborg N (2015) Influence of nitrogen sources on growth and fermentation performance of different wine yeast species during alcoholic fermentation. *Applied Microbiology and Biotechnology*, **99**, 10191–10207. https://doi.org/10.1007/s00253-015-6835-3

Kylilis N, Tuza ZA, Stan G-B, Polizzi KM (2018) Tools for engineering coordinated system behaviour in synthetic microbial consortia. *Nature Communications*, **9**, 2677. https://doi.org/10.1038/s41467-018-05046-2

Lages F, Silva-Graça M, Lucas C (1999) Active glycerol uptake is a mechanism underlying halotolerance in yeasts: a study of 42 species. *Microbiology*, **145**, 2577–2585. https://doi.org/10.1099/00221287-145-9-2577

Lambrechts MG, Pretorius IS (2000) Yeast and its importance to wine aroma-a review. *South african journal of enology and viticulture*, **21**, 97–129. https://doi.org/10.21548/21-1-3560

Lee S, Lim WA, Thorn KS (2013) Improved blue, green, and red fluorescent protein tagging vectors for S. cerevisiae. *PLOS ONE*, **8**, 67902. https://doi.org/10.1371/journal.pone.0067902

Lleixà J, Manzano M, Mas A, Portillo MDC (2016) *Saccharomyces* and non-*Saccharomyces* Competition during Microvinification under Different Sugar and Nitrogen Conditions. *Frontiers in Microbiology*, **7**. https://doi.org/10.3389/fmicb.2016.01959

Longin C, Petitgonnet C, Guilloux-Benatier M, Rousseaux S, Alexandre H (2017) Application of flow cytometry to wine microorganisms. *Food Microbiology*, **62**, 221–231. https://doi.org/10.1016/j.fm.2016.10.023

Magyar I, Soós J (2016) Botrytized wines – current perspectives. *International Journal of Wine Research*, **Volume 8**, 29–39. https://doi.org/10.2147/IJWR.S100653

Marcus A, Raulet DH (2013) A simple and effective method for differentiating GFP and YFP by flow cytometry using the violet laser. *Cytometry Part A*, **83**, 973–974. https://doi.org/10.1002/cyto.a.22347

Mateus D, Sousa S, Coimbra C, S Rogerson F, Simões J (2020) Identification and Characterization of Non-Saccharomyces Species Isolated from Port Wine Spontaneous Fermentations. *Foods*, **9**. https://doi.org/10.3390/foods9020120

Maturano YP, Mestre MV, Esteve-Zarzoso B, Nally MC, Lerena MC, Toro ME, Vazquez F, Combina M (2015) Yeast population dynamics during prefermentative cold soak of Cabernet Sauvignon and Malbec wines. *International Journal of Food Microbiology*, **199**, 23–32. https://doi.org/10.1016/j.ijfoodmicro.2015.01.005

Mbuyane LL, Bauer FF, Bloem A, Camarasa C, Ortiz-Julien A, Divol B (2022) Species-Dependent Metabolic Response to Lipid Mixtures in Wine Yeasts. *Frontiers in microbiology*, **13**, 823581. https://doi.org/10.3389/fmicb.2022.823581

Mbuyane LL, De Kock M, Bauer FF, Divol B (2018) *Torulaspora delbrueckii* produces high levels of C5 and C6 polyols during wine fermentations. *FEMS Yeast Research*, **18**. https://doi.org/10.1093/femsyr/foy084

Medina K, Boido E, Dellacassa E, Carrau F (2012) Growth of non-*Saccharomyces* yeasts affects nutrient availability for *Saccharomyces cerevisiae* during wine fermentation. *International Journal of Food Microbiology*, **157**, 245–50. https://doi.org/10.1016/j.ijfoodmicro.2012.05.012

Mencher A, Morales P, Curiel JA, Gonzalez R, Tronchoni J (2021) *Metschnikowia pulcherrima* represses aerobic respiration in *Saccharomyces cerevisiae* suggesting a direct response to co-cultivation. *Food microbiology*, **94**, 103670. https://doi.org/10.1016/j.fm.2020.103670

Mendoza LM, Vega-Lopez GA, Fernández de Ullivarri M, Raya RR (2019) Population and oenological characteristics of non-*Saccharomyces* yeasts associated with grapes of Northwestern Argentina. *Archives of Microbiology*, **201**, 235–244. https://doi.org/10.1007/s00203-018-1601-4

Moenne MI, Saa P, Laurie VF, Pérez-Correa JR, Agosin E (2014) Oxygen Incorporation and Dissolution During Industrial-Scale Red Wine Fermentations. *Food and Bioprocess Technology*, **7**, 2627–2636. https://doi.org/10.1007/s11947-014-1257-2

Moreno-Beltrán M, Gore-Lloyd D, Chuck C, Henk D (2021) Variation among *Metschnikowia pulcherrima* Isolates for Genetic Modification and Homologous Recombination. *Microorganisms*, **9**, 290. https://doi.org/10.3390/microorganisms9020290

Niccum BA, Kastman EK, Kfoury N, Robbat A, Wolfe BE (2020) Strain-Level Diversity Impacts Cheese Rind Microbiome Assembly and Function (PD Cotter, Ed,). *mSystems*, **5**, e00149-20. https://doi.org/10.1128/mSystems.00149-20

Nisiotou AA, Spiropoulos AE, Nychas G-JE (2007) Yeast community structures and dynamics in healthy and *Botrytis*-affected grape must fermentations. *Applied and environmental microbiology*, **73**, 6705–13. https://doi.org/10.1128/AEM.01279-07

Nissen P, Nielsen D, Arneborg N (2003) Viable *Saccharomyces cerevisiae* cells at high concentrations cause early growth arrest of non-*Saccharomyces* yeasts in mixed cultures by a cell-cell contact-mediated mechanism. *Yeast*, **20**, 331–341. https://doi.org/10.1002/yea.965

Ocón E, Gutiérrez AR, Garijo P, Tenorio C, López I, López R, Santamaría P (2010) Quantitative and qualitative analysis of non-*Saccharomyces* yeasts in spontaneous alcoholic fermentations. *European Food Research and Technology*, **230**, 885–891. https://doi.org/10.1007/s00217-010-1233-7

Peng B, Williams TC, Henry M, Nielsen LK, Vickers CE (2015) Controlling heterologous gene expression in yeast cell factories on different carbon substrates and across the diauxic shift: a comparison of yeast promoter activities. *Microbial cell factories*, **14**, 91. https://doi.org/10.1186/s12934-015-0278-5

Peter J, De Chiara M, Friedrich A, Yue J-X, Pflieger D, Bergström A, Sigwalt A, Barre B, Freel K, Llored A, Cruaud C, Labadie K, Aury J-M, Istace B, Lebrigand K, Barbry P, Engelen S, Lemainque A, Wincker P, Liti G, Schacherer J (2018) Genome evolution across 1,011 *Saccharomyces cerevisiae* isolates. *Nature*, **556**, 339–344. https://doi.org/10.1038/s41586-018-0030-5

Petitgonnet C, Klein GL, Roullier-Gall C, Schmitt-Kopplin P, Quintanilla-Casas B, Vichi S, Julien-David D, Alexandre H (2019) Influence of cell-cell contact between *L. thermotolerans* and *S. cerevisiae* on yeast interactions and the exo-metabolome. *Food microbiology*, **83**, 122–133. https://doi.org/10.1016/j.fm.2019.05.005

Piccardi P, Vessman B, Mitri S (2019) Toxicity drives facilitation between 4 bacterial species. *Proceedings of the National Academy of Sciences of the United States of America*, **116**, 15979–15984. https://doi.org/10.1073/pnas.1906172116

Pourcelot E, Conacher C, Marlin T, Bauer F, Galeote V, Nidelet T (2023) Comparing the hierarchy of inter-and intraspecies interactions with population dynamics of wine yeast cocultures. *FEMS Yeast Research*, foad039. https://doi.org/10.1093/femsyr/foad039

Puig S, Pérez-Ortín JE (2000) Expression Levels and Patterns of Glycolytic Yeast Genes During Wine Fermentation. *Systematic and Applied Microbiology*, **23**, 300–303. https://doi.org/10.1016/S0723-2020(00)80018-1

R Core Team (2023) *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna, Austria.

Rantsiou K, Dolci P, Giacosa S, Torchio F, Tofalo R, Torriani S, Suzzi G, Rolle L, Cocolin L (2012) *Candida zemplinina* Can Reduce Acetic Acid Produced by *Saccharomyces cerevisiae* in Sweet Wine Fermentations. *Applied and Environmental Microbiology*, **78**, 1987–1994. https://doi.org/10.1128/AEM.06768-11

Renault PE, Albertin W, Bely M (2013) An innovative tool reveals interaction mechanisms among yeast populations under oenological conditions. *Applied Microbiology and Biotechnology*, **97**, 4105–4119. https://doi.org/10.1007/s00253-012-4660-5

Renault P, Miot-Sertier C, Marullo P, Hernández-Orte P, Lagarrigue L, Lonvaud-Funel A, Bely M (2009) Genetic characterization and phenotypic variability in *Torulaspora delbrueckii* species: Potential applications in the wine industry. *International Journal of Food Microbiology*, **134**, 201–10. https://doi.org/10.1016/j.ijfoodmicro.2009.06.008

Rigottier-Gois L, Le Bourhis A-G, Geneviève G, Rochet V, Doré J (2003) Fluorescent hybridisation combined with flow cytometry and hybridisation of total RNA to analyse the composition of microbial communities in human faeces using 16S rRNA probes. *FEMS Microbiology Ecology*, **43**, 237–245. https://doi.org/10.1111/j.1574-6941.2003.tb01063.x

Roudil L, Russo P, Berbegal C, Albertin W, Spano G, Capozzi V (2020) Non-*Saccharomyces* Commercial Starter Cultures: Scientific Trends, Recent Patents and Innovation in the Wine Sector. *Recent patents on food, nutrition & agriculture*, **11**, 27–39. https://doi.org/10.2174/2212798410666190131103713

Roy K, Marzorati M, van den Abbeele P, van de Wiele T, Boon N (2014) Synthetic microbial ecosystems: an exciting tool to understand and apply microbial communities. *Environmental microbiology*, **16**, 1472–81. https://doi.org/10.1111/1462-2920.12343

Rué O, Coton M, Dugat-Bony E, Howell K, Irlinger F, Legras J-L, Loux V, Michel E, Mounier J, Neuvéglise C, Sicard D (2023) Comparison of metabarcoding taxonomic markers to describe fungal communities in fermented foods. *Peer Community Journal*, **3**, e97. https://doi.org/10.24072/pcjournal.321

Ruiz J, de Celis M, Diaz-Colunga J, Vila JC, Benitez-Dominguez B, Vicente J, Santos A, Sanchez A, Belda I (2023) Predictability of the community-function landscape in wine yeast ecosystems. *Molecular Systems Biology*, **n/a**, e11613. https://doi.org/10.15252/msb.202311613

Sablayrolles JM (2009) Control of alcoholic fermentation in winemaking: Current situation and prospect. *Food Research International*, **42**, 418–424. https://doi.org/10.1016/j.foodres.2008.12.016

Schmidt SA, Kolouchova R, Forgan AH, Borneman AR (2020) Evaluation of *Saccharomyces cerevisiae* Wine Yeast Competitive Fitness in Enologically Relevant Environments by Barcode Sequencing. *G3*, **10**, 591–603. https://doi.org/10.1534/g3.119.400743

Seguinot P, Ortiz-Julien A, Camarasa C (2020) Impact of Nutrient Availability on the Fermentation and Production of Aroma Compounds Under Sequential Inoculation *With M. pulcherrima* and *S. cerevisiae*. *Frontiers in microbiology*, **11**, 305. https://doi.org/10.3389/fmicb.2020.00305

Shade A, Peter H, Allison SD, Baho DL, Berga M, Bürgmann H, Huber DH, Langenheder S, Lennon JT, Martiny JBH, Matulich KL, Schmidt TM, Handelsman J (2012) Fundamentals of

microbial community resistance and resilience. *Frontiers in microbiology*, **3**, 417. https://doi.org/10.3389/fmicb.2012.00417

Sheff MA, Thorn KS (2004) Optimized cassettes for fluorescent protein tagging inSaccharomyces cerevisiae. *Yeast*, **21**, 661–670. https://doi.org/10.1002/yea.1130

Simonin S, Alexandre H, Nikolantonaki M, Coelho C, Tourdot-Maréchal R (2018) Inoculation of Torulaspora delbrueckii as a bio-protection agent in winemaking. *Food research international (Ottawa, Ont.)*, **107**, 451–461. https://doi.org/10.1016/j.foodres.2018.02.034

Sternes PR, Lee D, Kutyna DR, Borneman AR (2017) A combined meta-barcoding and shotgun metagenomic analysis of spontaneous wine fermentation. *GigaScience*, **6**, 1–10. https://doi.org/10.1093/gigascience/gix040

Taillandier P, Lai QP, Julien-Ortiz A, Brandam C (2014) Interactions between *Torulaspora delbrueckii* and *Saccharomyces cerevisiae* in wine fermentation: influence of inoculation and nitrogen content. *World journal of microbiology & biotechnology*, **30**, 1959–67. https://doi.org/10.1007/s11274-014-1618-z

Tamang JP, Watanabe K, Holzapfel WH (2016) Review: Diversity of Microorganisms in Global Fermented Foods and Beverages. *Frontiers in Microbiology*, **7**. https://doi.org/10.3389/fmicb.2016.00377

Tofalo R, Schirone M, Torriani S, Rantsiou K, Cocolin L, Perpetuini G, Suzzi G (2012) Diversity of *Candida zemplinina* strains from grapes and Italian wines. *Food microbiology*, **29**, 18–26. https://doi.org/10.1016/j.fm.2011.08.014

Tondini F, Onetto CA, Jiranek V (2020) Early adaptation strategies of *Saccharomyces cerevisiae* and *Torulaspora delbrueckii* to co-inoculation in high sugar grape must-like media. *Food microbiology*, **90**, 103463. https://doi.org/10.1016/j.fm.2020.103463

Tourlousse DM, Narita K, Miura T, Sakamoto M, Ohashi A, Shiina K, Matsuda M, Miura D, Shimamura M, Ohyama Y, Yamazoe A, Uchino Y, Kameyama K, Arioka S, Kataoka J, Hisada T, Fujii K, Takahashi S, Kuroiwa M, Rokushima M, Nishiyama M, Tanaka Y, Fuchikami T, Aoki H, Kira S, Koyanagi R, Naito T, Nishiwaki M, Kumagai H, Konda M, Kasahara K, Ohkuma M, Kawasaki H, Sekiguchi Y, Terauchi J (2021) Validation and standardization of DNA extraction and library construction methods for metagenomics-based human fecal microbiome measurements. *Microbiome*, **9**, 95. https://doi.org/10.1186/s40168-021-01048-3

Vaquero C, Loira I, Bañuelos MA, Heras JM, Cuerda R, Morata A (2020) Industrial Performance of Several *Lachancea thermotolerans* Strains for pH Control in White Wines from Warm Areas. *Microorganisms*, **8**. https://doi.org/10.3390/microorganisms8060830

Varela C, Cuijvers K, van den Heuvel S, Rullo M, Solomon M, Borneman A, Schmidt S (2021) Effect of Aeration on Yeast Community Structure and Volatile Composition in Uninoculated Chardonnay Wines. *Fermentation*, **7**, 97. https://doi.org/10.3390/fermentation7020097

van de Velde CC, Joseph C, Biclot A, Huys GRB, Pinheiro VB, Bernaerts K, Raes J, Faust K (2022) Fast quantification of gut bacterial species in cocultures using flow cytometry and supervised classification. *ISME Communications*, **2**. https://doi.org/10.1038/s43705-022-00123-6

Venturelli OS, Carr AC, Fisher G, Hsu RH, Lau R, Bowen BP, Hromada S, Northen T, Arkin AP (2018) Deciphering microbial interactions in synthetic human gut microbiome communities. *Molecular Systems Biology*, **14**, 8157. https://doi.org/10.15252/msb.20178157

Wolfe BE, Button JE, Santarelli M, Dutton RJ (2014) Cheese rind communities provide tractable systems for in situ and in vitro studies of microbial diversity. *Cell*, **158**, 422–433. https://doi.org/10.1016/j.cell.2014.05.041

Wolfe BE, Dutton RJ (2015) Fermented foods as experimentally tractable microbial ecosystems. *Cell*, **161**, 49–55. https://doi.org/10.1016/j.cell.2015.02.034

Zimmer M (2002) Green Fluorescent Protein (GFP): Applications, Structure, and Related Photophysical Behavior. *Chemical Reviews*, **102**, 759–782. https://doi.org/10.1021/cr010142r

Zott K, Miot-Sertier C, Claisse O, Lonvaud-Funel A, Masneuf-Pomarede I (2008) Dynamics and diversity of non-*Saccharomyces* yeasts during the early stages in winemaking. *International Journal of Food Microbiology*, **125**, 197–203. https://doi.org/10.1016/j.ijfoodmicro.2008.04.001