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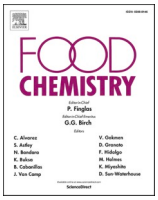

Novel *Saccharomyces cerevisiae* variants slow down the accumulation of staling aldehydes and improve beer shelf-life

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ABSTRACT

Beer quality generally diminishes over time as staling compounds accumulate through various oxidation reactions. Here, we show that refermentation, a traditional practice where *Saccharomyces cerevisiae* cells are added to beer prior to bottling, diminishes the accumulation of staling aldehydes. However, commonly used beer yeasts only show a limited lifespan in beer. Using high-throughput screening and breeding, we were able to generate novel *S. cerevisiae* hybrids that survive for over a year in beer. Extensive chemical and sensory analyses of the two most promising hybrids showed that they slow down the accumulation of staling aldehydes, such as furfural and *trans*-2-nonenal and significantly increased beer flavor stability for up to 12 months. Moreover, the strains did not change the original flavor of the beer, highlighting their potential to be integrated in existing products. Together, these results demonstrate the ability to breed novel microbes that function as natural and sustainable anti-oxidative food preservatives.

1. Introduction

Increasing the shelf-life of perishable products is one of the most promising strategies to increase the sustainability of food production. Beer in particular would strongly benefit from an increased shelf-life as the quality of beer generally declines as soon as it is produced, with exposure to light and higher temperatures accelerating this so-called aging process (Bamforth & Lentini, 2009). While every beer ages differently due to its unique chemical composition, aged beers across different beer styles generally lack freshness and are often described using descriptors such as 'sweet', 'caramel', 'madeira' and 'cardboard' (Dalgliesh, 1977). The development of such flavors is mainly attributed to the formation of staling carbonyl compounds or to their release from a bound state (Baert, De Clippeleer, Hughes, De Cooman, & Aerts, 2012), and is further intensified by the gradual hydolysis of desirable compounds such as fruity esters (Lettisha, Balakrishna, & Ademola, 2013; Neven, Delvaux, & Derdelinckx, 1997) and bittering *iso*-α-acids (Caballero, Blanco, & Porras, 2012; De Cooman, Aerts, Overmeire, & Keukeleire, 2000). Carbonyl compounds, including aldehydes such as *trans*-2-nonenal, 3-methylbutanal and phenylacetaldehyde, are notorious beer staling compounds because of their unpleasant flavor combined with a low flavor threshold in the parts per billion (ppb) range (Saison et al. 2009). Among the aldehydes, furfural is often used as an indicator of beer aging, although its aroma threshold is rarely exceeded in beer (Brenner, 1976; Malfliet et al., 2008).

Myriad factors influence the aging process, including raw materials, brewing process parameters, and storage conditions (Bamforth & Lentini, 2009). Specifically, residual oxygen in the bottle and elevated temperatures during transport and storage have been linked to accelerated beer aging. Residual oxygen catalyzes oxidative breakdown reactions during storage, while elevated storage temperatures increase the reaction rate of all aging reactions (Bamforth & Lentini, 2009). Several solutions have been proposed to tackle these problems. These include minimizing oxygen ingress during brewing and packaging processes, reducing aging precursors by using different barley varieties (Yu et al., 2014) or reducing the thermal load during wort production (De

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Schutter, 2008) and utilizing a yeast strain with a high reductive power (Xu et al., 2020), or with the ability to produce increased amounts of the antioxidants sulfur dioxide and glutathione during the main fermentation (Chen et al. 2012). While all these strategies may positively influence beer flavor stability, they are not always easy to implement and do not offer sufficient protection to prevent beer staling, especially when beers are stored at or above room temperature instead of being cooled (Bamforth & Lentini, 2009). This is particularly relevant in the increasingly globalized market, where transport and storage happen year round and cooling is costly and environmentally unsustainable, while product freshness is crucial to remain competitive with locally produced beers.

In beer brewing, yeast is primarily used for converting the fermentable sugars in wort, such as glucose, sucrose, maltose and maltotriose into ethanol and carbon dioxide. In addition, the yeasts also generate a broad range of important aroma compounds. After this main fermentation, yeast cells are often removed from the beer through a combination of sedimentation, filtration and centrifugation. However, in the production of some beers, fresh yeast is again added just prior to bottling. This process is also known as refermentation or bottle conditioning and was originally developed to impart carbonation to the beer (Derdelinckx, Vanderhasselt, Maudoux, & Dufour, 1992). Interestingly, refermentation has also been shown to mitigate beer staling, likely through the yeast's reductive metabolism (Saison et al. 2011). Moreover, yeast has also been suggested to positively influence flavor stability by scavenging residual oxygen and by producing sulfite (Ahrens et al., 2018; Baert et al., 2012). Sulfite not only acts as an antioxidant, but can also covalently bind carbonyl compounds, thereby trapping them as non-volatile adducts (Baert et al., 2012). However, despite the notion that refermentation may reduce beer ageing, the anti-staling potential of refermentation remains largely underexplored and unknown to many brewers.

Importantly, the duration of the protective effect of yeast used for refermentation remains unknown, but is likely limited in time as the number of metabolically active, living yeast cells rapidly declines during beer storage (Dekoninck, 2012; Vanbeneden, Vanderputten, Vanderhaegen, Derdelinckx, & Van Landschoot, 2006). In fact, apart from losing its potential to reduce staling compounds, yeast autolysis gives rise to negative staling compounds or precursors thereof, such as free fatty acids, as well as hydrolases and proteases that can impact beer foam stability, and esterases which can hydrolyze acetate esters and thus result in a loss of fresh fruity flavors (Neven et al., 1997; Van Landschoot, Vanbeneden, Vanderputten, & Derdelinckx, 2007). Hence, to exploit the potential of yeast as a preservative, it is crucial to extend the yeast's lifespan as much as possible.

In this study, we track the evolution of 41 key aroma compounds during the aging of a refermented beer. Our results show how staling compounds start accumulating as the viability of an industrial benchmark strain rapidly declines after bottling. Screening a large set (146) of diverse *Saccharomyces cerevisiae* strains indicates that some yeast strains that are not typically associated with beer brewing are able to survive the stressful beer medium and fluctuating temperatures. Using these strains as a starting point, we employed our high-throughput yeast breeding pipeline to generate new hybrid yeasts that show best-parent heterosis, enabling them to survive for over a year in bottled beer and withstand the extreme temperatures associated with beer transport. We show that using these yeasts for refermentation does not change the initial flavor profile of the fresh beer, but does protect the beer from staling. Finally, we link these sensory results to the beer's chemical profile and show how long-lived strains slow down the accumulation of specific staling compounds.

2. Main research hypothesis

Long-lived yeast strains can increase the flavor stability of beer.

3. Materials and methods

3.1. Yeast strains and storage

Strains used in this study are listed in supplementary table 1. Strains were maintained on solid YPD medium containing 1 % [wt/vol] yeast extract, 2 % [wt/vol] peptone, 20 % [wt/vol] glucose and 2 % [wt/vol] agar. Strains were stored at −80 °C using a glycerol-based storage medium 25 % [wt/vol] glycerol, 1 % [wt/vol] yeast extract, 2 % [wt/vol] peptone and 20 % [wt/vol] glucose.

3.2. Analytical methods

Before analysis, the beer was centrifuged for 5 min at 3000 rpm and filtered over a paper filter (Macherey Nagel 713 ¼). The cell pellet was used for viability measurements (see further) while the supernatant was used to analyze the beers chemical composition. Sulfite was measured using a Gallery Plus discrete photometric analyzer (Thermo Fisher Scientific, USA). Quantification of beer esters and higher alcohols was carried out using headspace gas chromatography coupled with flame ionization detection (HS-GC-FID) based on a method described earlier (Steensels, Meersman, Snoek, Saels, & Verstrepen, 2014). Results were analyzed using GCsolution software (Shimadzu).

Quantification of aldehydes and terpenes was carried out using headspace gas chromatography coupled with mass-spectrometric detection, following solid-phase micro-extraction with on-fiber derivatization (HS-SPME-GC–MS). The method was adapted from Saison and coworkers (Saison, De Schutter, Delvaux, & Delvaux, 2008). The GC–MS system (Shimadzu QP2010 Ultra Plus) was equipped with a non-polar DB-5MS column (length, 30 m; internal diameter, 0.25 mm; thickness, 0.25 μm). Helium was used as carrier gas with a linear velocity of 48.1 cm/s. A Combi PAL autosampler (PAL system, CTC analytics, Switzerland), containing an SPME-arrow unit with a DVB-PDMS fiber (65 μm, Supelco Co., Bellefonte, PA, USA), was installed on the GC–MS. Before each analysis, the fiber was conditioned for 5 min at 250 °C. Afterwards, the fiber was coated for later derivatization, by exposing it for 10 min to a vial containing 10 mL of 1 g/l pentafluorobenzyl hydroxylamine solution (40 °C, stirring at 250 rpm). During this time, beer samples were preincubated in the agitator (40 °C, shaken at 250 rpm). After the equilibration time, the fiber was exposed to the beer sample for 30 min (40 °C, stirred at 250 rpm). Subsequently, compounds trapped on the fiber were desorbed in the injection port of the GC–MS by heating the fiber for 3 min at 250 °C (splitless). The oven temperature was held at 36 °C for 5 min, after which it was increased to 160 °C at 5 °C/min. Next it was increased to 200 °C at 10 °C/min and then to 260 °C at 20 °C/min, where it was held for 5 more minutes. Total GC runtime was 41.8 min. The mass detector was operated in scan mode (35–500 amu), using electron impact ionization (70 eV). For derivatized compounds, the areas of both isomers were summed after the analysis. In each GC batch, identical QC samples, containing 10 mL of fresh reference beer, were included at the start and end of a batch and in between the samples, for a total of 4 references. The area of each compound in each beer sample was then corrected using a linear regression model of the corresponding compound in the QC samples. This not only allowed for correction of the observed gradual signal increase within each batch, but also allowed for correction between different batches.

3.3. Viability measurements

For most refermentation experiments, viability was assessed by plating a serial dilution of the yeast pellet onto solid YPD 2 % glucose plates and counting the colony forming units (CFU). This number was then compared to the number of CFU at the start of the experiment to calculate viabilities. Alternatively, the fraction of cells that was still able to form microcolonies (growth assay) was measured using timelapse microscopy. To this end, aging cultures were diluted in synthetic

complete medium and subsequently seeded into 384 well plates coated with concanavalin A (Sigma Aldrich). Using an inverted Nikon Eclipse Ti microscope, micrographs were taken from 5 positions within each well, every hour using Metamorph (Version 7.8.0.0; Molecular Device, LLC) to track cells growing into microcolonies. The micrographs were processed with cellprofiler in order to identify and track micro-colony size over time. The fraction of cells that could grow into microcolonies was used as measure for viability. Finally, dead cells were stained with alkaline methylene violet (Sigma Aldrich) as described by Smart and colleagues (Smart, Chambers, Lambert, Jenkins, & Smart, 1999). The ratio of unstained cells to total cells was then used as measure for viability.

3.4. Sporulation and mass mating

Yeast strains were mass-sporulated and the resulting spores were subjected to mass-matings as described previously (Meersman et al., 2015). Five separate mass-matings were initiated, in which the spores of five 'best natural' strains were pooled in varying ratios to obtain as many different hybrids as possible:

Combination 1: 20 % WI011, 20 % WI036, 20 % WI018, 20 % CO001, 20 % CO002.

Combination 2: 25 % WI011, 15 % WI036, 15 % WI018, 30 % CO001, 15 % CO002.

Combination 3: 25 % WI011, 15 % WI036, 15 % WI018, 15 % CO001, 30 % CO002.

Combination 4: 20 % WI011, 10 % WI036, 10 % WI018, 30 % CO001, 30 % CO002.

Combination 5: 40 % WI011, 10 % WI036, 10 % WI018, 20 % CO001, 20 % CO002.

3.5. Temperature tolerance assays

Yeast strains were tested for thermotolerance using two different methods. Firstly, they were grown at 41 °C in a spot assay as described previously (Meersman et al., 2015). Colony-areas at 41 °C were normalized to the colony-areas of the same strain grown at 30 °C. Secondly, the strains were subjected to a 47.9 °C heat shock in a thermocycler, in a setup adapted from Gemayel and coworkers (Gemayel et al., 2017). Briefly, the strains were grown overnight in YPD at 30 °C, washed with and subsequently diluted in PBS to $OD_{600} = 0.1$. Then, 50 µl of each sample was transferred to a thermocycler, which was used to induce a rapid shift from a physiological growth temperature (30 °C for 1 h) to the heat stress temperature of 47.9 °C (3 h) on multiple samples at once. From a pilot experiment in which twelve different temperatures were tested, 47.9 °C was chosen because it was severe enough for the viabilities to drop drastically, but not severe enough to kill off all cells. Survival rates were calculated by dividing the number of CFU after heat shock by the number of CFU from a non-shocked control plate.

3.6. Refermentation assays

To obtain a comprehensive view on the aging profile of a refermented beer, bottles of refermented blond ale of 8.5 % ABV (reference beer) ranging from 1 to 19 months old were obtained from a Belgian brewery at the time they were released to the market. To obtain a view on the viability of the newly tested yeasts and/or the resulting chemical composition of the resulting refermented beer, refermentations were set up as described below: Yeast strains were grown overnight in 5 mL of YPD 2 % glucose at 30 °C. For refermentations in GC vials, this pellet was used to inoculate 10 mL of degassed 'base beer' to a final density of $2x10^6$ cells/mL. The 'base beer' is a non-refermented blond ale in which the ABV was adjusted with 100 % ethanol to reach the same ABV (8.5 %) as the reference beer. The vials were closed with a screwcap with a PTFE-silicone septum and sampled with a needle syringe, allowing frequent sampling with minimal sample evaporation and disruption. For bottle refermentations, yeast strains were grown overnight in 5 mL of YPD 2 % glucose at 30 °C. One mL of this preculture was used to inoculate 50 mL of YPD 2 % glucose for a second overnight at 30 °C. The pellet of this preculture was subsequently used to inoculate 200 mL of 18°P wort to a final density of $1x10^7$ cells/mL. Fermentation in wort was done for 6 days at 24 °C at 200 rpm. Finally, the pellet was used to start refermentations. For bottle refermentations that were set up in the lab, the yeast was pitched to a final density of $2x10^6$ cells/mL in the alcohol-adjusted 'base beer'. Afterwards, a 50 % glucose solution was added to a final density of 0.2°P before flushing the headspace with CO_2 and capping the bottles again. The industrial bottle refermentations were set up in the Reference beer on an industrial filling line in order to limit the oxygen ingress during filling. To this end, the pellet of two long-lived strains (Hyb1.22 and CO001) was used to prepitch empty bottles, right before they were filled with fresh reference beer. The long-lived strains were tested at 'low' ($2x10^3$ cells/mL) and 'high' ($1x10^6$ cells/mL) final pitching density. The fresh reference beer also contained the reference yeast of the brewery.

3.7. Storage conditions

Throughout the research project, different storage profiles were used depending on the goal of the experiment. During the initial survival screening in vials, samples were stored at 30 °C continuously. During the second screening in bottle refermentations, we mimicked long-term transportation conditions. To this end, samples were stored at 24 °C for 2 weeks to allow the refermentation to take place. This was followed by a 5-week maturation stage at 7 °C before the temperature was increased to 35 °C over a period of 3 weeks. To conclude, the temperature was fluctuated diurnally between 35 °C and 18 °C for 8 weeks. For selecting the hybrids resulting from the mass mating, a shorter and more stringent variant of the previous storage profile was used: 1 week at 24 °C followed by 1 week at 7 °C. Then the temperature was increased to 37 °C over 2 weeks after which it was fluctuated diurnally between 37 °C and 18 °C for 2 more weeks. Finally, for the industrial bottle refermentations, samples were first allowed to referment at 24 °C for 2 weeks, before maturating at 4 °C for 5 weeks. After this, the samples were moved to warehouse conditions of ~ 20 °C for the remainder of the storage period.

3.8. Sensory analyses

Tastings were performed as described previously (Sinkinson, 2017; Yang & Ng, 2017). Briefly, beers were served in black glasses labeled with three-digit codes, generated randomly. The glasses were covered with a plastic lid to avoid evaporation between pouring and serving. Testing for similarity was done via a set of forced-choice triangle tests in a randomized block design. Each taster was provided with 5 different triangles of beer. In each triangle, 2 samples were identical and 1 one was different. The taster was then asked to indicate the odd one out for each triangle. The 5 triangles covered the comparisons between the reference beer and the beers with long-lived yeast (CO001_high, CO001_low, Hyb1.22_high and Hyb1.22_low) as well as a force-aged control sample (Aged_control) to confirm the sensitivity of the tasting panel. Testing for difference was done via a forced-choice multiple paired comparison tasting in a randomized block design. Each taster was provided with 6 pairs of beer (Reference vs CO001_high, Reference vs CO001_low, Reference vs Hyb1.22_high, Reference vs Hyb1.22_low, Hyb1.22_high vs CO001_high and Hyb1.22_low vs CO001_low). For each pair, the taster was asked to indicate which of the two beers tasted most aged. The panel consisted of 33, 32 and 38 trained tasters for the tasting after 2, 6 and 12 months respectively.

3.9. Statistical analyses

All analyses were carried out in R (version 3.6.0). For the heatmap,

the 'pheatmap' function from the 'pheatmap' R-package was used on the batch-corrected, average values of three biological replicates, centered and scaled over rows. Instead of clustering, compounds were organized according to their functional groups. For PCA analysis, the 'PCA' function from the 'FactoMineR' R-package was used on the batch-corrected, average values of three biological replicates, scaled to unit variance. The variables 'timepoint', 'strain' and 'timepoint_strain' were used as qualitative supplementary variables. The two principal components with the highest explanatory value were plotted using the 'fviz_pca_biplot' function from the 'factoextra' R-package in combination with the R-package 'ggplot2' for customization. For the sensory analyses, we used the R-package 'sensR' to calculate the minimum number of tasters required to draw statistically sound conclusions with a power ≥ 0.95 for similarity testing (maximum proportion of distinguishers, Pd, was set at

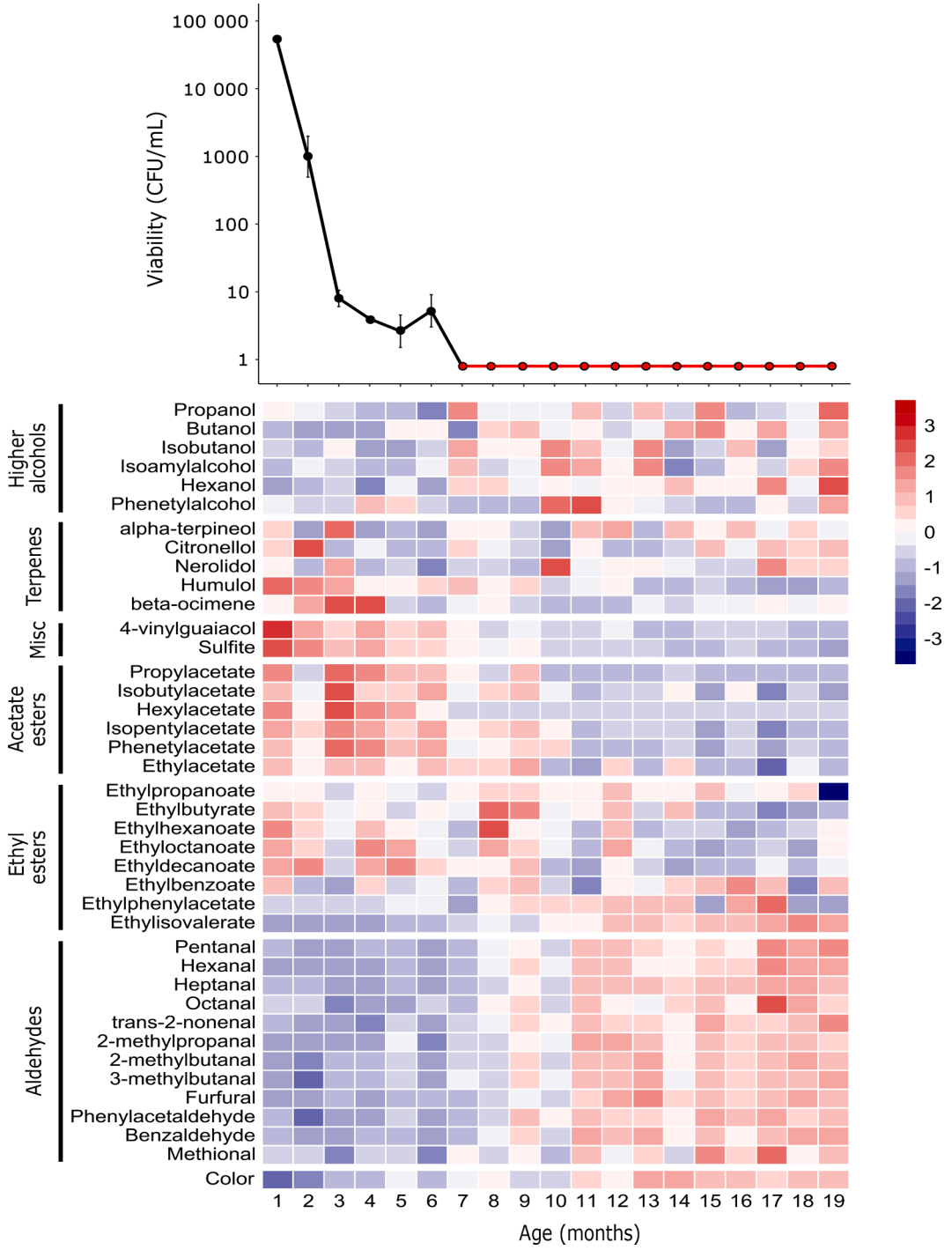

Fig. 1. The composition of refermented beer changes over time. Characterization of a commercial refermented beer during storage at 24 °C for a period of 19 months. Samples were taken every month. Yeast viability (top panel) during storage was determined by CFU per mL of beer. Red datapoints indicate that no viable cells could be recovered from the bottle. Note how staling aldehydes start accumulating as soon as the last yeast cells have died. Because a log-scale does not allow for zero values, the CFU at these timepoints was manually set to 0.1. Data points and error bars represent the means and standard deviations of 2 biological replicates (n = 2). Chemical changes (bottom panel) are displayed in a heatmap in which values were scaled (z-scores) over rows. Compounds were organized according to their functional groups. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

0.35) and with a significance $\leq$ 0.05 (and power of 0.9) for difference tests (meaningful departure from equal intensity, Pmax, was set at 0.75). The counts from the similarity tests were checked for significance by comparing them with available statistical tables (Sinkinson, 2017). The counts of the difference tests were further converted to a single 'preference' score (alongside its statistical significance) for each sample using a Bradley-Terry model (Yang & Ng, 2017). To this end, we used the 'BTm' function from the R-package 'BradleyTerry2′ and selected the reference beer as reference category for the modeling.

4. Results

4.1. Yeast viability rapidly decreases in refermented beer during storage, whereas staling compounds accumulate

To obtain a comprehensive view on the effect of yeast viability on the chemical profile of refermented beer during long term storage, we measured cell viabilities and concentrations of 41 different aroma compounds and aging markers (Fig. 1) in a standard Belgian ale beer for a period of 19 months after release from the brewery. The majority of the cells had died after only 3 months of storage, with less than 10 cells/mL remaining from a starting density of $2x10^6$ cells/mL (i.e. 0.0005 % survival). Importantly, this sharp drop in viability is not unique for the chosen reference beer or reference yeast. For example, previous studies also reported low viabilities (less than4%) for eight different strains that were aged at 25 °C for 3 months in a blond beer of 9.9 % ABV (Dekoninck, 2012). Similarly, Vanbeneden and coworkers found that 5 of the 7 refermentation yeasts in their study dropped to a viability of below 10 % after only 1.5 months of aging (Vanbeneden et al., 2006). This shows that yeast cells rapidly lose viability in finished beer.

In the chemical profile throughout the storage period (41 compounds, Fig. 1), compounds can roughly be divided into three groups based on their changes over time. The first group contains compounds of which the concentration does not seem to depend on storage time, including higher alcohols such as phenethyl alcohol and isoamyl alcohol and hop-derived terpenes such as nerolidol and citronellol. The second set consists of compounds that increase over time in a pattern that roughly mirrors the loss of viable yeast cells. This group includes compounds that have received little attention in the context of beer aging such as ethyl phenylacetate (honey aroma) and ethyl isovalerate (sweet fruity aroma), but also includes well-known aging aldehydes such as *trans*-2-nonenal (cardboard) and methylpropanal (grainy). It also includes the marker aldehyde furfural, which increased > 30-fold in concentration over 19 months. The third group contains compounds that decrease over time and mainly includes volatile acetate esters with fresh and fruity aroma's such as isoamylacetate (banana) and phenethylacetate (roses) and certain ethyl esters such as ethyl hexanoate (pineapple) and ethyl octanoate (fruity). Importantly, the concentration of sulfite, an important antioxidant and preservative in beer, also declines over time, likely because it reacts with some oxidized staling products.

4.2. The ability of different S. Cerevisiae yeasts to survive in beer is highly variable

To identify yeast with high survival in beer, we screened 146 *S. cerevisiae* strains and measured their viability in beer over time. This collection includes 51 different strains that originate from the beer industry, but also contains 95 yeasts derived from very different environments, such as winemaking, spontaneous cocoa-fermentation, and the bioethanol industry. The strains were screened in glass GC vials containing 10 mL of degassed beer and were stored at 30 °C. The yeast strains were analyzed for their viability on a weekly basis for a one month period, using both methylene violet staining and timelapse microscopy. The former is typically used in breweries while the latter offers an accurate, high-throughput alternative for counting CFU, which is a commonly used method for assessing survival in a lab-environment (Longo, Shadel, Kaeberlein, & Kennedy, 2012). Fig. 2A shows the viability of all tested yeast strains following 4 weeks of storage as a correlation between both viability methods ($R^2 = 0.6401$, p-value less than 2.2e-16). The results reveal that the ability of *S. cerevisiae* to survive in beer is highly variable, ranging from 0 to 58 % based on the growth assay. Moreover, staining cells with methylene violet resulted in consistently higher viabilities than when the cells were checked for their ability to grow into colonies, suggesting that a fraction remains viable but is unable to resume growth. For this reason, CFU was used in further experiments to measure viability.

From this screening, 26 strains were chosen for further screening in bottle refermentations. For this selection, we prioritized long-lived strains from different origins, but also included a few short-lived strains to validate the results of the screening in GC-vials. Furthermore, we added five additional strains derived from cocoa fermentations as this origin yielded the most long-lived strains in the GC-vial screening. Compared to vial refermentations, bottle refermentations more closely mimick industrial storage conditions of beer, including the anaerobic environment and high CO_2-pressure. Moreover, bottles were also stored at a temperature profile that mimics the more extreme temperature conditions associated with shipping and storage (Marquez, Dunstall, Bartholdi, & MacCawley, 2012). For 12 out of 31 strains, viable cells were recovered after this extreme storage profile (Fig. 2B). Of the 12 surviving strains, the absolute survival rates are low, with only 3 strains (CO001, CO002 and WI011) showing a viability higher than 1 % at the end of the experiment. It is worth pointing out that out of the 51 ale strains included in the initial vial screening, only one managed to survive the bottle refermentations, with a viability below 0.01 %. Strains related to cocoa-fermentations, on the other hand, showed consistently higher survival rates than most other tested strains. Taken together, these results suggest that there are yeast strains much more suited for refermentation than the standard ale strains currently used in the brewing industry.

4.3. Newly developed hybrid S. Cerevisiae strains show increased temperature resistance

The two most promising cocoa strains (CO001 and CO002) and the three most promising wine strains (WI011, WI018, WI036) were used as parent strains to create hybrids that could potentially show an even higher ability to survive for extended periods in beer stored at higher temperatures. Mass matings were set up in 5 different pools, where the spores coming from the 5 parents were mixed in different ratios to increase the chance of obtaining crosses between all parental strains, even in the face of possible biases in mating preference between the different parents. The viability profiles in Fig. 3A show that some cells in each of the hybrid pools managed to outlive their parental strains, which could be an indication of best-parent-heterosis in the hybrids. Importantly, the stringency of the selection allowed to narrow the initial ~ $1x10^8$ different hybrids down to the last 50 that remained viable throughout the entire storage period. The best hybrids among these 50 strains were then further selected using two different phenotypic screens. First, a spot assay was used to test the ability of the hybrids to grow at elevated temperature (41 °C), a phenotype that the best-surviving parental strain, CO001, excells at (Fig. 3B). While the hybrids showed a large diversity in their ability to grow at 41 °C, none could match the performance of CO001. However, while they did not match the temperature resistance of the most robust parental strain, some of the novel hybrids did show best-parent heterosis for other properties, including the 3 h survival test at 47.9 °C, where one of the novel hybrids still showed a remaining viability of 78 %, whereas all parental strains showed less than 5 % survival (Fig. 3C).

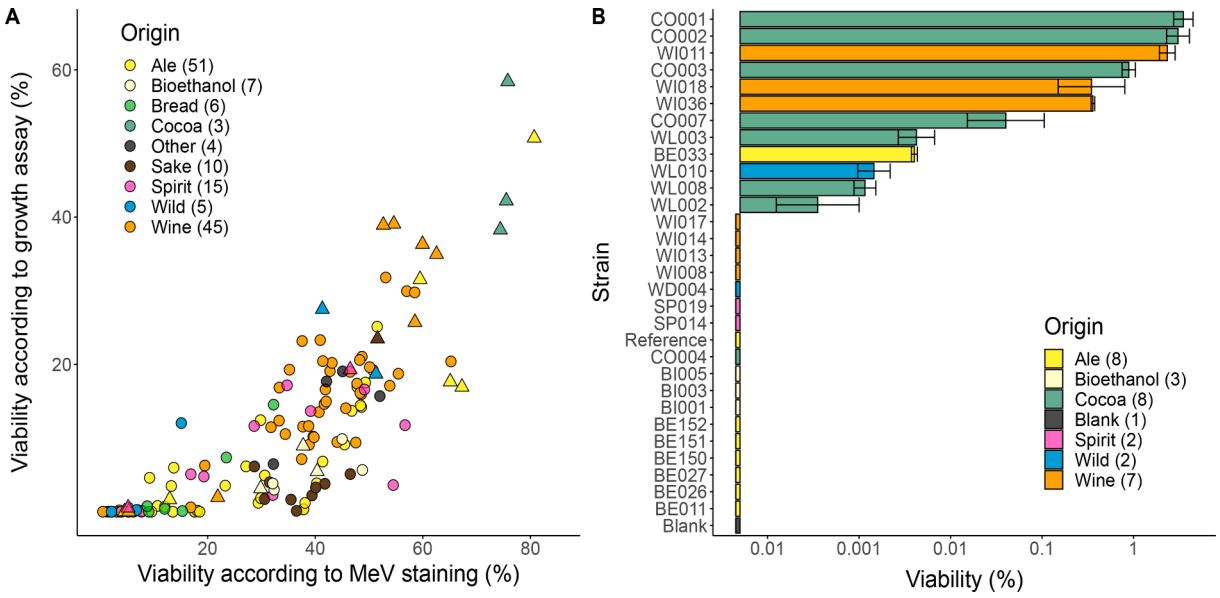

Fig. 2. Different *S. cerevisiae* strains show different lifespan in beer. A) Viability of 146 genetically distinct *S. cerevisiae* strains after one month of storage in beer at 30 °C. Viability was measured by growth assay (vertical axis) or methylene violet staining (horizontal axis). Data shown are the mean of 2 biological replicates (n = 2). Strains depicted with a triangle symbol were selected for further screening. Color of the symbol indicates their origin, and total number of each origin that was tested is indicated between brackets. Note that most traditional beer yeasts show poor viabilities. B) Further characterization of 26 strains selected from the screening depicted in panel A, along with five additional strains derived from cocoa fermentations. Reference indicates the industrial benchmark strain from Fig. 1, while Blank indicates that no viable cells were added to the bottle. Bottles were stored for 2 weeks at 24 °C, followed by 5 weeks at 7 °C. Then the temperature was increased to 35 °C over a period of 3 weeks after which it was fluctuated diurnally between 35 °C and 18 °C for 8 more weeks. Viability was calculated by dividing the number CFU at the end of the experiment by the number of CFU at the start of the experiment. Data points and error bars represent the mean and standard deviation of 3 biological replicates (n = 3). Colour of the bars indicates their origin, and total number of each origin that was tested is indicated between brackets. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

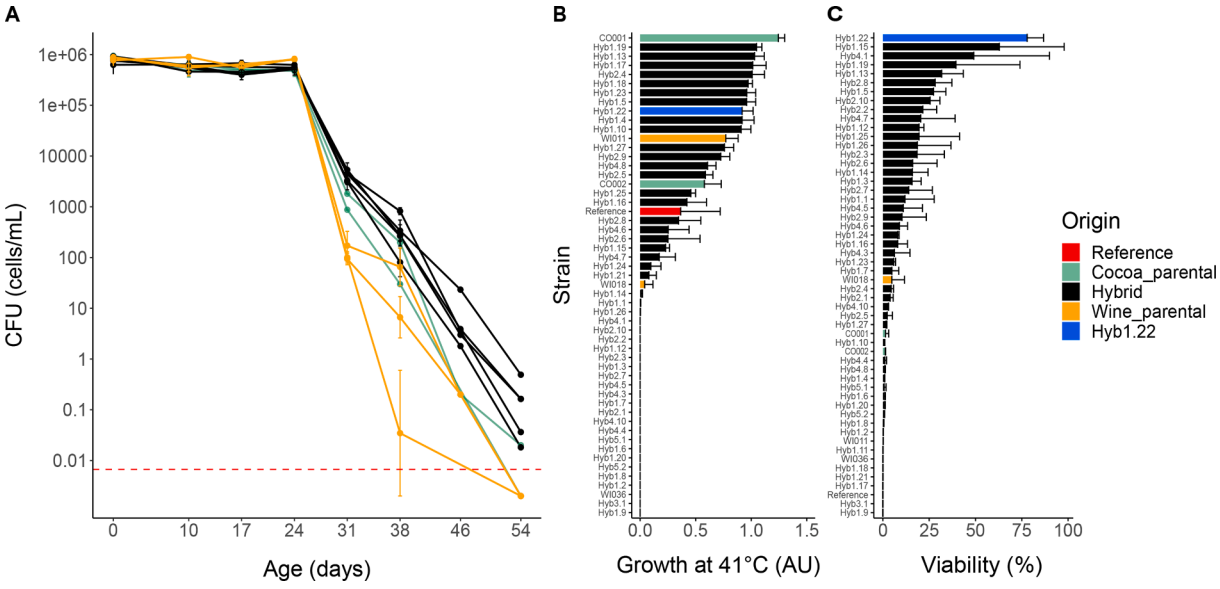

Fig. 3. Hybrids outperform best parental strains. A) Yeast survival during beer storage as determined by CFU per mL of beer. Newly developed hybrids (black lines) were inoculated as five different pools of genetically different yeast strains obtained after a high-throughput breeding step. The five parental strains (orange and green lines) were inoculated as pure cultures. The red dashed line represents the limit of detection. Datapoints and error bars represent the mean and standard deviation of 3 biological replicates (n = 3). B) Temperature tolerance of selected hybrid yeasts assessed by growth at 41 °C. Data shown is the mean and standard deviation of 6 biological replicates (n = 6). C) Temperature stress resistance of selected hybrids assessed by their survival following a 47.9 °C heat shock. Data shown are the mean and standard deviation of 2 biological replicates (n = 2). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

4.4. Newly developed S. Cerevisiae strains show increased survival and aroma preservation during beer refermentation

Following this round of breeding, the longest-lived parental strain (CO001) and the most promising hybrid (Hyb1.22) were selected for further testing in long-term beer aging experiments. As a reference, an otherwise identical beer containing the industrial reference beer yeast was used. Since we anticipated that some brewers might prefer to keep

their current yeast for the refermentation process itself and add only a small amount of long-lived yeast to slow down beer aging, we investigated two different inoculation concentrations. Specifically, for each yeast to be tested, we pitched either at low density ($2x10^3$ cells/mL) or high density ($1x10^6$ cells/mL) in addition to the reference beer yeast that was added at the usual inoculation rate of $1x10^6$ cells/mL.

After a standard 10 day refermentation period at 24 °C, followed by a 5 week maturation period at 7 °C, the beers were stored at room temperature (20 °C) for the remainder of the year-long aging experiment. As expected, the reference yeast rapidly lost viability as soon as the beers were brought to room temperature (Fig. 4A). After 3 months most of the cells (>99.9 %) of the reference yeast had died, while CO001 and Hyb1.22 still showed viabilities well over 50 % and 80 %, respectively, with Hyb1.22 still having 3 % viable cells after 1 year of storage.

4.4.1. *Addition of long-lived S. Cerevisiae helps preserve the fresh taste of beer*

To assess whether the presence of long-lived yeasts during beer storage had an influence on flavor, the beer samples were assessed sensorially by a large panel (N > 30) of trained tasters at three different timepoints throughout the aging process: after 2 months, 6 months and 12 months. Two months is the timeframe within which a refermented, maturated beer would typically reach the consumer and as such serves as a measurement for 'fresh' beer. At this timepoint, the beers pitched with the long-lived strains were compared with the reference beer in a set of triangle tests that were set up to test for similarity between the samples. A force-aged sample ('Aged_control') was also compared to the fresh reference beer to validate the tasting panel. To avoid false negatives, the false-negative rate (β) was set at a maximum of 5 % to draw significant conclusions. With a maximum proportion of distinguishers (Pd) of 0.35, a difference in flavor between the long-lived strains and the industry reference could be rejected (Fig. 4B). In other words, adding our novel long-lived yeast strain did not impact the flavor profile of the existing reference beer in any way.

After 6 and 12 months of aging, the samples were assessed in a forced-choice multiple paired comparison test. In this test, the tasters were asked to indicate the sample they perceived as 'most aged' for each pair of samples. The results of the different comparisons were converted to a single 'preference' score for each sample using a Bradley-Terry model (Yang & Ng, 2017), a commonly used approach allowing for a directional comparison of all samples at once. As can be seen from the preference scores in Fig. 4C and Fig. 4D, beers refermented with a high density of the selected long-lived yeast were perceived as significantly fresher than the reference beer after both 6 and 12 months of aging. It is worth noting that the beer refermented with a high density of CO001 is still perceived as more fresh than the reference at the 12-month mark, despite that at that time, no viable cells could be detected anymore. This indicates that the positive flavor effect of viable yeast is not immediately lost after the last cells die off. Unexpectedly, the beers pitched with a low density of long-lived yeast seemed to be perceived as slightly more aged

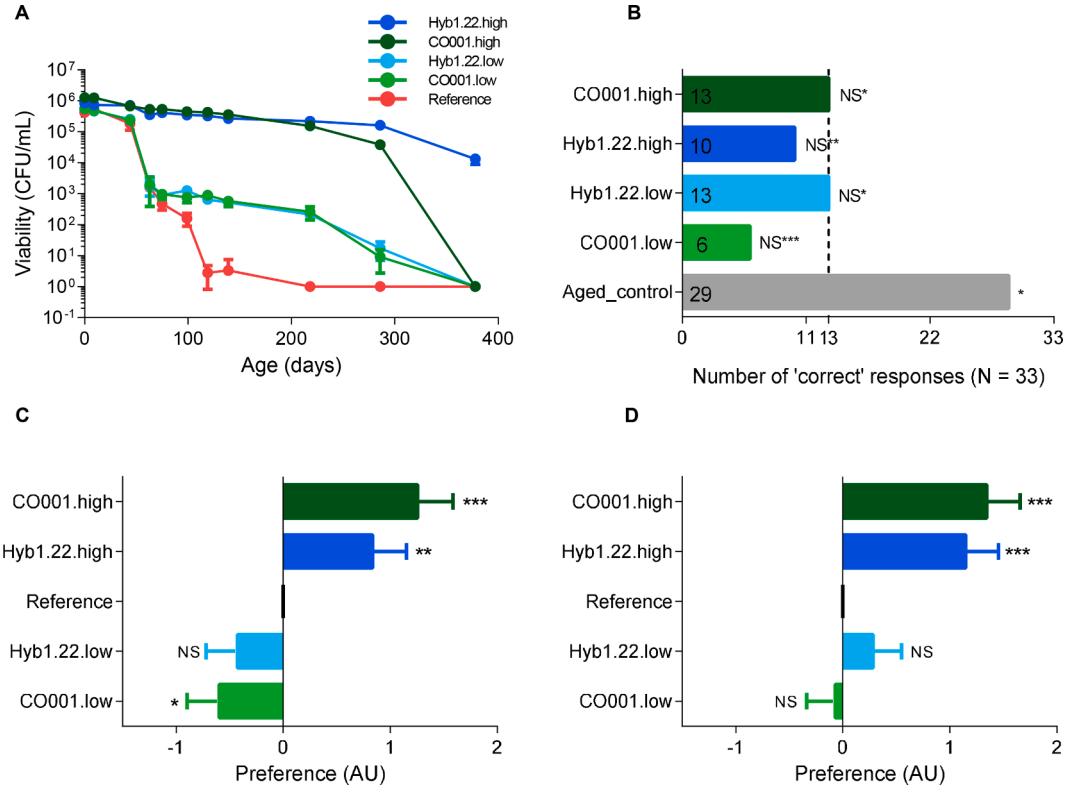

Fig. 4. A newly developed *S. cerevisiae* hybrid yeast strain survives for more than one year in beer and slows down beer aging. A) Survival of the industrial benchmark (Reference, red curve), the longest-lived parental strain (CO001) and the longest-lived hybrid (Hyb1.22) strain in beer. The long-lived strains were pitched at both high ($1x10^6$ cells/mL) and low density ($2x10^3$ cells/mL). Data shown are the means and standard deviations of 3 biological replicates (n = 3). B) Triangle tests to investigate whether consumers detect differences in the aroma of fresh beers refermented with an industry reference ale yeast and beers refermented with different selected long-lived yeast strains (CO001 and Hyb1.22). The beer with the industry benchmark (Reference) strain was also compared with a sample force-aged at 60 °C for 2 days (Aged_control) to test the sensitivity of the tasting panel. '*' means that the samples are significantly different using a triangle test, while 'NS*' 'NS**' or 'NS***' means that the samples are not significantly different (at a maximum proportion of distinguishers (Pd) of 0.35) with a type II error smaller than 0.05, 0.01 or 0.001, respectively. The tasting panel consisted of 33 members (n = 33). C) Multiple paired comparison test to investigate differences between beers refermented with different yeast strains following 6 months of aging. Counts were converted to preferences using the Bradley-Terry model. '*' '**' or '***' means that the samples are different from the Reference at a significance level of 0.05, 0.01 or 0.001, respectively. 'NS' means that the samples are not significantly different. The tasting panel consisted of 32 members (n = 32). D) Identical as panel C, but after 12 months of aging. The tasting panel consisted of 38 members (n = 38). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

than the reference beer after 6 months of storage. However, it has to be noted that several tasters stated that there were no noticeable differences between the reference beer and the beers containing a low density of long-lived yeast, but they were forced to make a preference selection. Moreover, this trend was no longer observed after 12 months of storage. Nevertheless, it suggests the addition of 2000 cells/mL of long-lived yeast is insufficient to drastically impact the beer flavor.

4.4.2. *Addition of long-lived S. Cerevisiae slows down the accumulation of staling compounds*

At the different tasting points, samples were also subjected to a detailed chemical analysis. To more easily visualize the changes in the concentrations of a large set of aroma compounds in the different samples during storage, the dimensionality of the dataset was reduced using a principal component analysis. In Fig. 5, the samples are plotted in terms of the two principal components (Dim1 and Dim2) that explain the largest part of the variation in the dataset. Dim1 accounts for>60 % of the observed variation. Dim1 is partly driven by positive contributions of known aging compounds such as benzaldehyde, 2-methylbutanal, hexanal, *trans*-2-nonenal and furfural, but also includes negative contributions from compounds associated with fresh beer such as isoamylacetate, sulfite and ethyl octanoate. As such, Dim1 increases with aging and captures two essential aspects of aging concurrently. As expected, the 2-month old samples show low values for Dim1, which indicates relatively high amounts of compounds associated with fresh beer. For later sampling points, these compounds decreased in concentrations, while staling aldehydes increased, causing the samples of 6 and 12 months to gradually move towards higher Dim1 values. The explanatory value of Dim2 is lower and driven mainly by the variation in 'CFU' (colony forming units, i.e. the concentration of living yeast cells in the beer samples), which partly explains the separation between the 2-month old samples, despite their similarity in taste. However, Dim2 also contains contributions from aldehydes such as furfural, *trans*-2-nonenal, 3-methylbutanal and hexanal. These compounds allow Dim2 to separate the samples refermented with a high density of long-lived yeast (Hyb1.22.high and CO001.high) from the Reference beer and the samples pitched with a low density of yeast (Hyb1.22.low and CO001.low) after both 6 and 12 months of aging. They are shown separately in Fig. 6, from which it becomes clear that the accumulation of staling aldehydes during storage is slowed down or even reduced by a high density of long-lived yeast. This observation is consistent with the sensorial data in Fig. 4C and Fig. 4D, in which the beers containing a high density of long-lived yeasts were rated as significantly fresher than the other samples. After 12 months of aging, the difference between the samples to which long-lived yeasts were added at a high concentration and the other samples is even clearer than after 6 months, with a firm separation along both Dim1 and Dim2. This more evident separation can partly be attributed to lower amounts of the aldehydes 2-methylbutanal and pentanal in the former (Fig. 6).

5. Discussion

In this study we analyzed if and how living yeast cells might protect refermented beer from staling as it ages. Most beer aging research to date has focused on the market-dominant lager beers, while only few studies have investigated the aging profiles of specialty beers (Barnette & Shellhammer, 2019; Bart Vanderhaegen, Delvaux, Daenen, Verachtert, & Delvaux, 2007). However, the aging of a refermented ale has not been investigated. Our results demonstrate that once all yeast cells in a reference refermented ale beer die, a rapid increase in staling compounds and decrease in certain fresh aroma compounds such as acetate esters is observed. These chemical changes resemble and confirm previously reported aging reactions (Saison et al., 2008; Vanderhaegen et al., 2003). It has to be noted however that in the study of Saison and coworkers (2008), the beers were force-aged for 2 weeks at 40 °C. The continuous exposure to this elevated temperature might have catalyzed aging reactions not observed under milder, more 'natural' storage conditions. Our dataset also reveals compounds that have not yet received

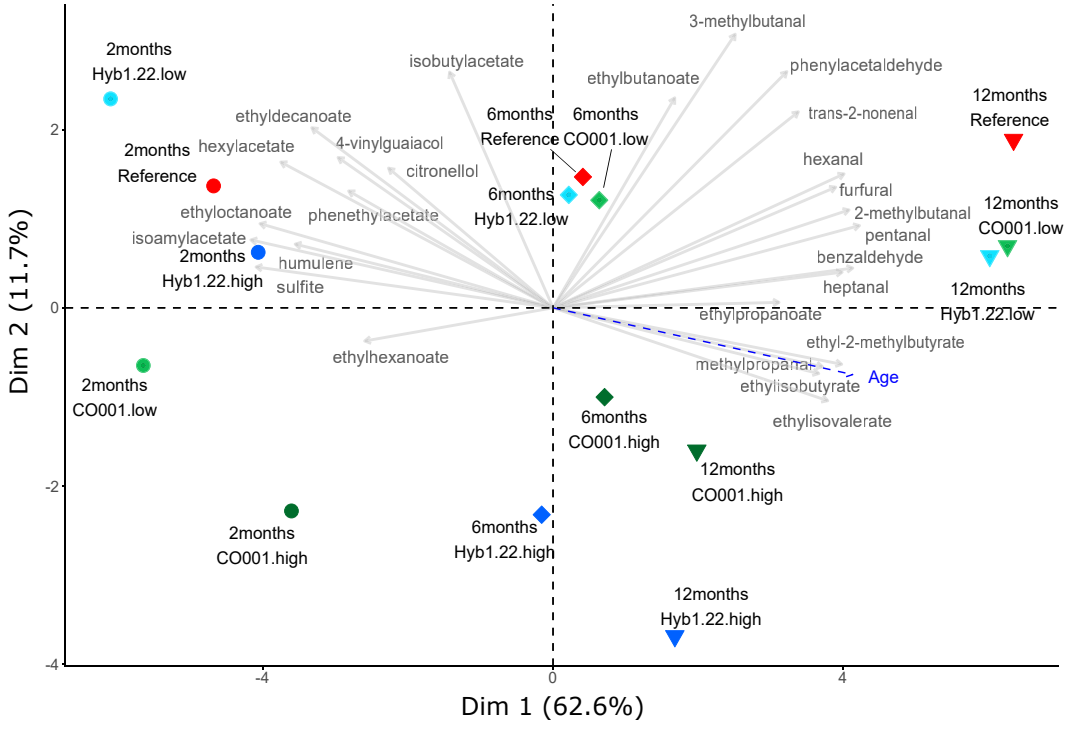

Fig. 5. Evolution of the chemical profile of beers refermented with different yeast strains. Samples (points) and variables (arrows) are plotted in a biplot. The axes represent two new orthogonal variables that explain the largest part of the variation in the dataset, as obtained from a PCA-analysis. Colors indicate the strain used for refermentation. Shape of the symbols indicates the age of the beer at the timepoint of chemical analysis. Data shown are based on the mean 3 biological replicates (n = 3).

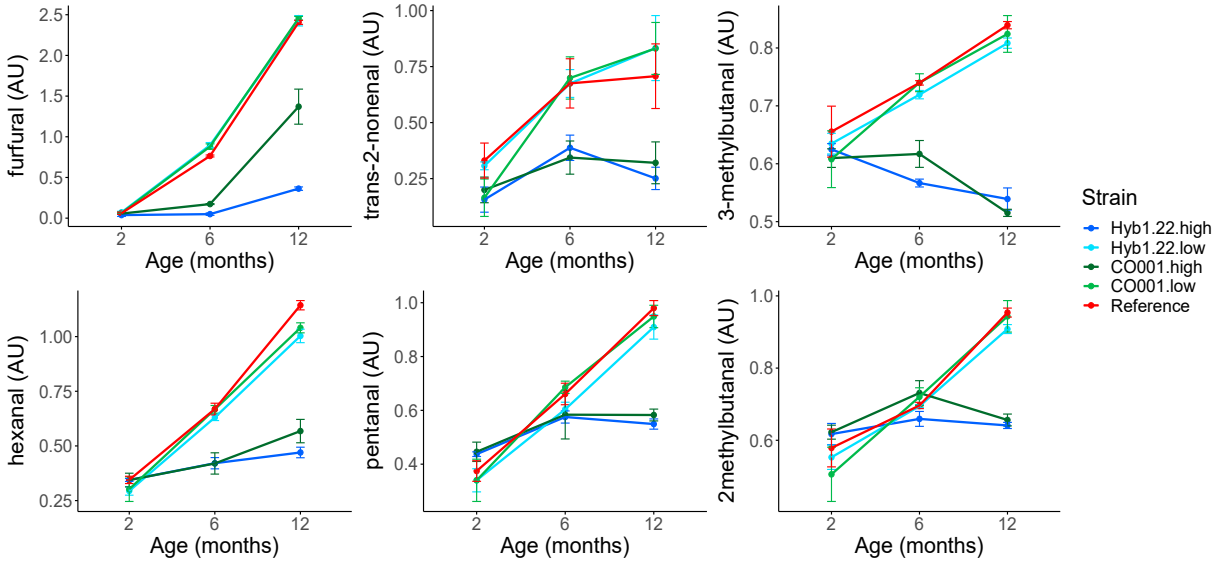

Fig. 6. Long-lived yeast slow down accumulation of staling aldehydes when pitched at high density. Concentration of select staling aldehydes (furfural, *trans*-2-nonenal, 3-methylbutanal, hexanal, pentanal and 2-methylbutanal) over time as measured by GCMS. To account for positional effects observed within a GC run, the area of each compound was corrected using a linear regression model of the corresponding compound in four quality control samples included in the same GC-run. Data shown represents the mean and standard deviation of 3 biological replicates (n = 3).

much attention in the context of beer aging, including ethyl phenylacetate and 4-vinylguaiacol (4VG). 4-VG has a spicy, clove-like aroma and decreases over time while ethyl phenylacetate is associated with a honey-like aroma and gradually increases over time. The presence of ethyl phenylacetate is likely the result of an esterification reaction between ethanol and phenylacetic acid (Vanderhaegen et al. 2003). The latter is a byproduct from yeast metabolism, and ethyl phenylacetate is therefore particularly interesting as a potential aging marker in the context of refermentation, where yeast cells gradually lyse and release their contents to the beer matrix (Chen, Jamieson, and Van Gheluwe 1980). Another notable example is sulfite. While sulfite has a known role as antioxidant and aldehyde-scavenger (Baert et al., 2012), its evolution during storage has not been described in detail before. Here we show that sulfite gradually decreases during storage and drops to a third of its initial concentration after 1 year. This is consistent with its role as antioxidant, in which it is expected to counteract the gradually oxidizing beer matrix. Importantly, sulfite can be measured spectrophotometrically. This makes it a promising aging marker as it does not rely on chromatographic separation which is required for other aging markers such as furfural and 5-hydroxymethylfurfural.

Interestingly, the transition from a fresh beer to a beer with more aging notes showed a marked acceleration around 3 months post-packaging, which is the point where most or all of the yeast cells in the beer had died. The observation that yeast cell die relatively rapidly during beer storage can partially be explained by the harsh environmental conditions, a combination of low nutrient and oxygen availability, temperature fluctuations, low pH, and high alcohol and CO_2 concentrations (Dekoninck, Mertens, Delvaux, & Delvaux, 2013; C. M. Rogers, Veatch, Covey, Staton, & Bochman, 2016). Moreover, brewers tend to use the same yeast for refermentation as they do for the primary fermentation or even opt for a yeast that is readily available in dry form, typically baker's yeast (Derdelinckx et al., 1992). This claim is supported by genetic data, where many refermentation yeasts fall in the same clade as many of the baker's yeasts (Gallone et al., 2016). As such, it seems likely that most current refermentation strains were selected out of convenience, rather than optimal performance during refermentation. While most refermentation yeasts survive poorly in beer (Dekoninck, 2012; Vanbeneden et al., 2006), our screening of 146 different yeast strains revealed that some yeasts related to wine and especially cocoa fermentations are better suited for long-term survival in packaged beer. This is consistent with recent observations from De Chiara and co-workers, who found West-African cocoa strains to have the highest survival rate following growth in rich, synthetic complete medium out of>1000 yeast isolates (De Chiara et al., 2020) and indicates an origin-dependent nature of survival.

Two of the longest-lived strains were tested in a bottle refermentation experiment (8.5 % ABV ale, 20 °C storage) alongside a reference strain from the brewing industry. The industry reference only managed to survive for three months, while some of the longest-living hybrid cells survived for over a year. Moreover, some of the yeasts exhibited an increased resistance to extreme temperatures and temperature fluctuations, which are known to represent a challenge for standard beer yeasts (Gallone et al., 2016). Sensorial analysis of the aged beers to which either standard beer yeast or long-lived yeast strains had been added, showed that the beer with the long-lived strains preserved far better and showed fewer signs of staling after both 6 and 12 months after production. Detailed chemical analyses revealed that a large part of the observed flavor difference could be attributed to the long-lived yeasts' ability to slow down the accumulation of staling aldehydes, including 2-methylbutanal, 3-methylbutanal, furfural, *trans*-2-nonenal, pentanal and hexanal. A likely explanation for the decreased accumulation of these aldeydes is that *S. cerevisiae* reduces these compounds to their corresponding alcohols, as was shown previously for 2-methylpropanal and furfural (Saison et al., 2010). These results show for the first time that the type of refermentation yeast strain strongly affects the flavor profile of the resulting beer. Interestingly, the flavor stabilizing effect at both the chemical and sensorial level was only observed when long-lived yeast strains were inoculated at a density of $1x10^6$ cells/mL, but not when they were inoculated at $2x10^3$ cells/mL. This indicates that apart from the strain itself, its concentration during refermentation also strongly influences the flavor profile of the resulting beer. This is consistent with earlier observations where increasing concentrations of refermentation yeast were shown to result in decreasing concentrations of the staling aldehydes furfural and 2-methylpropanal (Saison et al. 2011). It has to be noted that the authors did not observe this trend for all compounds tested and even saw an increase in *trans*-2-nonenal with increasing yeast concentrations.

Importantly, as long as the beers were fresh (i.e., up to 2–3 months of

storage), the beers with long-lived yeast proved sensorially and chemically indistinguishable from the reference beer, indicating that they do not alter the original character of the beer. An aspect crucial for brewers, as they might not be inclined to implement long-lived yeast if it came at the cost of altering the signature flavor for which their beer is known. The absence of sensory differences between beers refermented with different yeast strains could be due to the limited amount of sugar added for refermentation. Indeed, 0.2°P glucose seems insufficient for the added *S. cerevisiae* to produce sufficient levels of flavor-active metabolic intermediates to alter the beer's typical flavor profile. This is in contrast with yeast strains from the genus *Brettanomyces* for example. On top of the sugar added for refermentation, *Brettanomyces* species can utilize leftover dextrins in the beer, a carbon source that *S. cerevisiae* cannot ferment (Steensels et al., 2015). In doing so, they provide the beer with acidity and a whole range of unique flavors, making them prime candidates for bioflavoring through refermentation (Vanderhaegen et al. 2003), for example in lambic-style beers, but less suited to be used for refermentation when a consistent beer flavor is desired.

An important detail of the current experimental setup is that the long-lived yeast strains were added in addition to the industrial reference strain. As such, gradual lysis of the industrial reference strain and the accessory release of esterases and fatty acids may have contributed to the flavor deterioration in the samples containing long-lived yeast. It is tempting to speculate that adding pure cultures of long-lived yeast could further enhance the preservative potential of refermentation. However, further research is necessary to investigate whether this is really the case.

Finally, it seems that the presence of viable, but not necessarily actively fermenting yeast is key to slow down beer aging during storage. This provides opportunities to increase the flavor stability of beer styles that do not have 'refermentation' as part of their manufacturing process, including the market-dominant lager beers.

6. Conclusion

Taken together, our results show how the use of long-lived refermentation yeast is an interesting new route to slow down the staling of beer and prolong its shelf life, without the need for cooling. Screening a large number of yeasts isolated from different niches yielded specific strains that show an extreme natural capacity to survive in beer during storage. The generation and selection of superior hybrids derived from these natural strains yielded hybrid lineages that showed even stronger longevity and stress resistance and proved able to slow down beer staling for up to one year. As such, these results provide a foundation for a sustainable, natural method to increase the shelf life of beers stored at ambient temperature. Interestingly, similar procedures might also be applied to other products to which living microbes can be added, including for example certain dairy and meat products.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2022.133863.

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