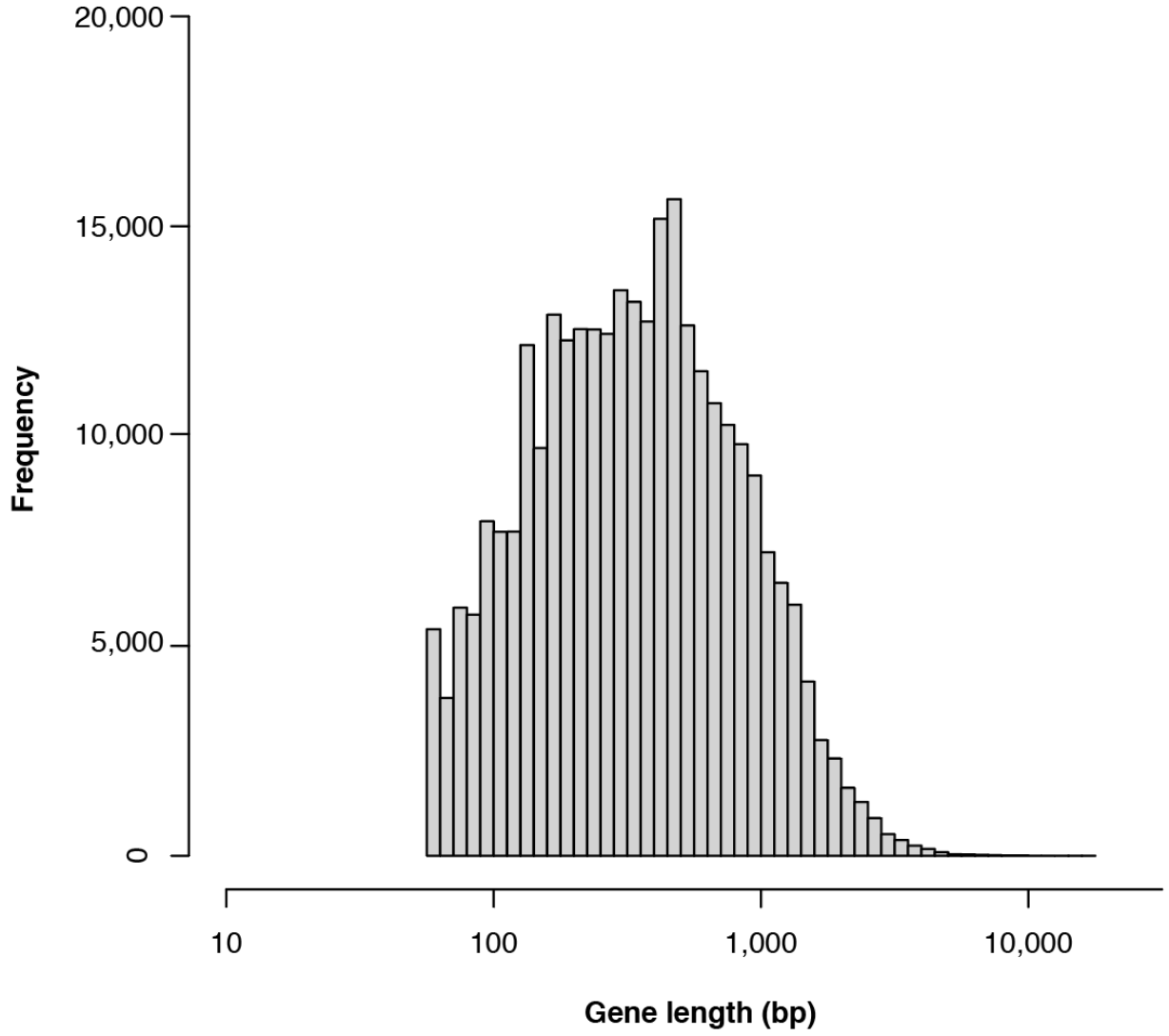
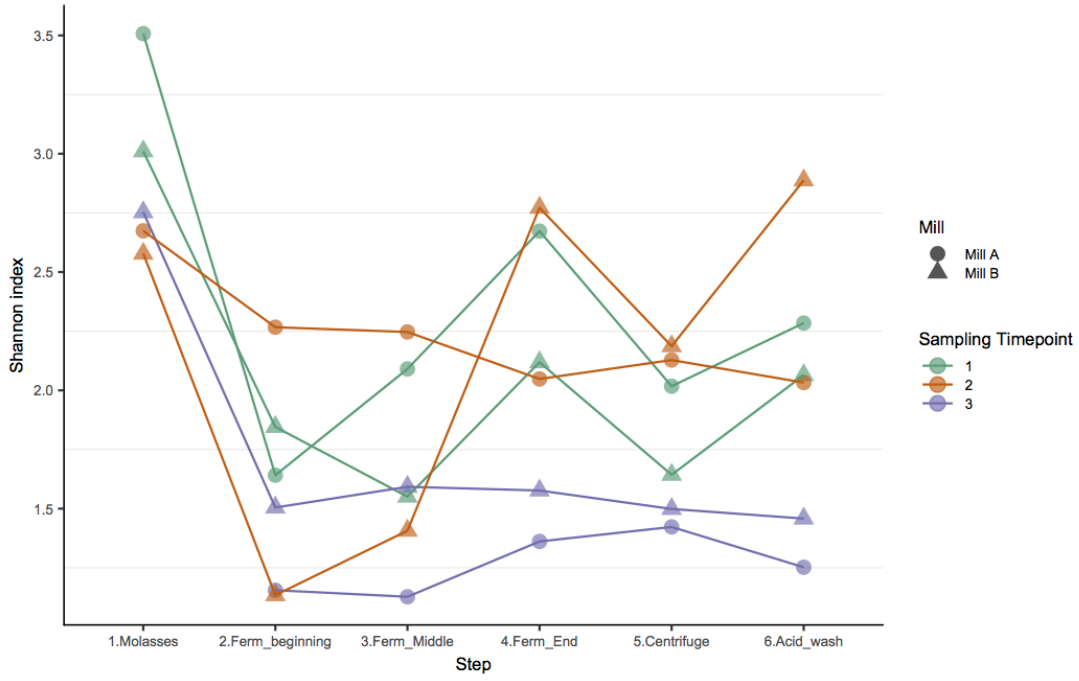
# Strain dynamics of contaminating bacteria modulate the yield of ethanol biorefineries

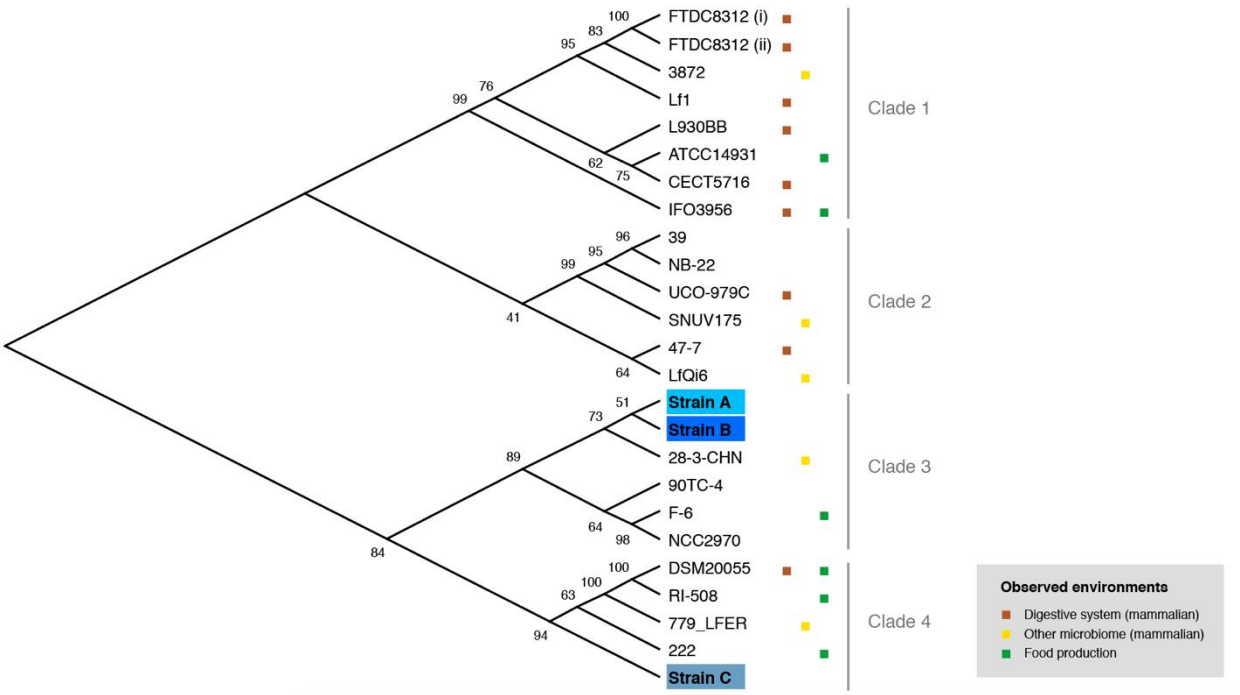
Lino *et al.*



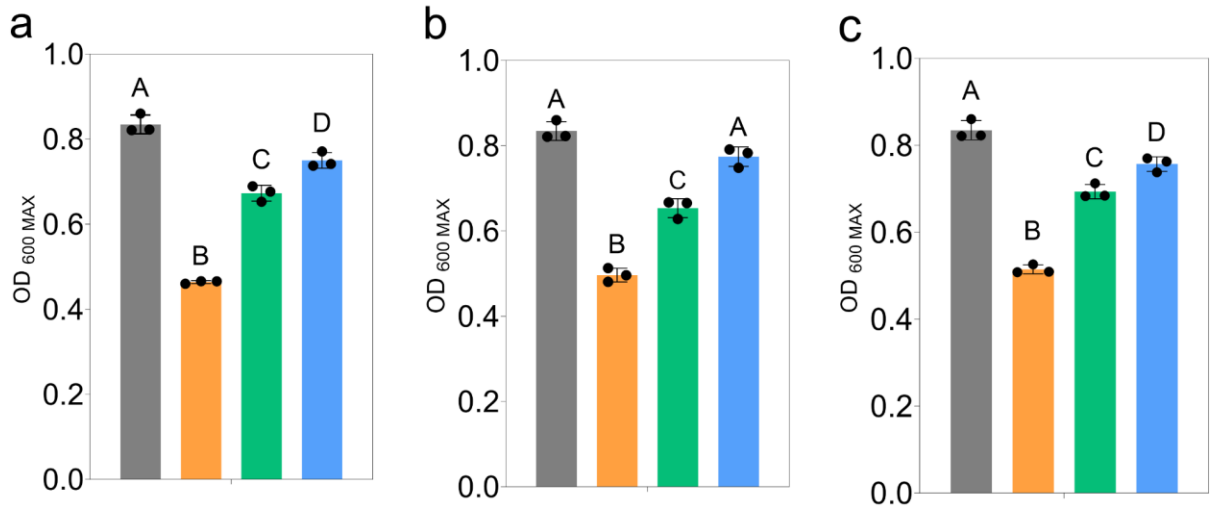

**Supplementary Figure 1. Distribution of sequence lengths in the bioethanol production gene catalogue.** Gene lengths ranged from 57 – 17,469 bp, with median of 336 bp. Source data are provided as a Source Data file.



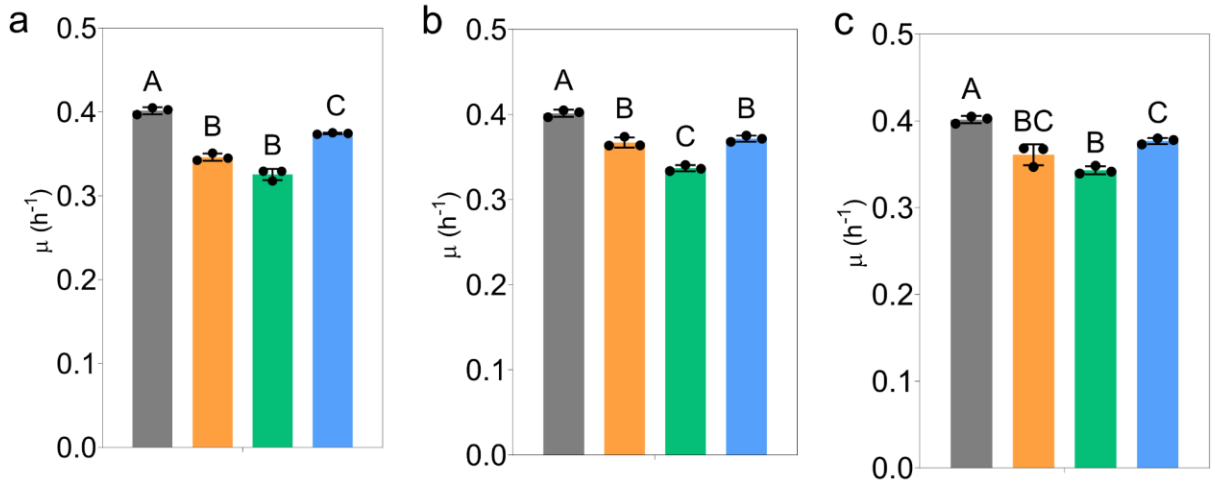

**Supplementary Figure 2. Compositional changes within the bacterial community, across process steps in a single production season.** Comparison of Shannon diversity index values for each collected sample: 1) molasses (or broth); 2) beginning, 3) middle, and 4) end of fermentation; 5) centrifuge; and 6) acid wash, in Mills A (circle) and B (triangle). Samples from the same collection timepoint share colour; line connects those from the same mill. Fermentation drastically reduces bacterial diversity in the starting molasses, which recovers as the bioprocess continues. Community diversity remains relatively stable in samples collected at the end of the production season (purple). Source data are provided as a Source Data file.



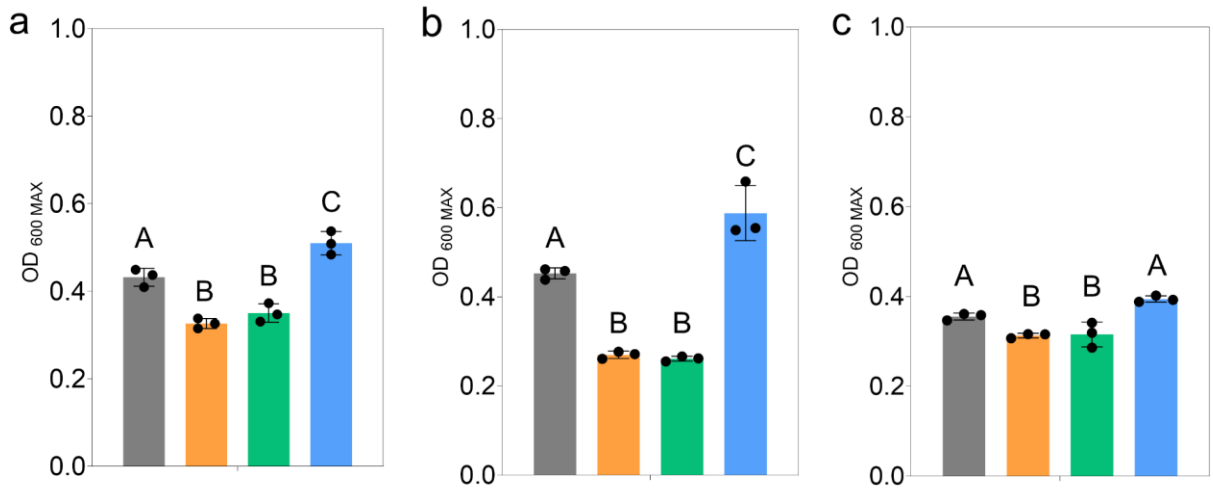

**Supplementary Figure 3. Strains A, B and C cluster with *L. fermentum* strains isolated from other industrial processes.** Cladogram is identical to Fig. 4B in the manuscript (here, with no adjustments to the root). Each strain is marked with coloured boxes denoting the environments it has been observed in (from other studies). While clades 1 and 2 primarily consist of *L. fermentum* strains associated with the mammalian gut microbiome, strains in clades 3 and 4 (containing our isolates A and B, and C, respectively) originate from an industrial setting, e.g. food production. No information was available for *L. fermentum* strains 39, NB-22, 90TC-4 and NCC2970. Source data are provided as a Source Data file.



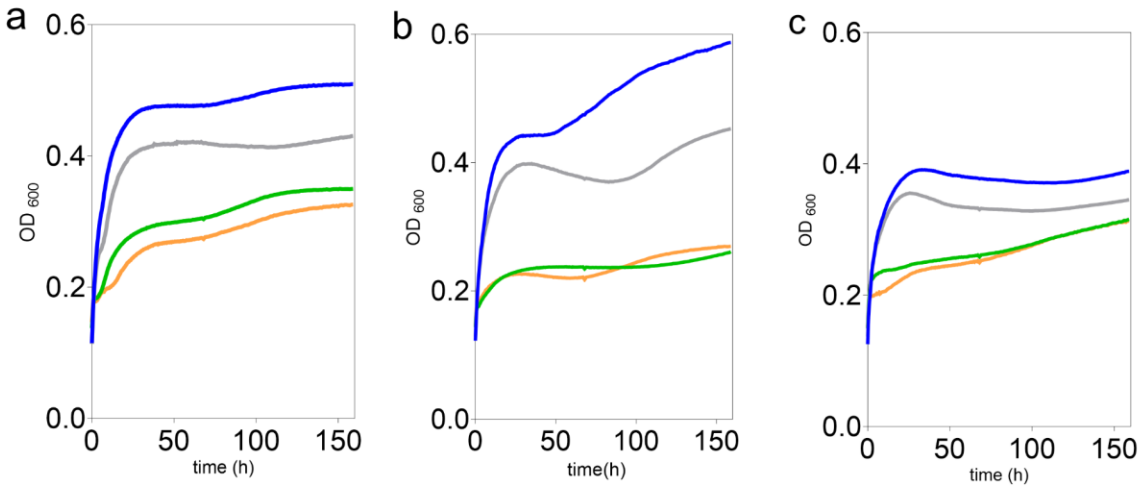

**Supplementary Figure 4. Statistical comparison of maximum optical density (OD600) of *Saccharomyces cerevisiae***. OD600 observed for each *L. fermentum* a) strain A, b) strain B and c) strain C in 4 conditions: diluted sugarcane molasses (gray), molasses previously fermented with *L. fermentum* strain (orange), molasses previously fermented with added sugar (to restore the original sugar titres) (green), fresh molasses spiked with key bacterial metabolites (blue). In each graph, treatments sharing the same letter have no significant difference in maximum optical density and treatments with different letters have significant difference in maximum optical density. Source data are provided as a Source Data file.



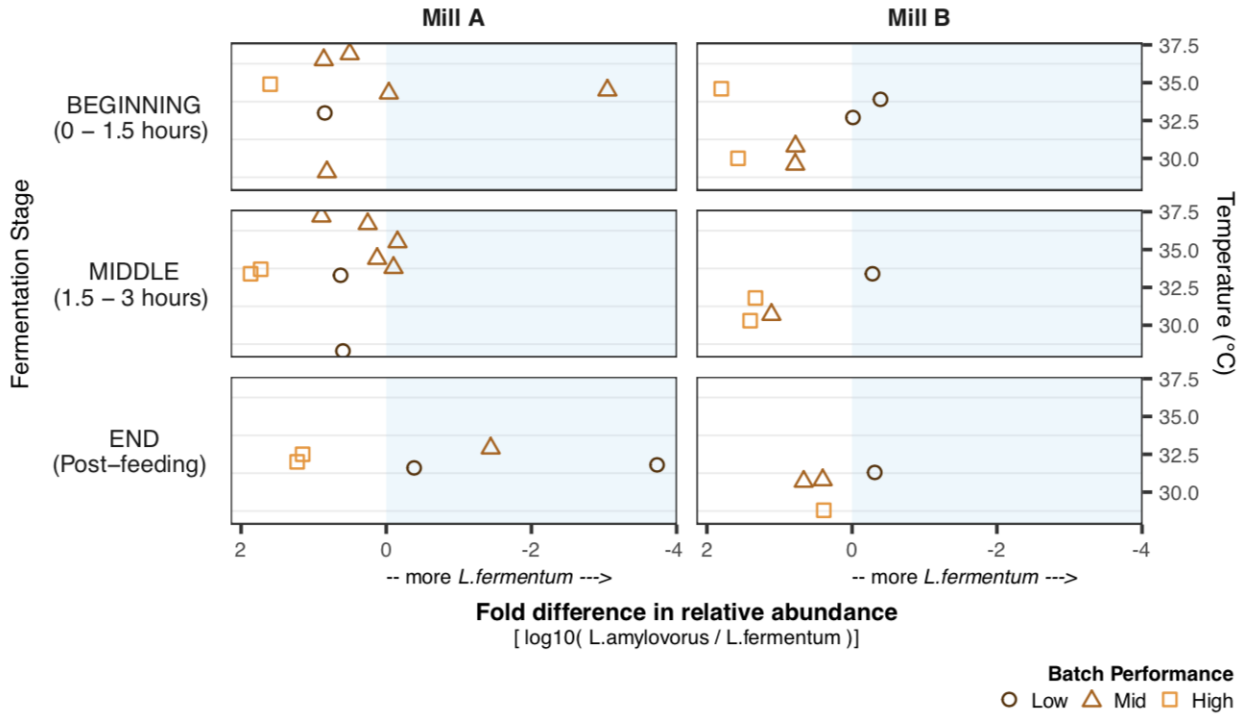

**Supplementary Figure 5. Statistical comparison of maximum specific growth rates of *Saccharomyces cerevisiae*.** Specific growth rates observed for each *L. fermentum* a) Strain A, b) Strain B and c) Strain C in 4 conditions: diluted sugarcane molasses (gray), molasses previously fermented with *L. fermentum* strain (orange), molasses previously fermented with added sugar (to restore the original sugar titres) (green), fresh molasses spiked with key bacterial metabolites (blue). In each graph, treatments sharing the same letter have no significant difference in maximum specific growth rate and treatments with different letters have significant difference in maximum specific growth rate. Tukey's multiple comparison test - $P < 0.05$. Source data are provided as a Source Data file.



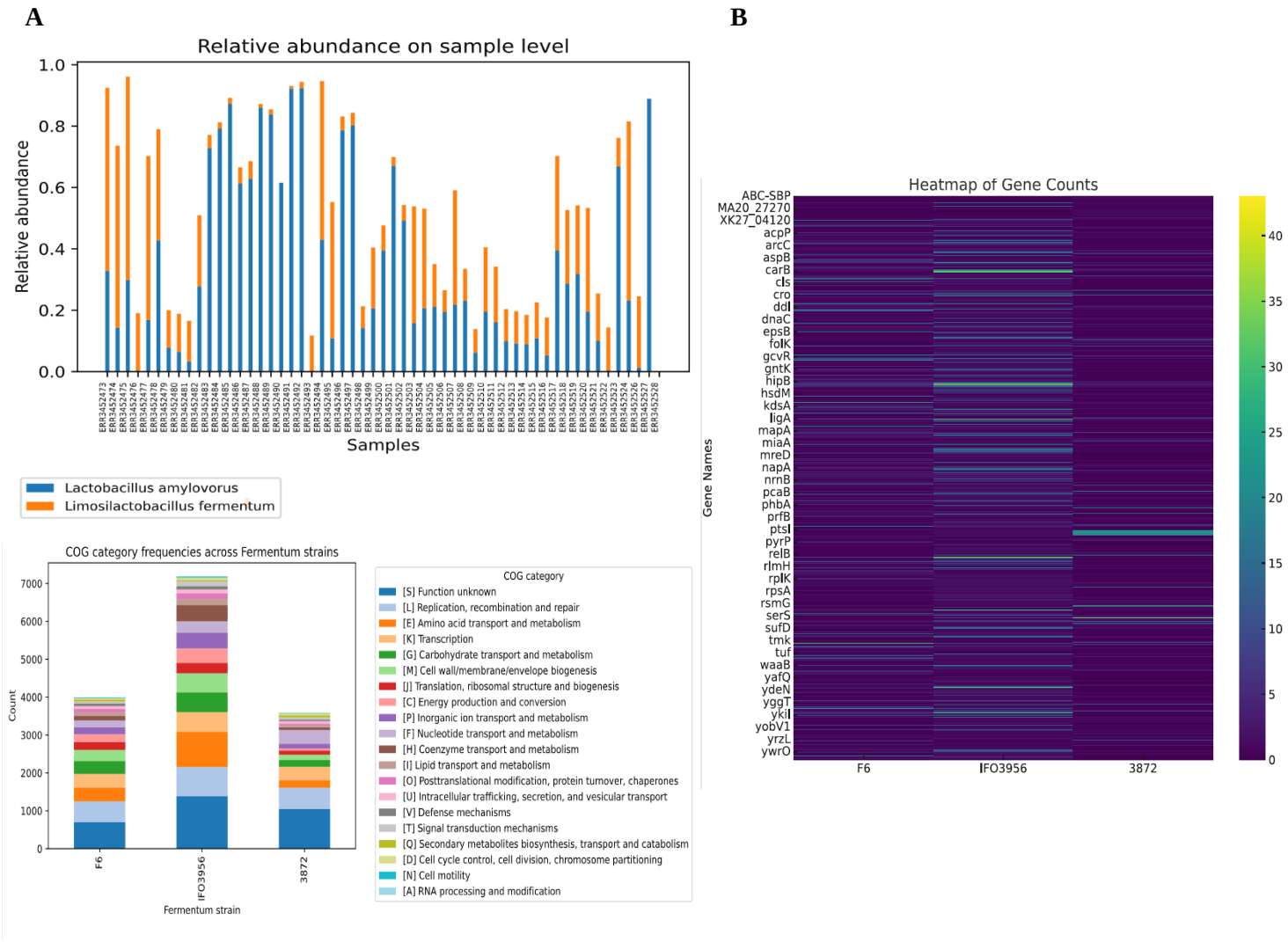

**Supplementary Figure 6. Statistical comparison of maximum optical density (OD600) of each *L. fermentum* strain.** a) Strain A, b) Strain B and c) Strain C observed for in 4 conditions: diluted sugarcane molasses (gray), molasses previously fermented with *S. cerevisiae* strain (orange), molasses previously fermented with added sugar (to restore the original sugar titres) (green), fresh molasses spiked with key yeast metabolites (blue). In each graph, treatments sharing the same letter have no significant difference in maximum optical density (OD600) and treatments with different letters have significant difference in maximum optical density (OD600). Tukey's multiple comparison test - $P < 0.05$



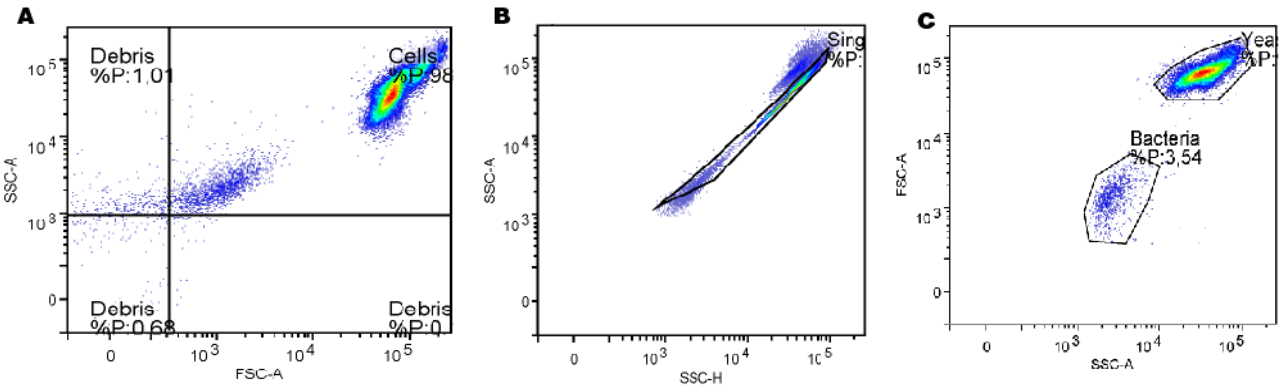

**Supplementary Figure 7. Growth curve of *L. fermentum.*** a) Strain A, b) Strain B and c) Strain C. Growth curve obtained in the following media: diluted sugarcane molasses (gray), molasses previously fermented with *S. cerevisiae* strain (orange), molasses previously fermented with added sugar (to restore the original sugar titres) (green), fresh molasses spiked with key yeast metabolites (blue). Growth conducted in triplicate on the Tecan Infinite® 200 PRO microplate reader at a temperature of 30°C for 150 hours with an initial OD of 0.1




**Supplementary Figure 8. Temperature does not influence *L. fermentum* and *L. amylovorus* interplay or batch performance.** Comparison of temperature and *L. fermentum* and *L. amylovorus* populations on fermentation performance in Mills A and B (columns). Zero-fold difference denotes equal amount of the two bacteria; blue region highlights batches with greater numbers of *L. fermentum*. In both mills, batches displaying poorer performance are characterised by a dominance of *L. fermentum* over *L. amylovorus* at the final fermentation step, after media feeding has stopped (circles and triangles in the blue region, bottom row). Fermentation performance is not directly impacted by vat temperature.




**Supplementary Figure 9. Metagenome-assembled genomes (MAGs) analyses.** A: Relative bacterial abundance values obtained from MAGs for *L.fermentum and L.amylovorus* across all samples. B: Using MAGs from all samples, gene annotations of three *L.fermentum* strains along with their metabolic characterisation: (top) COG category frequencies across strains; (bottom) Heatmap of gene annotation counts across strains. Source data are provided as a Source Data file.




**Supplementary Figure 10. The gating strategy applied to resolve both yeast and bacterial populations.** A: Initially cells are separated from media debris, using side scatter (SSC-A) and front scatter (FSC-A) parameters. The gating named "Cells" contain all the yeast and bacteria cells, and not the media debris. B: By comparing area versus height parameters (SSC-A and SSC-H, respectively) from the gated events from the gate "Cells", the singlets are separated from duplets, in the gate "Singlets". C: Yeast and bacterial cells are separated based on their volume and granularity, using SSC-A and FSC-A, respectively, from the gate "Singlets". Source data are provided as a Source Data file.

**Supplementary Table 1. Taxonomic profiling of bacterial populations across study samples.**

| Species | Phylum | Relative abundance (average %) | st.dev. | Relative abundance (minimum %) | Relative abundance (maximum %) | Samples detected (of 56) | |
|---|---|---|---|---|---|---|---|
| *Lactobacillus amylovorus* | Firmicutes | 45,2 | 24,8 | 0,008 | 76,3 | 56 | |
| *Lactobacillus fermentum* | Firmicutes | 19,6 | 17,6 | 0,945 | 60,5 | 56 | |
| *Lactobacillus helveticus* | Firmicutes | 5,3 | 3,6 | 0,029 | 16,2 | 56 | |
| *Pediococcus claussenii* | Firmicutes | 4,7 | 2,0 | 0,148 | 8,0 | 56 | Top 10 |
| *Lactobacillus buchneri* | Firmicutes | 2,0 | 5,1 | 0,048 | 33,0 | 56 | most |
| *Zymomonas mobilis* | Proteobacteria | 2,0 | 4,3 | 0,005 | 26,6 | 55 | abundant |
| *Lactobacillus plantarum* | Firmicutes | 1,5 | 1,9 | 0,121 | 12,7 | 55 | species |
| *Lactobacillus mucosae* | Firmicutes | 1,4 | 1,7 | 0,032 | 6,3 | 55 | |
| *Acetobacter pasteurianus* | Proteobacteria | 1,3 | 7,6 | 0,001 | 56,9 | 51 | |
| *Bacillus cereus* | Firmicutes | 1,2 | 2,8 | 0,003 | 18,6 | 56 | |
| *Leuconostoc mesenteroides* | Firmicutes | 1,1 | 4,1 | 0,03 | 29,8 | 56 | |
| *Streptococcus infantarius* | Firmicutes | 0,9 | 1,8 | 0,002 | 11,8 | 56 | |
| *Lactobacillus paracasei* | Firmicutes | 0,9 | 1,0 | 0,061 | 5,3 | 55 | |
| *Lactobacillus amylolyticus* | Firmicutes | 0,8 | 1,4 | 0,002 | 7,1 | 56 | |
| *Lactobacillus gallinarum* | Firmicutes | 0,7 | 0,4 | 0,011 | 2,1 | 56 | |
| *Lactobacillus rhamnosus* | Firmicutes | 0,6 | 1,5 | 0,008 | 6,6 | 56 | |
| *Geobacillus stearothermophilus* | Firmicutes | 0,5 | 1,1 | 0,001 | 5,5 | 41 | |
| *Lactobacillus casei* | Firmicutes | 0,4 | 0,3 | 0,01 | 2,2 | 56 | |
| *Lactobacillus parabuchneri* | Firmicutes | 0,4 | 0,5 | 0,008 | 2,3 | 56 | |
| *Bacillus coagulans* | Firmicutes | 0,4 | 0,7 | 0,001 | 3,0 | 52 | |
| *Geobacillus lituanicus* | Firmicutes | 0,4 | 0,8 | 0,006 | 4,0 | 37 | |
| *Lactobacillus delbrueckii* | Firmicutes | 0,3 | 0,5 | 0,048 | 2,5 | 56 | |
| *Parageobacillus thermoglucosidar* | Firmicutes | 0,3 | 0,7 | 0,003 | 2,9 | 35 | |
| *Geobacillus thermoleovorans* | Firmicutes | 0,3 | 0,6 | 0,005 | 3,2 | 34 | |
| *Lactobacillus acetotolerans* | Firmicutes | 0,2 | 0,4 | 0,004 | 2,4 | 56 | |
| *Acetobacter senegalensis* | Proteobacteria | 0,2 | 0,8 | 0,001 | 5,1 | 36 | |
| *Weissella paramesenteroides* | Firmicutes | 0,2 | 0,4 | 0,003 | 1,9 | 56 | |
| *Geobacillus kaustophilus* | Firmicutes | 0,2 | 0,3 | 0,002 | 1,8 | 32 | |
| *Acetobacter aceti* | Proteobacteria | 0,2 | 1,1 | 0,001 | 8,2 | 24 | |
| *Lactobacillus agilis* | Firmicutes | 0,1 | 0,3 | 0,001 | 1,5 | 52 | |
| *Cutibacterium acnes* | Actinobacteria | 0,1 | 0,3 | 0,001 | 1,8 | 49 | |
| *Weissella cibaria* | Firmicutes | 0,1 | 0,3 | 0,008 | 2,2 | 56 | |
| *Escherichia coli* | Proteobacteria | 0,1 | 0,2 | 0,001 | 1,3 | 51 | |
| *Stenotrophomonas maltophilia* | Proteobacteria | 0,1 | 0,3 | 0,001 | 2,6 | 34 | |
| *Mycobacterium tuberculosis* | Actinobacteria | 0,0 | 0,3 | 0,001 | 2,3 | 56 | |
| *Gluconobacter oxydans* | Proteobacteria | 0,0 | 0,2 | 0,001 | 1,4 | 35 | |
| *Leuconostoc citreum* | Firmicutes | 0,0 | 0,2 | 0,001 | 1,6 | 50 | |
| *Enterobacter cloacae* | Proteobacteria | 0,0 | 0,2 | 0,001 | 1,1 | 53 | |
| *Pantoea ananatis* | Proteobacteria | 0,0 | 0,2 | 0,001 | 1,7 | 29 | |
| *Lactobacillus pentosus* | Firmicutes | 0,0 | 0,2 | 0,001 | 1,3 | 50 | |
| *Xanthomonas albilineans* | Proteobacteria | 0,0 | 0,1 | 0,001 | 1,1 | 33 | |
| *Burkholderia gladioli* | Proteobacteria | 0,0 | 0,2 | 0,001 | 1,3 | 27 | |
| *Gluconacetobacter diazotrophicus* | Proteobacteria | 0,0 | 0,2 | 0,001 | 1,1 | 31 | |
| *Pseudomonas tolaasii* | Proteobacteria | 0,0 | 0,2 | 0,001 | 1,2 | 27 | |
| *Cupriavidus metallidurans* | Proteobacteria | 0,0 | 0,2 | 0,001 | 1,3 | 11 | |
| *Methylobacterium radiotolerans* | Proteobacteria | 0,0 | 0,2 | 0,002 | 1,2 | 13 | |
| *Enterobacter asburiae* | Proteobacteria | 0,0 | 0,1 | 0,001 | 1,1 | 23 | |
| *Mycobacterium* sp. MS1601 | Actinobacteria | 0,0 | 0,2 | 0,001 | 1,2 | 21 | |

**Supplementary Table 2. Yeast and bacteria cell counts in pairwise cultivations.**

| **Pairwise cultivation/ yeast bacteria ratio** | **Change in yeast population (% against control)** | **Change in bacteria population (% against control)** |
|---|---|---|
| PE-2/*L. fermentum* strain A/100:1 | -7,4 | -94,8 |
| PE-2/*L. fermentum* strain B/100:1 | -6,5 | -93,9 |
| PE-2/*L. fermentum* strain C/100:1 | -23,5 | -93,4 |
| PE-2/*L. amylovorus*/100:1 | 1 | -48,9 |
| PE-2/*L. helveticus*/100:1 | -1,4 | -84,2 |
| PE-2/*P. claussenii*/100:1 | -5 | -85,3 |
| PE-2/*L. buchneri*/100:1 | -2 | -93,5 |
| PE-2/*Z. mobilis*/100:1 | -3 | -96,3 |

Control: Standalone yeast and bacteria cultivations.

**Supplementary Table 3. *L.fermentum* strains used in phylogenomic analysis.**

| Clade | Strain name | Name used | NCBI BioSample ID | Origin |
|---|---|---|---|---|
| 1 | FTDC8312 | FTDC8312 (i) | SAMN02469912 | Human digestive tract |
| | FTDC 8312 | FTDC8312 (ii) | SAMN06703219 | Human digestive tract |
| | 3872 | - | SAMN02314197 | Human breastmilk |
| | Lf1 | - | SAMN02053534 | Human digestive tract |
| | L930BB | - | SAMEA3158477 | Human digestive tract |
| | ATCC 14931 | ATCC14931 | SAMN00001473 | Human vaginal tract |
| | CECT 5716 | CECT5716 | SAMN02604100 | Human digestive tract; Human oral cavity |
| | IFO 3956 | IFO3956 | SAMD00060917 | Human digestive tract; Bird digestive tract |
| 2 | 39 | - | SAMN03452287 | |
| | NB-22 | - | SAMN02470787 | |
| | UCO-979C | - | SAMN04100088 | Human digestive tract |
| | SNUV175 | - | SAMN06174220 | Human vaginal tract |
| | 47-7 | - | SAMN05893390 | Human digestive tract |
| | LfQi6 | - | SAMN03372370 | Human breastmilk |
| 3 | 28-3-CHN | - | SAMN02463745 | Human vaginal tract |
| | 90 TC-4 | 90TC-4 | SAMN03452288 | |
| | F-6 | - | SAMN02603935 | Dairy product |
| | NCC2970 | - | SAMN05510874 | |
| 4 | DSM 20055 | DSM20055 | SAMN02797777 | Sugar beet fermentation process |
| | RI-508 | - | SAMN05717758 | Cacao bean fermentation process |
| | 779_LFER | - | SAMN03197989 | Human respiratory tract |
| | 222 | - | SAMEA3158475 | Cacao bean fermentation process |

**Supplementary Table 4. Description of the concentrations in $g.l^{-1}$ of the sugars and metabolites present in each of the media used in the microplate assays.**

| Medium | Glucose | Fructose | Sucrose | Ethanol | Acetic acid | Lactic acid |
|---|---|---|---|---|---|---|
| **Molasses** | 1.668 | 1.608 | 15.284 | 0 | 32 | 101 |
| **Fermented LAB A** | 0 | 545 | 11.239 | 545 | 891 | 2.602 |
| **Fermented LAB B** | 0 | 500 | 12.04 | 422 | 886 | 2.687 |
| **Fermented LAB C** | 0 | 337 | 12.570 | 771 | 804 | 2.384 |
| **Fermented A + Sugar** | 1.668 | 1.762 | 18.484 | 541 | 884 | 2.584 |
| **Fermented B + Sugar** | 1.668 | 1.777 | 18.094 | 416 | 883 | 2.696 |
| **Fermented C + Sugar** | 1.668 | 1.884 | 17.929 | 763 | 797 | 2.377 |
| **Molasses + Metabolites A** | 1.668 | 1.608 | 15.284 | 557 | 883 | 2.338 |
| **Molasses + Metabolites B** | 1.668 | 1.608 | 15.284 | 475 | 847 | 2.392 |
| **Molasses + Metabolites C** | 1.668 | 1.608 | 15.284 | 871 | 808 | 2.144 |

Molasses refers to diluted molasses at a concentration of 20g l-1. Fermented refers to molasses fermented by lactic bacteria (LAB A,B and C). Fermented + sugar refers to bacteria fermented with the addition of sugars at a level equivalent to that found in the initial molasses. Molasses + metabolites refers to the initial molasses with the addition of metabolites at the level produced by bacteria during fermentation.

**Supplementary Table 5. Description of the concentrations in g.l-1 of the sugars and metabolites present in each of the media used in the microplate assays.**

| Medium | Glucose | Fructose | Sucrose | Ethanol | Glycerol |
|---|---|---|---|---|---|
| Molasses | 1.676 | 1.623 | 15.738 | 0 | 21 |
| Fermented | 1.208 | 3.482 | 5.814 | 4.634 | 813 |
| Fermented + Sugar | 1.270 | 3.329 | 15.157 | 4.445 | 801 |
| Molasses + Metabolites | 1.676 | 1.623 | 15.738 | 4.171 | 847 |

Molasses refers to diluted molasses at a concentration of 20g/l-1. Fermented refers to molasses fermented by Saccharomyces cerevisiae PE-2. Fermented + sugar refers to yeast fermented with the addition of sugars at a level equivalent to that found in the initial molasses. Molasses + metabolites refers to the initial molasses with the addition of metabolites (ethanol and glycerol) at the level produced by yeast during fermentation.