Answer to the reviewers

We want to give our thanks to the editor and the reviewers for the time and effort dedicated to provide their feedbacks on our manuscript. We hope that the modifications made to the manuscript answer your suggestions and questions.

Answer to reviewer 1

The manuscript by Pourcelot et al. is a nice and valuable contribution to the growing field of applied microbial ecology, particularly focusing on wine fermentations as a model system. This research is notable for offering a refined model system, which utilizes a set of species tagged with fluorescence for precise population monitoring. It also investigates key research questions, such as how taxonomic diversity influences the performance of ecological communities and how varying environmental conditions may impact this performance.

> We thank you for your appreciation of the article and the valuable comments to improve the manuscript.

Major observations:

- The theoretical background supporting the ecological questions addressed in this work could be better elaborated in the introduction, and more importantly, in the discussion of results. The current discussion primarily focuses on previous observations in wine research rather than drawing connections to other studies in theoretical ecology that have addressed similar questions.

*> Further background information were added in the introduction **(lines 77-82)**. We agree that the theoretical frame of this work was not well discussed and accordingly we added few lines in the discussion **(lines 623-647).***

- Regarding the two environmental conditions tested (S200 vs. S280), it is unclear whether they genuinely represent contrasting conditions of stress versus non-stress. Does 280 g/L of sugar truly constitute an osmotic stress condition compared to 200 g/L, or does it simply present a more challenging medium for sugar consumption due to cell exhaustion or limited nitrogen availability? While it is evident that osmotic stress persists longer at 280 g/L compared to 200 g/L, it is uncertain if the initial conditions significantly differ in terms of osmotic stress at an ecological or molecular level. If the authors have evidence of stress response induction at 280 g/L versus 200 g/L of sugar, this should be justified in the introduction.

*> We agree with the reviewer that the osmotic stress difference was probably not enough to observe strong differences between S200 (200 g/L) and S280 (280 g/L). We did not run preliminary tests to check whether there was a significant difference in stress induction since previous experiments in our lab found differences in the lag phase of Sc using these two concentrations. These two concentrations have also been used in studies focusing on osmotic stress done in other labs. Thus, we expected to find greater differences. The decision on these two test concentrations was also motivated to represent the evolution of sugar concentration in relation to climate change. We better explained our choice in the Results section **(lines 434-436)** and in the discussion **(lines 590-593)**. We have also clarified how this difference may be a*

limitation to the results we obtained ***(lines 487-490)*** *since 200 g/L of sugar already represent a high osmotic stress.*

- As the authors propose this consortium as a model system, it would be beneficial to conclude the manuscript (at the end of the discussion) with a clear statement of its strengths and weaknesses. Additionally, outlining the oenological and ecological issues that this model system can address, which previous models could not, would provide valuable context and direction for future research.

> The end of the discussion was reorganized to include a paragraph stating pros and cons of the strategy used ***(lines 650-671).***

Minor comments:

Abstract

Lines 33-34: I suggest to rewrite this sentence not to give the impression that this result is a limitation, as this is an actual and important result. Maybe removing the word “Although” is enough, but, please, consider rephrasing.

> ***lines 33-34***

Introduction

Lines 68-72: It would be interesting to mention here some relevant works that already used wine microbial consortia to explore fundamental ecological questions (see: https://www.cell.com/action/showPdf?pii=S2405-4712%2817%2930390-3; https://www.nature.com/articles/s42003-023-05284-1; https://www.embopress.org/doi/full/10.15252/msb.202311613)

> The introduction was completed ***(lines 75-77 and 82-83)***

Material and methods

Line 120: While the assays in mock communities effectively demonstrate that the fluorescent tag accurately indicates yeast cell abundance, it would be beneficial to use qPCR to verify that TDH3 expression remains constant during wine fermentation in monocultures of the six species studied. Perhaps another promoter is needed for Mp.

> Confirming the TDH3 *gene expression by qPCR would be a great addition. It was not performed since the monocultures showed that the expression was enough to be able to discriminate the different species during fermentation (clarified in the results,* ***lines 357-359****). For* M. pulcherrima*, the issue was not due to the expression level but related to the difficulty of doing targeted integration into its genome (despite the use of long homology arms or repression of the NHEJ). Although for* S. cerevisiae *different integration sites might lead to differences in transformation yield, in* M. pulcherrima*, targeting other sites (HIS3 or URA genes) was also unsuccessful (Gordon et al., 2019; Moreno-Beltrán et al., 2021). The main issue of random integration was clarified in the result section* ***(lines 305-309).***

Line 135: Why was pasteurization chosen over filtration? Did you measure the concentration of residual sugars after pasteurization? Could pasteurization be reducing the actual differences between the S280 and S200 trials?

> We performed pasteurization since it is an easier sterilization method to implement for large media batch within our lab. Media analysis (by HPLC) done at T0 were done simultaneously to the T0 for cell numbering, so the measure is performed after pasteurization. We found that the average sugar concentration after pasteurization were of 202 ± 1.5 g/L in S200 and 287 ± 1.5 g/L in S280, which is consistent with the theoretical sugar concentration.

Line 142: Change "physiological water" to "saline solution."

> ***Line 167***

Line 145: Is there a rationale for using different scales for different trials, or was this simply due to standard laboratory practices and to reduce the cost of the second batch of experiments? If there is a specific reason, please state it here.

> It was indeed an issue of reducing the cost when dealing with many fermenters, as well as a lower availability of 1-L fermenters in the lab. In addition, 1L-scale fermentation allow for more sampling points (total sampling volume is kept under 10% total volume) and better fermentation kinetics measurements, which is why it was preferred for the consortium initial characterization. These precisions were added ***(lines 170-173).***

Line 171: There is a typo here: change "functionnality" to "functionality."

> ***Line 198***

Line 190: Please revise this sentence. There are some unexpected symbols between numbers and units.

> ***Line 217*** *- PDF generated before the BioRxiv submission to avoid formatting errors.*

Line 204: please, write “1·105 to 5·105”.

> ***Lines 196-197 and 232***

Lines 212-213: Are there any data available to support this statement?

> Supplementary data was added with a comparison with PBS or YPD+PBS incubation for S. cerevisiae *monocultures and consortium after 160 h of fermentation* ***(line 241).***

Line 234: When stating “They included four CO_2 kinetics…,” isn’t it three?

> ***Line 262***

Results

Lines 296-298: This sentence is incomplete.

> Sorry for this elementary mistake. The sentence was modified and completed ***(lines 325-328).***

Line 302: How did you measure and adjust the cell concentration from the pre-inoculum to later test theoretical vs. observed cell counts?

*> One milliliter of pre inoculum was resuspended in PBS. The pre-inoculum cell concentration was measured by flow cytometry in order to prepare single-cell suspension at 10^6 cells/mL (in PBS). Defined volume of each single-species cell suspension were then mixed to a final volume of 200µL that make the undiluted mock community (final concentration of 10^6 cells/mL). Before the measure, this undiluted cell suspension was then diluted 3-fold to be in the reading range of the cytometer ($1 \cdot 10^5$ to $5 \cdot 10^5$ cells/mL.). Details were added in the results **(lines 330-333)** and the material and method rephrased **(lines 218-220).***

Line 323: If I am interpreting Supplementary Figure S3 correctly, how do the authors explain the detection of cells from other species in monocultures? Are these contaminations or cross-identifications by fluorescence? In addition to the online repository, which is excellent for raw data and scripts, the supplementary material would be better organized in a single file with the corresponding legends to the figures (which have been difficult for this reviewer to find, complicating the interpretation of some results).

> We attributed this detection of other cells to rare noise events as we never managed to have PBS having absolutely 0 events despite filtration on 0.2µm filter. Cross-contamination could also be an explanation but seems less likely since these events often correspond to non-fluorescent cells (thus being identified as M. pulcherrima*) or sometimes as mCherry positive when applying the PI stain (thus being identified* as L. thermotolerans*). Should it be a matter of cross-contamination during the cytometry reading, we would observe as many GFP/mCitrine/GFP+mCherry events as non-fluorescent and mCherry events, which was not the case. As for fermenters cross-contamination, we think it is very unlikely since sampling is done with separate syringe, and such cross contamination would very probably lead to actual noticeable change in population dynamics. We are aware it is a limitation of our method, since it mostly limit our system to be based on relative abundance or high cell concentration (to be out of the "noise" background range). Using more conservative gatings might maybe reduce the inclusion of noisy events too.*

*We figures in the results section **(lines 357-358)** and stated more precisely this limitation in the conclusion **(lines 665-667).***

> For supplementary data, we put the corresponding folder as the 1st folder of the repository and indicated early in the ReadMe that single files for supplementary data can be found in this folder. If it is still unclear, we will use a dedicate repository for the additional information files.

Line 341: When comparing the performance of Sc in monoculture versus as part of consortia, was the concentration of Sc in monoculture 10^6? If so, this is 20 times higher than its concentration in the consortia. This should be mentioned to avoid erroneous conclusions about the potential role of the consortia in reducing fermentation kinetics. It is indeed expected that the consortia ferments more slowly compared to S. cerevisiae alone, as the abundance of the primary fermenting agent is reduced.

*> Thank you for the reminding, the longer latency is indeed related to the inoculation rate of Sc. This was made clear **(line 380)**.*

Lines 373-375: It is interesting that *T. delbrueckii* does not consume pyruvic acid (a similar

observation is made with Sb, but since Sb is much less fermentative, the observation is less relevant). This should be highlighted as a result.
*> **Lines 416-421***

· Figure 6: It would be beneficial to include a Supplementary Figure to Fig. 6 that shows absolute cell numbers rather than relative abundance. This would allow for a better understanding of whether changes in the relative abundance of a species are due to direct increases or decreases in its absolute cell count, or if they result from changes in other populations while a species remains unaffected by sugar concentration. In this context, did the higher sugar concentration lead to higher maximum cell concentrations of the total consortium and/or individual species? The results should be discussed accordingly.
*> A supplementary figure was added **(line 439).***

· Lines 446-447: As mentioned earlier in the "Major comments" section, another possible explanation for this result is that the conditions do not represent significantly different osmotic pressures.
*> It was indeed one of our hypothesis. We made it more explicit in the discussion section **(lines 489-490)***

Line 481: Remove the ")" after "value" at the end of the sentence.
*> **Line 529***

Lines 579-581: Why is the system described as low complexity? Please explain this more clearly and establish a reference system for comparison (there are examples of both more complex and simpler model systems).
*> The low complexity was referring to the synthetic media which include less molecules than natural must that may contain rare nutrients and molecules causing additional stress to the cells (polyphenols, …). We specified "synthetic media" instead of "environment" to be clearer **(line 636)***

Answer to reviewer 2

The article by Pourcelot E. et al. aims to design yeast microbial communities that represent the diversity of wine fermentation environments. Along with this, the aim is to develop a high-accuracy method to monitor the population dynamics of microbial consortia during the fermentation process. The work fluorescently labels *S. cerevisiae* and 6 non- *Saccharomyces* species. The work is well written and of high quality and shows great rigour in each of the methodologies used. It should be noted that the authors provide the scientific community with five new strains of fluorecently labelled yeast, a valuable material that can be used for future research. In addition, the clarity and quality of each of the scripts provided in the supplementary material is appreciated.

> We thank you for your review and the nice comments on the article.

In the abstract it is mentioned that mixed fermentations were performed with two sugar concentrations (200 and 280 g/L). What is the result of this experiment. Is it relevant? If so, mention the result; if not, eliminate it from the abstract.

> We referred to the osmotic stress in the abstract ***(line 34)***

Eliminate sentence (L33-35) and change it to one that starts from a positive perspective. This helps and motivates the reader to continue reading.

> This part of the abstract was amended ***(lines 33-35)***

The introduction makes clear the need to explore population dynamics and the role of microbial diversity in the wine fermentation process. The use of differential cultures, qPCR and metabarcoding are mentioned as examples. However, no clear examples are given as to why the use of flow cytometry is superior to the techniques mentioned above. Clear arguments or examples should be added as to why this experimental strategy was selected.

This will give value and weight to the article, motivating researchers to use this experimental design over others.

Again, I consider this to be possible only due to the clarity of the methodology presented in the paper.

> Additional information on the pros of this strategy were added in the introduction ***(lines 87-90)***

Across the text, some words present typing errors (e.g. L89, L190, this could be due to a format issue in the platform).

> PDF generated before the BioRxiv submission to avoid formatting errors.

Include at the bottom of Table 1 the meaning of the acronym CIRM and SPO.

> Lines 115-120

L113- Add the full name of the *TDH3* gene.

*> **Line 138**, also added for ENO2 **(lines 144)***

In supplementary figure 1, given the low variability of the measurements of auc, k, and r, it is not possible to differentiate the colors of the boxplots. Add in the figure caption meaning of D6, B8, A1, BA1 and C3.

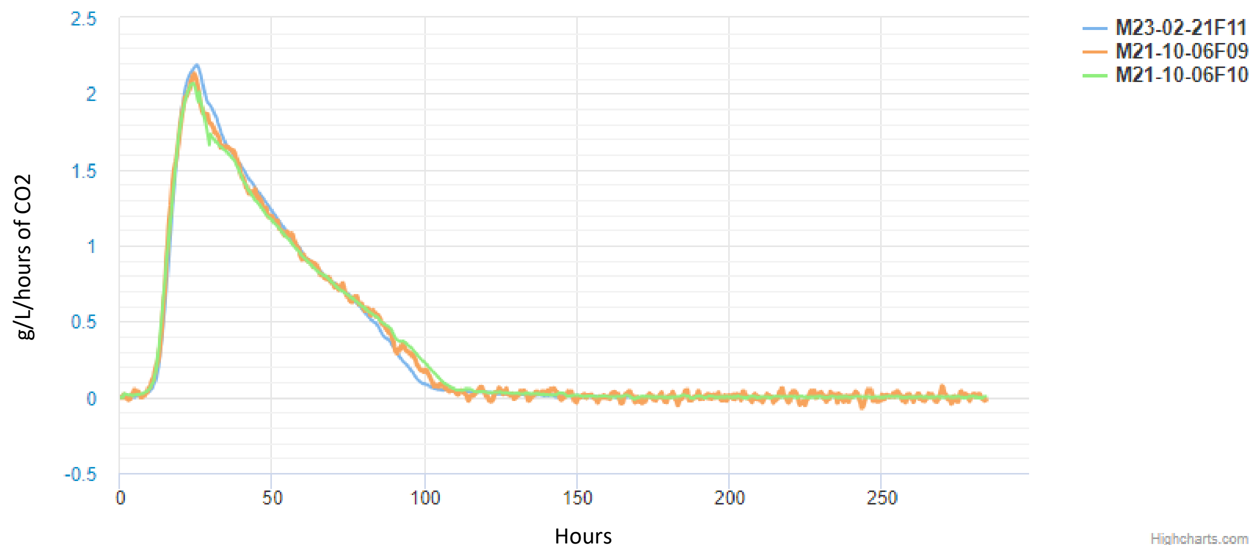
*> The graph was amended – Supplementary figure file **(line 9)***

Figure 1 shows how to separate each of the strains according to the different markers incorporated. What was the inoculation ratio in the example set up?

*> Precision were added **(lines 318-319)***

In the fermentations, 6 ml (scaled) is collected per time point and 1,5 ml in the 250, does this volume affect the fermentation itself?

> In total, the sampling amounts to less than 10% of total volume (for both fermentation scales). Even though we don't have specific data for this experiment, comparison between Sc monocultures with or without sampling do not show variations in the kinetics (see figure below). It may cause slight background noise on the CO_2 kinetics graph, but nothing impairing the results obtained.



Examples of CO_2 production rate in S. cerevisiae *monocultures. Blue: Sc MTF4798 (TDH3-mCherry) with no sampling, orange and green Sc MTF4798 (TDH3-mCherry) with sampling*

Answer to reviewer 3

The current manuscript describes the development of a model wine yeast consortium by tagging different yeast species with different fluorescent proteins. The concept is very interesting and relevant to the wine research community. The manuscript is generally well written, results are well presented, and conclusions are sound. I only have minor suggestions for the authors to consider.

> We thank you for your review and nice comments on the article.

L89 check the alpha symbol after DH5, also in L102

> The PDF was generated before the BioRxiv submission to avoid formatting errors.

L164 saline solution instead of physiological water

> Line 167

L190 check symbols for units

> PDF generated before the BioRxiv submission to avoid these formatting errors.

L211-212 it is not clear if this incubation in YPD for 1 hour and then PBS for another hour was the protocol followed by the authors for all samples or only for S. cerevisiae monocultures. Authors should indicate clearly the final protocol followed.

> We added precision in the material and method section ***(lines 239-240);***

L225 were measured

> Line 253

L271-279 Authors mention in the results section that the phenotype of the transformants was assessed. It would be great if the authors could comment on how many transformants were tested and what were the results.

> PCR testing for target integration was run on at least 8 clones (additional clones were tested if we did not find 2 positive clones). For the growth phenotyping in microplates, 2 clones were tested for each transformation. We did not include all the clones in the supplementary data to allow for better readability. Precision on the number of testing was added in the material and method section ***(lines 150-153)*** *as well as in the Supplementary Figure 1 caption.*

L279 what is in accordance with previous studies? Please include more information

> This segment was rephrased to be clearer ***(lines 305-309)***

L298-299 since they…..?

> Sorry for this elementary mistake. The sentence was modified and completed ***(lines 325-328)***

L299 overnight cultures in synthetic grape must (S200)

> Line 331

L318-319 the word fermentation is repeated

> Line 350-351

L323-328 it would be interesting to see if there is a correlation between the drop in abundance for some non-*Saccharomyces* and ethanol concentration or dissolved oxygen during the first days of fermentation

> We added a supplementary figure showing the ethanol production in the consortium compared to the ethanol concentration reached in monocultures (before viability drops in monocultures, as well as the ethanol concentration reached at the end). This data show that the NS drop at 24h in the consortium is not directly related to the ethanol concentration reached at this time, since it is inferior to those reached in all monocultures. This has been added in the results ***(lines 367-369)*** *and in the discussion* ***(lines 569-571)****. For the oxygen, we do not have data on the dissolved oxygen in the media so we cannot conclude. We can hypothesized* O_2 *depletion shouldn't be a direct cause of cell concentration decrease, since this drop is not observed in the monocultures, while NS cells alone consume quickly the oxygen present in the media as well. Our current methodology do not allow for O2 measurements to confirm or infirm this hypothesis.*

Figure 3 It seems that the symbols in the figures are diamonds while in the legend symbols are circles.

> The figure was corrected ***(line 371)***

L375-377 please indicate that this sentence refers to pyruvic acid

> Information added ***(lines 416-417)***

L469 fluorescent proteins

> ***Line 516***

L500-501 could this be the result of using different *S. bacillaris* strains?

> The strain effect was added as an explanation ***(lines 549-551);***

L510 why is *S. cerevisiae* the likely cause of yeast viability drop? Please provide more information

> The paragraph was reorganized ***(lines 561-566).***

L542 'to be promoted' is not the right expression, authors may use 'dominate', 'have a competitive advantage', etc. also in L546 and L549

> We replaced "to be promoted" by "have a competitive advantage" ***(lines 605, 608).***

L561-563 this sentence is not clear, please check grammar and rewrite.

> The sentence was replaced ***(lines 618-620).***

L591 alcoholic

> ***Line 685***

Discussion. In general, we are tempted to think that one particular strain represents the species it belongs to. However, this is not necessarily the case as we know how different S. cerevisiae strains are to each other. This is likely the case with non-Saccharomyces species. I encourage the authors to consider this point and include it in the discussion section.

*> Intra-specific variations are also known in non-*Saccharomyces*. We thus added some discussion on this point* ***(lines 549-551).***

Additional edits:

> Corrected the inversion of species/fluorescent proteins combination for Td (mCitrine) and Sb (EGFP) in figure 1 and their fluorescence characteristics in table 3.

> Origin of the strains specified in the material and method.