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Complex yeast-bacteria interactions affect the yield of industrial ethanol fermentations

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Abstract

Microbial communities affect several natural processes, from nutrient cycling to human health. Nevertheless, the mechanisms of interaction between microorganisms and their influence on community functions are not well understood. Sugarcane ethanol fermentations represent a simple microbial community dominated by *S. cerevisiae* and co-occurring bacteria with a clearly defined functionality. In this study, we dissected the microbial interactions in sugarcane ethanol fermentation by combinatorically reconstituting every possible combination of species, comprising approximately 80% of the biodiversity in terms of relative abundance. Functional landscape analysis showed that higher-order interactions counterbalance the negative effect of pairwise interactions on ethanol yield. In addition, we found that *Lactobacillus amylovorus* improves the yeast growth rate and ethanol yield by cross-feeding acetaldehyde, as shown by flux balance analysis and laboratory experiments. Our results suggest that *Lactobacillus amylovorus* could be considered an industrial probiotic with the potential to improve sugarcane ethanol fermentation yields by more than 10%. These data highlight the biotechnological importance of comprehensively studying microbial communities and could be extended to other microbial systems with relevance to human health and the environment.

Introduction

Microorganisms live in complex communities that span from simple multicellular aggregates to complex microbiomes composed of thousands of different species¹. Microbial communities are ubiquitous in nature and contribute to many crucial functions, from nutrient recycling in soil to the maintenance of human health². Microbial communities are shaped and stabilized by the interactions between their constituent members³. These interactions define the composition, dynamics and functionality of the microbial community⁴.

Further understanding microbial interactions is crucial from microbiological, ecological and biotechnological perspectives⁵, but progress is hindered by the enormous complexity of most natural communities. Synthetic microbial communities represent important tools for studying microbial interactions⁶⁻⁸, as they can be directly manipulated and their responses can be precisely quantified^{9,10}.

Sugarcane ethanol fermentations with synthetic microbial communities represent a potentially interesting real-world microbial community that is tractable for comprehensive assessment. The microbial community from sugarcane ethanol fermentations is reproducible, and simplified versions can be established in the laboratory¹¹⁻¹³, providing researchers with a wealth of data regarding fermentation parameters that are directly linked to microbial physiology and community functionality (e.g., ethanol titre, biomass growth, and organic acid titres)¹¹. Additionally, bioethanol production has both economic and environmental relevance. In Brazil, sugarcane refineries produced more than 34 billion litres of ethanol in 2019, employing over 1 million people¹⁴. During the last 13 years, bioethanol has been responsible for avoiding the release of more than 500 million tons of CO₂ in the atmosphere, and bioethanol replaced more than 40% of the gasoline consumed in Brazil¹⁵.

This industry makes use of the Melle-Boinot fermentation process, which is based on high cell density fed-batch fermentations¹⁶. The fermentation broth is typically composed of sugarcane molasses diluted with water or sugarcane juice and usually has a sugar concentration of *ca.* 18 – 22% (w/v basis)¹⁷. Large

fermenters, with volumes normally exceeding 500 thousand litres, are fed fresh broth for approximately 4 – 6 h¹⁸. Selected strains of *Saccharomyces cerevisiae* convert the sugars present in the broth into ethanol, reaching final concentrations of 7 – 11%, with fermentation yields as high as 92% of the total theoretical yield¹⁶. The high cell density of yeast cells in these fermentations (approximately 10%, wet weight/volume) allows for very fast fermentations, usually lasting for 6 – 12 h¹⁶. After the fermentation is complete, cells are separated from the beer via centrifugation¹⁶. After centrifugation, these cells are diluted with water and acid-washed for preventive control of contamination for approximately 1 h, after which cells are pumped back into the fermenters for a new batch of fermentation¹⁸. This yeast biomass can be continuously recycled through the production season, resulting in almost 600 fermentation cycles¹⁸.

The Melle-Boinot process is operated under non-sterile conditions, which makes contamination commonplace^{16,18}. The most common contaminants are lactic acid bacteria, which are able to form stable populations throughout the production season^{19–22}. It is considered that such contaminants are mostly detrimental to the production process, since they will compete with yeast for the available nutrients, and their metabolic products are inhibitory towards yeast cells²¹. We have previously shown that these communities have similar compositions across different biorefineries and follow similar community adaptations during the industrial process²³.

In this study, we used a combination of flux balance analysis and combinatorial microbial culture to assess the functional landscape of microbial assemblies reflecting the sugarcane ethanol process.

Results

Competition shapes the microbial community of sugarcane biorefineries

We sought to develop a simplified yet realistic microbial consortium that could be used as a model system for studying and understanding microbial interactions in industrial sugarcane ethanol biorefineries. To that end, we constructed a synthetic consortium composed of *Lactobacillus amylovorus*, *Lactobacillus*

fermentum, *Lactobacillus helveticus*, *Pediococcus claussenii* and *Zymomonas mobilis*, in addition to the dominant species *Saccharomyces cerevisiae*, which is responsible for ethanol fermentation (**Figure 1A**). These bacterial species represent more than 80% of the contaminant community found in industrial sugarcane ethanol fermentations²³.

To unravel how each species and their interactions contribute to the desired performance output (ethanol yield), we combinatorically reconstituted every possible microbial consortium that contained yeast as well as any number (from zero to six) of the bacterial species growing together⁷ (**Figure 1B; Methods**). Each synthetic community was inoculated with yeast/bacteria at a ratio of 100:1 to replicate actual industrial conditions¹⁶. All bacterial species in the inoculum were included at equal proportions. Microbial consortia were incubated for 24 h, after which both cells and media were separated and harvested to analyse community structure (yeast and bacterial population size and relative abundance) via flow cytometry and function (final ethanol yield) via HPLC analysis (**Figure 1C, Methods**). Three replicates for each condition were run, leading to a total of 258 fermentations (**Figure 1D**).

Figure 1: Schematics of the combinatorial assembly experimental setup. A: Microbial species used in this study. These species make up more than 80% of the diversity found in fuel ethanol fermentations in relative abundance. **B:** Initially, pairwise fermentations between yeast inoculum and all available bacteria were performed. This results in a combinatorial assembly of order 1. In parallel, every pairwise assembly was also mixed with every other bacterial monoculture, resulting in a set of 51 different fermentations or order 2. This was performed for every possible combination of bacterial species and yeast, resulting in 258 fermentations, and a total order of 6 (i.e., a community containing up to 6 different bacterial species and yeast). The ratio between all bacterial species was kept at 1 for every cultivation, and the yeast:bacteria

ratio was 100:1¹⁶. **C:** After fermentation was completed, the community structure was analysed via flow cytometry, and the fermentation metabolites were quantified via HPLC. **D:** An overview of the microbial compositions generated via the combinatorial assembly throughout the 258 fermentations. This figure was created using Biorender (<https://app.biorender.com/>).

The population size of both yeast and bacteria generally decreases in total cell count following the increase in the number of different species added to the consortia, suggesting that competition is a major interaction shaping these communities²⁴⁻²⁶. The yeast population dropped by 16% from *ca.* $1.1 \times 10^7 \pm 1 \times 10^6$ cells ml⁻¹ when grown in monoculture to approximately $9.1 \times 10^6 \pm 9.3 \times 10^5$ cells ml⁻¹ when grown in co-culture with all six bacterial species. The decline in population size was statistically significant ($p < 0.0001$, one-way ANOVA; **Figure 2A**). The bacterial population also dropped almost 50% when comparing the cell counts when a single species of bacteria was inoculated with yeast to when all six bacterial species were present in the consortium ($p = 0.0002$, one-way ANOVA; **Figure 2A**).

While both yeast and bacterial populations declined as diversity increased, the relative abundance of yeast grew with increasing diversity (**Figure 2B**). Yeast relative abundance shifted from $90.3 \pm 1.6\%$ in pairwise consortia to $98.8 \pm 0.3\%$ in consortia containing 6 different bacterial species. In contrast, the relative abundance of the bacterial population fell from $9.7 \pm 1.8\%$ from one species to $1.2 \pm 0.3\%$ in cultivations where the 6 species were present. The changes in population structure were both statistically significant ($p < 0.0001$; one-way ANOVA in both cases).

Figure 2: The community structure of yeast and bacteria co-cultivations. **A:** Final cell counts of yeast (blue) and bacteria (red) from different community structures. The final yeast cell counts dropped from $1.1 \times 10^7 \pm 1 \times 10^6$ to *ca.* $9.1 \times 10^6 \pm 9.3 \times 10^5$ cells ml^{-1} as the number of bacterial species in the community increased (one-way ANOVA, $p < 0.0001$). The final bacterial cell counts dropped from $1.5 \times 10^7 \pm 1.5 \times 10^6$ to *ca.* $8 \times 10^5 \pm 1.4 \times 10^5$ cells ml^{-1} (one-way ANOVA, $p = 0.0002$). Symbols and error bars represent the mean $\pm$ standard deviation, $N = 3$. **B:** The relative abundance of yeast and bacterial populations in different community structures. The relative abundance of the yeast population (blue bar) increased from $90.3 \pm 1.6\%$ in pairwise cultivations to $98.8 \pm 0.3\%$ in the presence of all 6 different bacterial species (one-way ANOVA, $p < 0.0001$). The bacterial relative abundance (red bar) decreased from $9.7 \pm 1.8\%$ initially in pairwise cultivations to $1.2 \pm 0.3\%$ in the most complex community tested, which contained yeast and 6 different bacterial species (one-way ANOVA, $p < 0.0001$).

Higher-order interactions stabilize the performance of yeast and bacterial fermentations

In order to better understand the influence of interactions between different bacterial species on ethanol yield, we studied the functional landscape of our communities, a method used to assess how the function of a community relates to its composition⁷. To that end, we characterized the community function (F) as

the logarithm of the ratio between the ethanol yield (Y) observed when yeast was grown in a mixed culture with a given bacterial consortium (**equation (2), Methods**) and the ethanol yield for yeast in monoculture, i.e., $F = \log(Y_{\text{community}}/Y_{\text{alone}})$. In this definition, $F=0$ means that the bacterial consortium has no effect on ethanol production compared to yeast growing alone. The measured functional landscape shows a non-monotonic trend in which communities with either very low or very high diversity are less detrimental to ethanol yield than communities with intermediate diversity (**Figure 3A**).

To quantitatively characterize the interactions in our communities, we propose a null model that the effects of bacterial species on ethanol yield combine additively, such that the impact of adding an additional species to the community is equal to its effect when co-cultured alone with yeast. Such a null model was previously shown to be reasonable under a variety of different assumptions regarding the relation between species abundance and function⁷. In our communities, this null model correctly predicted the function of 36/76 communities (47.4%, **Figure 3A and 3B; Supplementary Data**). We next attempted to improve the predictions in communities with 3 or more species by including pairwise interactions in our null model, which can be computed by comparing the one- and two-species co-cultures with yeast (**Methods**). Given that most of the pairwise interactions were negative (**Figure 3C**), our model predicted that function would decrease as a consequence of the addition of new species (**Figure 3D**). However, this was not the case, and the model including pairwise interactions performed substantially worse than the simple, additive model (**Figure 3E**, compare to inset **Figure 3B**).

Effectively, the difference between the measured values and the predictions of the model including both single and pairwise effects (**Figure 3E**) measures the effect of functional high-order interactions (HOIs) (**Methods**). As expected, our results indicate that the influence of HOIs greatly increases with the addition of new species to the community. Quantitatively, the observed HOIs not only neutralize the effects of the negative pairwise interactions, in which case we would expect the detrimental effect observed in pairs to be kept equal when adding more species. The HOIs actually remove these detrimental effects, driving the function to become almost equal to that of yeast alone (**Figure 3A**). In other words, while the addition of

176 most pairs of bacterial species to the yeast cultures had generally adverse effects on ethanol yield, the
177 addition of a diverse community of contaminants with up to six species had beneficial effects, pushing
178 yields to values close to those found for yeast monocultures. This dissection of the interactions found in
179 this community suggests that HOIs play an important stabilizing role in sugarcane ethanol fermentations.

180

181 **Figure 3: The ecological interactions within the sugarcane biorefinery microbial community. A:**

182 Observed functional landscape. We plotted the measured function for communities of different species.

Each line represents the effect of the addition of one species to a community. The red line represents the average of all points for each community size. **B**: The expected functional landscape when considering the presence/absence (additive) null model. The inset shows the difference between the measured and predicted functions, plotted against community size, as a way to visualize the fit of the data to the null model. **C**: Heat map showing the interactions among pairs of bacterial species (asterisks represent significance taking $\pm 2SE$). These interactions were diverse and mostly detrimental to ethanol yield. The epistasis difference (epsilon) between these species also suggests that such interactions are strain driven. **D**: The functional landscape was predicted by adding up single and pairwise effects. This landscape predicts a rapid reduction in function as more species are added. **E**: The difference between the prediction of the model in panel **D** is plotted against community size. For visualization, a jitter was applied to points of different composition but the same community size. The plotted difference effectively measures the influence of high-order interactions.

***Lactobacillus amylovorus* benefits yeast via acetaldehyde production**

To pinpoint specific taxa that influenced community function, we performed correlation analysis of the community composition and ethanol yield. The most significant correlations between measured parameters were weak (**Supplementary Table 1**), suggesting that different species have similar impacts in the community, e.g., redundancy between species³². However, the presence of one of the species (*L. amylovorus*) in the consortia was significantly correlated with higher fermentation yields (Wilcoxon rank-sum analysis; $p=0.009$). Indeed, fermentations containing *L. amylovorus* had $3.02 \pm 1.06\%$ higher ethanol yields compared to those lacking this species. (**Figure 4A**).

In addition to its impact on the yeast population and ethanol yield, the presence of *L. amylovorus* was also positively correlated with yeast growth (Wilcoxon rank-sum analysis; $p = 0.043$. **Figure 4B**).

Figure 4: The effect of the presence of *L. amylovorus* on ethanol fermentations. A: Fermentations performed with *L. amylovorus* in the composition of the microbial community showed higher fermentation yields ($3.02 \pm 1.06\%$, $p=0.009$; Wilcoxon rank-sum analysis) in comparison to the others lacking this species. **B:** Fermentations with microbial communities containing *L. amylovorus* showed higher yeast cell counts (Wilcoxon rank-sum analysis, $p=0.043$).

The increase in yeast population and ethanol yield might be partly explained by the possible antagonistic effect of this species against other bacteria in the fermentation environment³³⁻³⁷. However, considering that this statistical analysis indicates only when this species is present, independent of the community's configuration, we decided to further analyse whether *L. amylovorus* may have a positive effect on yeast metabolism. One mechanism for such a positive impact might be through a cross-feeding interaction. To investigate the possibility of cross feeding between *S. cerevisiae* and *L. amylovorus*, we simulated the growth of *S. cerevisiae* via flux balance analysis (FBA), both in the presence or absence of *L. amylovorus*-secreted by-products. In this simulation, the growth of both species was simulated separately in a defined environment. The secreted by-products and the outer fluxes from *L. amylovorus* growth were used to

complement the media composition used in the *S. cerevisiae* growth simulations (**Methods, Supplementary Data**).

The yeast model³⁸ lacked the capacity to consume some metabolites produced from *L. amylovorus*. These metabolites, present in minute concentrations compared to the main metabolites, were not considered to simplify further analyses. The simulation predicted that yeast would consume the acetaldehyde produced from *L. amylovorus*, resulting in higher biomass and ethanol yields (**Supplementary Data**).

In order to validate these findings, we compared the growth and fermentation performance of *S. cerevisiae* in the presence of acetaldehyde with concentrations between 0 and 1000 mg l⁻¹³⁹. The results suggest that acetaldehyde was able to stimulate the growth rate (**Figure 5A**) of *S. cerevisiae*, in a range from 400 to 800 mg l⁻¹, as well as the ethanol yield, resulting in an increase of more than 10% (**Figure 5B**). The observed results also suggest that the stimulation of yeast growth and ethanol production had an optimum value in a particular acetaldehyde concentration range. Tested concentrations indicate that higher concentrations of acetaldehyde (from 700 to 1000 mg l⁻¹) become inhibitory for yeast growth rate (**Figure 5B**) and biomass production (**Supplementary Figure**) when compared to more intermediate concentrations. Indeed, acetaldehyde is a toxic compound to yeast, and its stimulatory effects are concentration dependent and can become inhibitory above certain threshold values under specific physiological conditions^{39,40}.

Figure 5: The effect of different concentrations of acetaldehyde on yeast physiology. A: Comparison between the growth rates (h^{-1}) of the yeast population in the presence of different concentrations of acetaldehyde. An optimal concentration of acetaldehyde (between 400 to 800 mg l^{-1}) results in growth rates higher than the control (0). **B:** Ethanol yield (%) of yeast fermentations in the presence of different concentrations of acetaldehyde. The highest values of ethanol yield were achieved when the acetaldehyde concentration fell within a range of 400 – 900 mg l^{-1} . Values over lines represent significant p-values from unpaired t-tests.

The redox potential of yeast cells is balanced by two main reactions during fermentations: via the reduction of dihydroxyacetone phosphate (DHAP) to glycerol-3-phosphate (G3P), a reaction catalysed by cytosolic NAD^{+} -dependent G3P dehydrogenase, or by the reduction of acetaldehyde into ethanol via alcohol dehydrogenase 1 (ADH1)⁴¹. This is in line with the FBA simulations, which predicted that 97% of the acetaldehyde flux would go towards ethanol production via alcohol dehydrogenase reduction

(**Supplementary Data**). Therefore, the provision of a higher acetaldehyde pool as an alternative electron acceptor could allow yeast cells to rebalance their NADH/NAD pools solely via ethanol production, allowing cells to distribute more carbon towards pyruvate and biomass production. This carbon rerouting would result in higher ethanol and biomass titres, with the expense of glycerol production. To test this hypothesis, we quantified the glycerol produced by yeast exposed to different acetaldehyde concentrations. As expected, we observed a continuous reduction in glycerol titres following acetaldehyde concentration (**Figure 6A**).

In light of these results, it was necessary to identify which species might contribute to the potential acetaldehyde pool in fermentation. When analysing the supernatants from bacterial cultivations, it is possible to identify *L. amylovorus* as the major aldehyde producer, corroborating the previous observations. The concentration of acetaldehyde in *L. amylovorus* supernatant was *ca.* 460 mg l⁻¹ (**Supplementary Table 2**). According to our experimental analysis, this value could provide both higher ethanol yield and growth rate for the yeast population (**Figure 5**).

Based on these results, we hypothesize that under the given fermentation environment, *L. amylovorus* secretes acetaldehyde, which is readily assimilated by yeast cells to balance the NAD/NADH cytosolic pool and reduced to ethanol. This implies that lower glycerol and higher yeast biomass and ethanol titres are produced, further increasing the ethanol titre (**Figure 6B**).

Figure 6: Acetaldehyde produced by *L. amylovorus* during fermentation is used by yeast as an electron acceptor. **A:** Glycerol production by yeast cells decreased linearly, following increased acetaldehyde concentrations in the fermentations. This decrease was not followed by other measured parameters, such as biomass production, growth rate and ethanol yield (**Figure 4**), suggesting a certain uncoupling of glycerol production and growth in the presence of acetaldehyde. **B:** Proposed mechanism of the metabolic interaction between *L. amylovorus* and *S. cerevisiae*. A representation of yeast growing alone (**without *L. amylovorus***) is shown in the left figure, and a representation of yeast growing in the presence of *L. amylovorus* (**with *L. amylovorus***) is shown in the right figure. During growth, yeast produces glycerol mainly to balance its redox potential by oxidizing NADH into NAD⁺ via the reduction of dihydroxyacetone-P into glycerol-P. This step is necessary due to the accumulation of NADH during fermentation⁴¹. Glycerol is then passively diffused to the extracellular environment, similar to ethanol. Glycerol is also important to maintain the osmo-tolerance of yeast cells, and its production can be induced by high salt and organic acid concentrations⁴³. *L. amylovorus* produces an extra pool of acetaldehyde, which can be reduced into ethanol via the ADH1 (alcohol dehydrogenase 1) enzyme in yeast. This extra pool of acetaldehyde pushes the equilibrium towards ethanol production, and most of the NADH will be oxidized in this step. This will result in lower glycerol production (faint arrows and words) and higher ethanol production (bold arrows and words). In addition, this extra acetaldehyde pool can lead to pyruvate accumulation, resulting in higher

biomass production (bold arrows and words). Several biochemical steps were suppressed for the sake of clarity. Values over lines represent significant p-values from unpaired t-tests.

Discussion

In this study we show how synthetic microbial communities and functional landscape analysis can help resolve the interactions found in actual microbial communities^{5,6,8,46}. We demonstrate that bacterial competition is a major microbial interaction found in such communities and that higher-order interactions have a key role in buffering deleterious pairwise functional interactions between bacteria and yeast. These findings are in accordance with previous studies showing that competition is the major interaction found in microbial communities²⁶ and that higher-order interactions are common forces involved in shaping the functionality of microbial communities^{7,47}. Our findings also show that contamination control methods should be more precise⁴⁷ and should discriminate among different species of bacteria⁴⁹. Our analysis identified a key bacterial contaminant—*L. amylovorus*—whose presence was positively associated with higher ethanol yields. We found that this may be caused by two different mechanisms.

FBA simulation of yeast growing in the presence of the by-products secreted by *L. amylovorus* identified a second potential mechanism that may also lead to increased ethanol yield, and this mechanism was later validated via laboratory experiments. Such a mechanism is based on the reduction of acetaldehyde produced by bacteria into ethanol by yeast to balance the cytosolic redox potential^{39,41}. This results in lower glycerol production and higher ethanol yields by the yeast.

While *L. amylovorus* has a positive effect on yeast ethanol production, we found that this effect is contingent on the additional presence of other taxa in the consortia. Our findings related to the stabilizing effect of high-order interactions indicate that biodiversity in the bacterial community is positively associated with the ethanol function of our consortia and that the beneficial effect of *L. amylovorus* is exhibited when in

co-culture with other bacterial contaminants. This finding opens the way to identifying specific multispecies consortia that may optimally promote bioethanol production.

Maintaining a viable population of *L. amylovorus* seems advantageous for industrial processes. Moreover, yeast is capable of providing a niche to certain lactobacilli via its nitrogen metabolism⁴⁴. This could result in a mutualistic interaction between these two species, benefiting an industrial process that still regards and responds to bacterial contaminants as being solely detrimental⁴⁵.

In summary, our results suggest that multispecies consortia containing *L. amylovorus* could be considered a new class of “industrial probiotics”. Our findings predict that adding these consortia to a fermenter during industrial ethanol fermentations would benefit the yeast population and the overall industrial process performance. We hypothesize that this effect could potentially stem both from competition towards other bacteria⁴⁹, through stimulation of the yeast central carbon metabolism⁴⁴, or both.

Materials and Methods

Chemicals

Unless stated otherwise, all chemicals and reagents used were of analytical grade and were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Strains used in laboratory experiments

Saccharomyces cerevisiae strain PE-2 was kindly provided by Prof Thiago Olitta Basso. Strains of *Lactobacillus amylovorus* and *Lactobacillus fermentum* were isolated from stored industrial samples. Strains of *Pediococcus claussenii*, *Lactobacillus helveticus*, *Lactobacillus buchneri* and *Zymomonas mobilis* were purchased from ATCC (Manassas, VA, USA).

333

334 **Isolation and maintenance of industrial strains**

335 For strain isolation, a previously introduced protocol was used¹⁹. Briefly, industrial samples were serially
336 diluted in sterile PBS and plated on Man Rogosa Sharpe (MRS) agar medium plates containing
337 cycloheximide (0.1% v.v⁻¹) to inhibit yeast growth. Plates were incubated at either 30°C or 37°C statically.
338 A loopful of an isolated colony was grown in liquid MRS under the same conditions and stored at -80°C.
339 Yeast strains were cultured in yeast potato dextrose (YPD) medium at 30°C. Lactobacilli were cultured in
340 MRS medium, either at 30°C or 37°C, and *Zymomonas mobilis* was cultured in trypsin soy broth (TSB)
341 medium, at 30°C. All cultivations for inoculum preparation were performed statically, *ca.* 5 ml volume.

342

343 **Fermentation experiments and combinatorial assembly**

344 Fermentations were performed in 96 deep-well plates with either pairwise cultivations (yeast:bacteria at a
345 ratio of 100:1)⁸ or standalone yeast or bacteria co-cultivations. A semi-synthetic medium that is able to
346 simulate sugarcane molasses-based medium (SM) was used¹². Briefly, all strains were cultured in their
347 optimal media and conditions for up to 48 h. After that, the biomass was calculated via optical density (OD;
348 600 nm wavelength). All cells were pelleted via centrifugation (3400 x g, 4°C, 15 min) and washed twice
349 with sterile PBS. Subsequently, cells were diluted in SM diluted in sterile Milli-Q H₂O (10x, final sugar
350 concentration of 18 g l⁻¹) for an OD value of 1.0. Strains were later diluted in fresh SM medium in specific
351 wells in the 96 deep-well plate to a final OD value of 0.1.

352 All co-cultivations were performed statically overnight at 30°C in *ca.* 1 ml of medium, in triplicate.
353 Combinatorically assembled communities of the six bacterial species were prepared by inoculating the same
354 volume from each bacterial seed culture into 96 deep-well plates (VWR) containing 500 µL of 10x diluted
355 SM. The final yeast and bacteria ratio (OD) was maintained at 100. This means that the final cell count of
356 each individual bacterial population decreased as the number of species in the community increased.

The fermentation metabolites (glycerol, ethanol, lactic and acetic acid) were determined by high-performance liquid chromatography (HPLC) (UltiMate 3000, Thermo-Fischer Scientific, Waltham, Massachusetts, USA). The analytes were separated using an Aminex HPX-87H ion exclusion column (Bio-Rad, Hercules, California, USA) and were isocratically eluted at 60°C, with a flow rate of 0.6 ml.min⁻¹, using a 5 mM sulfuric acid solution as the mobile phase. The detection was performed refractrometrically.

Ethanol yield was calculated as follows:

$$\text{Ethanol yield} = \frac{(\text{EtOH}_{\text{obs}} \times 100)}{\text{EtOH}_{\text{theor}}}$$

Equation (1): Ethanol yield calculation. EtOH_{obs} = the observed ethanol titre for each sample. EtOH_{theor} = the maximum theoretical ethanol titre for each sample. The sugar titre from the broth solution was multiplied with the stoichiometric conversion factor for ethanol production (i.e., 0.5111)¹¹.

Community composition was resolved via flow cytometry (BD LSRFortessa™, BD Biosciences, Franklin Lakes, New Jersey, USA). A sample from each well (10 µl) was taken after overnight cultivation, transferred to a new microplate and diluted in 190 µl of PBS buffer (pH 7.4). Yeast and bacterial populations were resolved via front and side scatter comparison (SSC versus FSC).

Functional landscape analysis

To study the structure-function landscape of our communities, we expressed the function (F) of interest (ethanol yield) as shown below:

$$F = \log(\text{Yield}_{\text{yeast}} + \frac{\text{Community}}{\text{Yield}_{\text{yeast}}})$$

Equation (2): Community function (F) is expressed as total ethanol yield from the community. Here, F is defined as the log value of the ethanol yield of standalone yeast fermentations ($\text{Yield}_{\text{yeast}}$) and the sum of the ethanol yield from different community structures (Community) divided by yeast standalone fermentation values.

The ethanol yield ratio was expressed in a logarithmic manner, allowing us to use an additive null model to quantify the interactions among our bacterial species.

Using this model and following the rationale presented elsewhere⁷, we were able to decompose the function of every community, F_C , into single, pairwise and higher order effects:

$$F_C = \sum_i F_i + \sum_{i \neq j} \epsilon_{ij} + H_C$$

Equation (3): Community model integrating the effects of single, pairwise and higher-order interactions. F_C is the community function, F_i is the single effects functions, ϵ_{ij} is the effect of pairwise interactions between species (i and j) and H_C is the higher order effects.

The single effects function F_i can be computed simply as the function of co-cultures of single species with yeast. Pairwise interactions can be estimated as the function of co-cultures of pairs of species with yeast that are not explained by single effects:

$$\epsilon_{ij} = F_{ij} - F_i - F_j$$

Equation (4): Calculation of pairwise effects (ϵ_{ij}), where the function values between yeast and different bacterial species in monoculture (F_i and F_j) are subtracted from the function value of co-culture pairs (F_{ij}).

Following the same logic, in communities with 3 or more members, higher-order interactions can be measured as a function that is unexplained by the sum of single and pairwise effects.

By truncating **equation (2)**, we can compute the expected structure function landscape for each community when considering only additive effects (**Figure 2C**) or a landscape that takes both single and pairwise effects into consideration (**Figure 2D**).

Construction of metabolic models

Genome-scale metabolic models from the used bacteria were constructed using the automated tool CarveMe⁵⁰. Reference genomes used to construct the models of the following bacteria were downloaded from the NCBI database: *L. amylovorus* 30SC; *L. fermentum* IFO 3956; *L. helveticus* CAUH18; *P. claussenii* ATCC BAA 344; *L. buchneri* CD034 and *Z. mobilis* ATCC 10988.

All models were gapfilled to allow anaerobic growth in minimal medium (M9). The command line used was “carve genome.faa --gapfill M9[-O2] -u grampos -o model.xml.gz”, where “genome.faa” stands for the annotated genome of each of the selected microorganisms, and model.xml.gz is the final file name. For *Z. mobilis*, the universal gram-negative model gramneg was used for top-down reconstruction.

Prior to any analysis, the models were used to simulate growth in defined conditions (see “Flux balance analysis” session) to corroborate the quality of the model.

For *S. cerevisiae*, the model used was the extensively curated iMM904³⁸.

Flux balance analysis

Flux balance analysis simulations were performed using COBRApy⁵¹. Briefly, a simple environment simulating defined minimal medium was created (**Supplementary Data**). In order to sustain yeast growth,

the minimal medium allowed the fluxes of NH₄, SO₄, Pi, H₂O, K, Na, and CO₂ to be unconstrained. The environment was also composed of ergosterol, zymosterol, palmitoleate (C16:1), stearate (C18:20), oleate (C18:1), and linoleate (C18:2), which were freely exchanged (lower bound = -1000). The lower bound of the exchange reaction for oxygen was set to 0 (anaerobic environment), and the carbon sources (glucose, fructose and sucrose) were each set to -10.

The growth of *Lactobacillus amylovorus* and *Saccharomyces cerevisiae* was simulated separately in this environment. To simulate the cross-feeding between both species, the secreted metabolites from *L. amylovorus* were included in the yeast environment, and their exchange fluxes were constrained based on the out fluxes from *L. amylovorus* (**Supplementary Data**)⁵². Any metabolite for which the yeast model lacked the capacity to assimilate was not considered in this simulation.

Acetaldehyde quantification

The different bacterial species were grown overnight at 30°C in 10x diluted SM. After this, the acetaldehyde concentration in the fermented media was measured enzymatically using the Acetaldehyde Assay Kit (K-ACHYD - Megazyme, Bray, Ireland), following the manufacturer's instructions.

Analysis of the effect of acetaldehyde on yeast physiology

Acetaldehyde was added to fresh Milli-Q-diluted SM at different concentrations³⁹, ranging from 100 to 1000 mg l⁻¹, with 100 mg l⁻¹ increments. All cultivations had an initial OD of 0.1.

The rate of yeast growth was analysed in the presence of different acetaldehyde concentrations at 30°C under agitation (double orbital, fast mode) in Synergy H1 plate readers (Biotek Instruments, Inc. Winooski, VT, USA). The OD was measured every 15 minutes for 24 h. The growth rate was later calculated using the R package *growthcurver*⁵³.

Biomass production and ethanol yield were measured from static fermentations in 10x diluted SM. Fresh medium (5 ml) was inoculated with yeast cells grown overnight in YPD medium. All fermentations were performed statically at 30°C for 24 h. After fermentation, a sample was taken, and the final OD value was measured. The remaining volume was centrifuged, and an aliquot of 1 ml was saved for HPLC analysis (see section “Fermentation experiments and combinatorial assembly”).

Statistical analyses

All the statistical analyses mentioned in this study were performed using either GraphPad Prism 8 or R software.

Data availability

All data from this study are available from the corresponding author upon request.

Code availability

All codes used in this study are available from the corresponding author upon request.

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Author contributions

FSOL, DB, JCCV, AS and MOAS designed the study. FSOL performed the fermentation experiments, combinatorial assemblies, analysis of acetaldehyde on yeast physiology and acetaldehyde quantification in bacterial supernatants, as well as data analysis. DB and AS performed the functional landscape

585 analysis. FSOL and JCCV built the metabolic models and performed the FBA simulations, as well as
586 analysed the results together with AS. MOAS supervised the entire study. All authors contributed to the
587 writing of this manuscript.

588

589 **Competing interests**

590 The authors declare no competing interests.

Figures

Figure 1

Schematics of the combinatorial assembly experimental setup. A: Microbial species used in this study. These species make up more than 80% of the diversity found in fuel ethanol fermentations in relative abundance. B: Initially, pairwise fermentations between yeast inoculum and all available bacteria were performed. This results in a combinatorial assembly of order 1. In parallel, every pairwise assembly was also mixed with every other bacterial monoculture, resulting in a set of 51 different fermentations or order 2. This was performed for every possible combination of bacterial species and yeast, resulting in 258 fermentations, and a total order of 6 (i.e., a community containing up to 6 different bacterial species and yeast). The ratio between all bacterial species was kept at 1 for every cultivation, and the yeast:bacteria

ratio was 100:C: After fermentation was completed, the community structure was analysed via flow cytometry, and the fermentation metabolites were quantified via HPLC. D: An overview of the microbial compositions generated via the combinatorial assembly throughout the 258 fermentations. This figure was created using Biorender (<https://app.biorender.com/>).

Figure 2

The community structure of yeast and bacteria co-cultivations. A: Final cell counts of yeast (blue) and bacteria (red) from different community structures. The final yeast cell counts dropped from $1.1 \times 10^7 \pm 1 \times 10^6$ to ca. $9.1 \times 10^6 \pm 9.3 \times 10^5$ cells ml^{-1} as the number of bacterial species in the community increased (one-way ANOVA, $p < 0.0001$). The final bacterial cell counts dropped from $1.5 \times 10^7 \pm 1.5 \times 10^6$ to ca. $8 \times 10^5 \pm 1.4 \times 10^5$ cells ml^{-1} (one-way ANOVA, $p = 0.0002$). Symbols and error bars represent the mean $\pm$ standard deviation, $N = 3$. B: The relative abundance of yeast and bacterial populations in different community structures. The relative abundance of the yeast population (blue bar) increased from $90.3 \pm 1.6\%$ in pairwise cultivations to $98.8 \pm 0.3\%$ in the presence of all 6 different bacterial species (one-way ANOVA, $p < 0.0001$). The bacterial relative abundance (red bar) decreased from $9.7 \pm 1.8\%$ initially in pairwise cultivations to $1.2 \pm 0.3\%$ in the most complex community tested, which contained yeast and 6 different bacterial species (one-way ANOVA, $p < 0.0001$).

Figure 3

The ecological interactions within the sugarcane biorefinery microbial community. A: Observed functional landscape. We plotted the measured function for communities of different species. Each line represents the effect of the addition of one species to a community. The red line represents the average of all points for each community size. B: The expected functional landscape when considering the presence/absence (additive) null model. The inset shows the difference between the measured and predicted functions,

plotted against community size, as a way to visualize the fit of the data to the null model. C: Heat map showing the interactions among pairs of bacterial species (asterisks represent significance taking $\pm 2SE$). These interactions were diverse and mostly detrimental to ethanol yield. The epistasis difference (epsilon) between these species also suggests that such interactions are strain driven. D: The functional landscape was predicted by adding up single and pairwise effects. This landscape predicts a rapid reduction in function as more species are added. E: The difference between the prediction of the model in panel D is plotted against community size. For visualization, a jitter was applied to points of different composition but the same community size. The plotted difference effectively measures the influence of high-order interactions.

Figure 4

The effect of the presence of *L. amylovorus* on ethanol fermentations. A: Fermentations performed with *L. amylovorus* in the composition of the microbial community showed higher fermentation yields ($3.02 \pm 1.06\%$, $p=0.009$; Wilcoxon rank-sum analysis) in comparison to the others lacking this species. B: Fermentations with microbial communities containing *L. amylovorus* showed higher yeast cell counts (Wilcoxon rank-sum analysis, $p=0.043$).

Figure 5

The effect of different concentrations of acetaldehyde on yeast physiology. A: Comparison between the growth rates (h^{-1}) of the yeast population in the presence of different concentrations of acetaldehyde. An optimal concentration of acetaldehyde (between 400 to 800 mg l^{-1}) results in growth rates higher than the control (0). B: Ethanol yield (%) of yeast fermentations in the presence of different concentrations of acetaldehyde. The highest values of ethanol yield were achieved when the acetaldehyde concentration fell within a range of 400 – 900 mg l^{-1} . Values over lines represent significant p-values from unpaired t-tests.

Figure 6

Acetaldehyde produced by *L. amylovorus* during fermentation is used by yeast as an electron acceptor. A: Glycerol production by yeast cells decreased linearly, following increased acetaldehyde concentrations in the fermentations. This decrease was not followed by other measured parameters, such as biomass production, growth rate and ethanol yield (Figure 4), suggesting a certain uncoupling of glycerol production and growth in the presence of acetaldehyde. B: Proposed mechanism of the metabolic interaction between *L. amylovorus* and *S. cerevisiae*. A representation of yeast growing alone (without *L. amylovorus*) is shown in the left figure, and a representation of yeast growing in the presence of *L. amylovorus* (with *L. amylovorus*) is shown in the right figure. During growth, yeast produces glycerol mainly to balance its redox potential by oxidizing NADH into NAD⁺ via the reduction of dihydroxyacetone-P into glycerol-3P. This step is necessary due to the accumulation of NADH during fermentation⁴¹. Glycerol is then passively diffused to the extracellular environment, similar to ethanol. Glycerol is also important to maintain the osmo-tolerance of yeast cells, and its production can be induced by high salt and organic acid concentrations. *L. amylovorus* produces an extra pool of acetaldehyde, which can be reduced into ethanol via the ADH1 (alcohol dehydrogenase 1) enzyme in yeast. This extra pool of acetaldehyde pushes the equilibrium towards ethanol production, and most of the NADH will be oxidized in this step. This will result in lower glycerol production (faint arrows and words) and higher ethanol production (bold arrows and words). In addition, this extra acetaldehyde pool can lead to pyruvate accumulation, resulting in higher biomass production (bold arrows and words). Several biochemical steps were suppressed for the sake of clarity. Values over lines represent significant p-values from unpaired t-tests.

Supplementary Files

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