Biotechnological exploitation of *Saccharomyces jurei* and its hybrids in craft beer fermentation uncovers new aroma combinations

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A B S T R A C T

Hybridisation is an important evolutionary mechanism to bring about novel phenotypes and may produce new hybrids with advantageous combinations of traits of industrial importance. Within the *Saccharomyces* genus, *Saccharomyces jurei* is a newly discovered species and its biotechnological potential has not yet been fully explored. This yeast was found to be able to grow well in unhopped wort and at low temperatures, qualities necessary in good candidates for fermented beverages. Here, we analysed its fermentation and aroma profile and created novel non-GMO hybrids between *S. jurei* and *S. cerevisiae* ale yeasts to develop new starter strains with interesting flavours for the craft brewing and beverage industry in general. Pilot beer fermentations with specific hybrids showed a good fermentation performance, similar to the ale parent strain, while eliminating the hyper-attenuation characteristic and a more complex flavour profile. This study exploits the genetic diversity of yeasts and shows how inter-specific hybridisation and clone selection can be effectively used in brewing to create new products and to eliminate or increase specific traits.

1. Introduction

The brewer's yeast is often synonymous with *S. cerevisiae*, a domesticated species and predominant in beer and wine fermentations (Gallone et al., 2016). In fact, *S. cerevisiae* offers outstanding fermentation capability together with the ability to consume the broad range of sugars present in wort and must (Mortimer, 2000).

Moreover, throughout their domestication, the *S. cerevisiae* strains employed in beer and wine diverged from the wild strains not only for their performances but also for their aroma profile. Wild strains often possess traits wich impart unusual, often unwanted, notes to the aroma, such as the production of phenolic off-flavours (Gallone et al., 2016).

The role of the *Saccharomyces* species in the production of fermented beverages is however not limited to that of *S. cerevisiae* alone. In particular, natural hybrids between *S. cerevisiae* and at least three other species, namely *S. eubayanus*, *S. uvarum*, and *S. kudriavzevii*, were also isolated from brewing and wine making processes (Alsammar and Delneri, 2020) (García-Ríos et al., 2019) (Krogerus et al., 2018). Hybridisation helped greatly to bridge the phenotypic gap between domestic

and wild species while adding traits of industrial relevance, such as cold tolerance and flocculation, to the *S. cerevisiae* backbone (Giannakou et al., 2020).

A clear example of the potential of interspecific hybridisation is represented by *S. pastorianus*, a *S. cerevisiae* and *S. eubayanus* hybrid, employed in the production of lager beers (Mertens et al., 2015) (Monerawela and Bond, 2018). Albeit less common, *S. cerevisiae* hybrids with *S. uvarum* or *S. kudriavzevii* have been associated with wine and cider fermentations while *S. cerevisiae* x *S. kudriavzevii* and *S. cerevisiae* x *S. uvarum* interspecific hybrids have also been isolated from brewing and winemaking fermentative environments (Krogerus et al., 2018) (García-Ríos et al., 2019). Much work has gone in the generation of novel hybrids, both to improve the fitness and to diversify the aroma profile of industrial strains to accommodate the consumer desire for a more complex sensory experience, (*i.e.* new combination of compounds that produce a unique aroma)(Bellon et al., 2013; Krogerus et al., 2017).

Recently, a new species in the genus, *S. jurei*, was discovered from *Quercus robur* bark in France. Phenotypic assays revealed *S. jurei* to possess interesting traits for industrial application. In fact, the strains

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characterized were resistant to a variety of stressors, from osmotolerance to high sugar concentrations, while presenting a relatively high fitness at low temperature (Naseeb et al., 2017, 2018). Recently, *S. jurei*'s application and performance in baking were assessed together with other cryotolerant species of the *Saccharomyces* genus, opening new avenues in the food market (Magalhães et al., 2021).

The craft beer market is constantly growing and is in high demand of new beer styles and flavours. Consumers demand more complex and stronger flavours from beer products with natural unconventional ingredients (Jaeger et al., 2020). This work is inspired from the need of expanding biotechnological applications to the brewing industry. Beer yeast strains can bring great complexity and novelties in future beer making and the need of new brewing candidates with unique traits is important. Here we evaluated *S. jurei* as a candidate for industrial applications studying its performance in wort media and providing a comprehensive characterization of its aroma profile through GCxGC-MS analysis. Moreover, we constructed non-GMO hybrids between *S. jurei* and a *S. cerevisiae* ale strain containing different combination of allelic traits. The novel hybrids were used in pilot beer fermentation revealing optimal sugar attenuation and different spectra of flavours. This study shows the efficacy of inter-specific hybridisation to create alternative cultures for beer fermentations.

2. Materials and methods

2.1. Yeast strains

The strains used in this study are the *S. cerevisiae* ale strains OYL200 (Tropical IPA; Omega Yeast lab; Sc-200) and OYL500 (Saisonstein's monster, Omega Yeast Lab, Sc-500), *S. jurei* D5088 (NCYC 3947; Sj-88) and D5095 (NCYC 3962; Sj-95)(Naseeb et al., 2017), the *S. cerevisiae* lab strain NCYC505 (Sc-505) (Martini and Kurtzman, 1985), the *S. cerevisiae* wild isolate 96.2 (Sc-96.2) (Paget et al., 2014) and the *S. cereivisae* x *S. kudriavzevii* allotetraploid hybrid PB7 (Morard et al., 2020) and BY4742 (Brachmann, 1998).

2.2. Hybridisation and hybrids confirmation

Sj-95 was crossed with ale strain Sc-200 and Sj-88 with strain Sc-500. The tetrads were formed by growing the parental strains in pre-sporulation media (yeast extract 0.8%, bacto-peptone 0.3% and glucose 10%, VWR,UK) at 30 °C for 16 h before plating on minimal sporulation medium (1% Potassium acetate, 0.125% yeast extract, 0.1% glucose and 2% bacto-agar, VWR,UK). The sporulation plates were incubated at 20 °C for 7–10 days for the formation of tetrads. Tetrad dissection and spore to spore mating was performed in fresh YPD plates (1% yeast extract, 2% peptone, 2% agar, 2% glucose, VWR, UK) using a 400MSM micromanipulator (Singer Instruments, UK). The plates were incubated at 30 °C till colony formation. Then, the colonies were spread plated in fresh YPM plates (1% yeast extract, 2% peptone, 2% agar, 2% maltose, Sigma Aldrich, UK) and a single colony was used for DNA extraction. DNA was extracted from an overnight grown culture of yeast strains by using the standard phenol/chloroform method described previously (Fujita and Hashimoto, 2000) with modifications described in (Naseeb et al., 2018). The hybrid status of potential hybrids was confirmed by PCR amplification using species-specific primers and genomic DNA template. Two primer pairs were used for the species-specific multiplex PCR, each targeting a specific part of one of the parental species' genome. Primers Scer_F (5′- GGTTTTATCTGG-CACTCAGGT -3′) and Scer_R (5′- GTTGCTGTTGCTGCAAAGGT -3′) amplify a 417 bp amplicon of the *S. cerevisiae* genome. Primers Sjur_F (5′- CTCAAATGGGAATGCCACCG -3′) and Sjur_R (5′- TCCTGA-TAGTGGTTGTTGCT -3′) generate a 233 bp *S. jurei* -specific amplicon (see Fig. S2 in the supplemental material). The PCR conditions were as follows: 2 min at 94 °C, 35 cycles of 1 min at 94 °C, 1 min at 55 °C, and 30 s of 72 °C, followed by a final cycle of 3 min at 72 °C and subsequent

cooling to room temperature. Candidate hybrids showing two bands were considered to be interspecific hybrids (see Fig. S2). PCR confirmed hybrids were streaked another six times in YPM plates to ensure strain purity and genome stability. To confirm the diploid nature of the hybrids, the ploidy was estimated by flow cytometry (Haase and Reed, 2002) using Amnis ImageStream X (ISX MKII) multispectral imaging flow cytometer; and the hybrids were sporulated to ensure no viable spores were detected. Furthermore, species-specific, chromosome-specific primers sets were designed for all the 16 chromosomes present in *S. cerevisiae* and *S. jurei*, respectively (Table S1) and PCR was performed to confirm the presence of both chromosomes sets in the hybrids.

2.3. Micro-fermentations and culture conditions

Growth kinetics in YPD, YP+ 2% maltose and YP+2% maltotriose were generated using BMG LabTech Omega series Microplate Readers. The experiment was held for 72h in 96 well plates with 4 biological replicates and initial OD_{600} was 0.1 in 200 μl of working volume. Growth was recorded via optical density at 600 nm measuring the absorbance at regular intervals.

The micro-fermentations in dry malt extract were carried out using a BioLector® I system (m2p Labs, Germany) with a 48 well FlowerPlate® (MTP-48-B, m2p Labs, Germany) for 72h. Unhopped wort, with starting gravity (OG) 1037, was made by using 104 g/L DME (Browland,UK) and boiled for 1 h. The media was filtered to remove any undiluted powder. The following BioLector settings were programmed: filter: biomass (gain 15), humidity: on (>85% using ddH2O), temperature: 20 °C, oxygen supply: 20.85% (atmospheric air), agitation speed: 800 rpm. The total volume of each well was 1500 μL, initial OD_{600} was 0.1. Scatter light at 620 nm was measured every 7.29 min and logged by the BioLector®.

The R package grofit was used to analyse the growth curve data of plate reader and micro-fermentation experiments (Kahm et al., 2010). Heat maps of the growth parameters were constructed using the R shiny app (https://kobchai-shinyapps01.shinyapps.io/heatmap_construction/) using min-max column scaling.

The 10L scale beer fermentations were carried out for 14 days in home brewing equipment (plastic ferment, lid and airlock) using pale wort OG 1055.4 according to the brewery's recipe (Table S2). Each day the gravity was measured to evaluate the fermentation rate using a density meter (DMA 48, Anton Paar). Samples from the final fermentation day (14th) were stored for GCxGC - MS and HPLC analysis.

2.4. PAD1, FDC1 transcript analysis by RT-qPCR

Strains were grown overnight in YP-glucose 2%, after which 3 biological replicates per strain were used to inoculate a 12°P unhopped wort to a starting OD_{600} of 0.1. Cultures were incubated overnight at 20 °C (OD_{600} varied between 6 and 8) after which RNA was isolated from pelleted yeast using RNeasy Mini Kit (Qiagen, Germany) The lysis was performed by enzymatic digestion of cell wall followed by lysis of spheroplasts. Between 0.25 and 0.5 μg of total RNA was transcribed into cDNA in a 20 μL reaction mix using QuantiTect Reverse Transcription Kit (Qiagen, Germany) according to the manufacturer's protocol. The resulting cDNA was diluted 5-fold and 2 μL of diluted cDNA (corresponding to 10 ng of total RNA) was used as template in 10 μL qPCR reactions. The qPCR reactions were prepared with 5 μl of iTaq Universal SYBR Green super Mix 2X and 0.3 μM of gene-specific primers (Table S1). In addition to using primers specific to *PAD1* and *FDC1*, reactions with primers for reference gene (*ACT*) were also performed. The qPCR reactions were performed on a LightCycler® 480 II instrument (Roche Diagnostics, Basel, Switzerland) in 3 technical replicates on the reverse-transcribed RNA isolated from 3 biological replicates. The following programme was used: pre-incubation (95 °C for 3 min), amplification cycle repeated 45 times (95 °C for 15s, 55 °C for 30s, 72 °C for 30s with a single fluorescence measurement), melting curve programme (65–97 °C with continuous fluorescence measurement), and

finally a cooling step to 40 °C. The relative expression of *PAD1* and *FDC1* in the 5 examined yeast strains was calculated using the 'delta-delta CT'-method by normalizing expression to that of the *ACT* gene. Statistical analysis and plots were produced using GraphPad prism with ANOVA.

2.5. Phenotypic assay test

To determine sensitivity to different growth temperatures, standard YPD and YP-maltose agar plates were incubated at 22 °C, 16 °C and 8 °C. The range of temperatures selected are according to temperatures used in the brewing industry. Strains were grown overnight in liquid YPD and 5 µl of 10 fold serial dilutions were spotted to plates starting with an OD_{600} 0.4.

2.6. HPLC and headspace – SPME GCxGC – TOF -MS

The substrate consumption and alcohol production were determined by ion-exchange HPLC. Prior to the HPLC analysis, the samples were filtered (pore size 0.45 µm). The injection volume was 10 µL, the eluent was 5 mM H_2SO_4 and the flow rate was 0.8 mL/min. A Hi-Plex H (7.7 × 300 mm × 8 µm) (Agilent, USA) was used and kept at 60 °C. Quantification was achieved using a RID-detector. Quantification of sugars and ethanol was achieved with usage of a calibration curve by plotting the instrument response of known standard concentrations of the compounds analysed.

Aroma composition of the final beer products was determined by HS-SPME-GCxGC - TOF- MS. All samples were kept frozen prior analysis to minimize changes occurring during storage. GC instrumentation, SPME extraction, GCxGC – TOF – MS protocol and data analysis was performed as described in (Carlin et al., 2016). Aroma profiling was visualized using R shiny app on heatmap construction (https://kobchai-shinyapps 01.shinyapps.io/heatmap_construction/). A Gerstel MultiPurpose Sampler autosampler (Gerstel GmbH & Co. KGMülheim an der Ruhr Germany) with an agitator and SPME fibre was used to extract the volatiles from the sample vial headspace. The GC x GC system consisted of an Agilent 7890 A (Agilent Technologies, Santa Clara, CA) equipped with a Pegasus IV time-of-flight mass spectrometer (Leco Corporation, St. Joseph, MI). A VF-Wax column was used as first-dimension (1D) column, and a RTX-200MS-column was used as a second-dimension (2D) column. The GC system was equipped with a secondary column oven and non-moving quadjet dual-stage thermal modulator. The injector/-transfer line was maintained at 250 °C. Oven temperature programme conditions were as follows: initial temperature of 40 °C for 4 min, programmed at 6 °C min⁻1 up to 250 °C, where it remained for 5 min. The secondary oven was kept 5 °C above the primary oven throughout the chromatographic run. The modulator was offset by +15 °C in relation to the secondary oven; the modulation time was 7s and 1.4s of hot pulse duration. Helium was used as carrier gas at a constant flow of 1.2 mL min⁻1. The MS parameters included electron ionisation at 70 eV with ion source temperature at 230 °C, detector voltage of 1317 V, mass range of m/z 35–450 and acquisition rate of 200 spectra s⁻1. SPME extraction was carried as follows: 5 mL of beer, sonicated for 2 min to remove the foam, were put into 20 mL glass headspace vials, 1.5 g of NaCl were added, the samples were spiked with 50 µl of alcoholic solution of 2-octanol at 2.13 mg L⁻1 and 50 µl of alcoholic solution of ethyl hexanoate (99%, Sigma-Aldrich) at 1.0 mg L⁻1 as internal standards. Samples were kept at 35 °C for 5 min and then extracted for 30 min at 35 °C. The headspace was sampled using 2-cm DVB/CAR/PDMS 50/30 lm fibre. The volatile and semi-volatile compounds were desorbed in the GC inlet at 250 °C for 4 min in splitless mode and the fibre was reconditioned for 7 min at 270 °C prior to each analysis.

2.6.1. Data processing and peak annotation

GCxGC-MS data acquisition and processing were achieved with LECO ChromaTOF (Version 4.71). The processing consists of peak picking, peak annotation, and statistic confirmation. During the peak

picking, signals which were just above the noise were taken into account (baseline offset = 1). Minimal expected peak width (on 2nd dimension) for deconvolution was 0.8 s. A peak was defined when at least 5 ions whose signal to noise ratio is above 100 (Stefanuto et al., 2017), can be grouped. A picked peak was annotated by matching its mass spectrum (MS) to the reference spectrum in the database. In this study, used MS databases were NIST/EPA/NIH 11, Wiley 8 and the FFNSC 2. The MS similarity threshold for the peak annotation was 700. Each sample was analysed with three technical replications. Inter-measurements peak alignment was performed based on the retention times (both 1st and 2nd dimensions) and mass spectrum. A minimal MS similarity of 600 was required. An analyte was further examined, only if it can be detected in all the technical replications. An inter-class comparison was performed between sample class and blank class. Fisher ratio threshold was used to eliminate artifact compounds (sorbent bleeding, column bleeding and other possible interferences). The applied significance level was 0.05. Peak identification was then completed by checking the linear temperature programmed retention index (LTPRI), which is available in the NIST RI database.

2.7. Sensory evaluation of beer products

Sensory analysis was carried out at Cloudwater Brew's laboratory by brewing experts trained to beer sensory analysis and off flavours. Finished beers from the 10L scale fermentation were used by a tasting panel of 6 assesors to evaluate on the differences and/or similarities on their aroma, flavour, taste/mouthfeel and overall impression. The samples were blind-coded by using 3-digit codes and presented to the trained assessors in random order and in duplicates. Palate cleansers were provided and used throughout the sensory testing. The panel was asked to score the intensity of different characteristics present in beer, and also to provide any descriptive profiling of the flavours presented in the samples. A list of descriptors was provided beforehand to the panel.

3. Results and discussion

3.1. Constructions of diploid non-GMO hybrids between S. jurei D5095, D5088 and ale strains S. cerevisiae OYL200 and OYL500

To expand the genetic and phenotypic diversity of yeasts used in the craft beer industry, we generated 8 new interspecific yeast hybrids between different *S. cerevisiae* and *S. jurei* strains.

A previous study showed that *S. jurei* is able to consume maltose, the main sugar found in wort, on solid media (Naseeb et al., 2017). We determined the growth kinetics of *S. jurei* and *S. cerevisiae* lab, wild and ale strains in liquid media containing maltose or maltotriose, as sole carbon source. All strains were able to utilize maltose and maltotriose as sole carbon source, although at different rates, with *S. cerevisiae* ale and lab strains performing better than *S. jurei* and *S. cerevisiae* wild isolates (Fig. S1). Sj-95 performed better than Sj-88 in both sugar sources. The different rates of maltose and maltotriose utilization observed in the two *S. jurei* strains could be due to their genomic divergence (Naseeb et al., 2018). The presence of maltose and maltotriose related genes (Baker and Hittinger, 2019) was evaluated using HybridMine software (Timouma et al., 2020). Both *S. jurei* strains have 100% identical *MAL13*, *MTT1* and *AGT1* genes in their genome. However, *MAL61*, *MAL33* and *MAL32* are diverged between the two strains both at nucleotide and protein level (Table S3), which could explain the phenotypic differences observed in the maltose and maltotriose uptake.

We attempted to obtain meiotic offspring from 16 different *S. cerevisiae* commercial strains to be used to construct hybrids via spore-to-spore mating (Table S4). Two strains, Sc-200 and Sc-500, had a good sporulation, produced four viable spores, and were chosen for the crossings. The other strains either they did not sporulate or had non-viable spores. Sj-95 was crossed with the commercial ale strain Sc-200 while Sj-88 was crossed with the *S. cerevisiae* strain Sc-500 to create

non-GMO hybrids in order to combine the fermentative ability of an ale strain and generate novel aroma profiles. The genome of the diploid parents possess a significant level of heterozygosis (Naseeb et al., 2018), therefore the hybrids will inherit different random combination of traits leading to phenotypic variation. We created eight hybrids (H1-8), via spore-to-spore mating: H1-3 from crossing Sc-200 x Sj-95, H4-8 from crossing Sc-500 x Sj-88. The hybrid nature of the spores was validated via species-specific PCR (Fig. S2). The DNA content of the hybrids used in pilot fermentations was analysed via FACS and compared to that of diploid control strain (Fig. S3). Meiosis was also triggered in the newly created hybrids and spore viability was checked. As expected for a inter-specific diploid hybrid there were no viable spores (*i.e.* aneuploid hybrid would produce some viable spores; data not shown). To check for the presence of both parental chromosome sets in the hybrids, we carried out a species-specific chromosome-specific PCR. All 16 chromosomes of *S. cerevisiae* and *S. jurei* were present in the hybrids ruling out chromosomal loss (Fig. S4).

3.1.1. Physiological characterization of the hybrids

The growth of the hybrids was tested in liquid medium in presence of the main wort sugars: glucose, maltose, and maltotriose, and compared to the parents (Fig. 1 and Table S5).

In YP + 2% maltose H1 mirrored the fermentation performance of Sc-200. H2 and H3 appeared to have a slower growth rate and elongated lag phase. In YP + 2% maltotriose a different growing pattern was observed. In this carbon source, H2 was mirroring the fermentation performance of Sc-200 while, H1 and H3 had a moderate performance. In YP + 2% Glucose, all strains behaved similarly with small variations in their growth rates. Interestingly, H3 generated the highest biomass in all 3 sugar sources (Fig. 1A).

In conclusion, H1 and H2 performed as well the OYL200 ale parent in maltose and maltotriose, respectively, while H3 reached the highest biomass compared to the other strains in both sugars. Thus, H1 and H2 inherited different characteristics from the parental ale strain in relation to the efficient utilization of the different brewing sugars.

Regarding the family of strains Sj-88 x Sc-500 different patterns were observed. In YP + 2% maltose, hybrids H4 and H8 had the maximum

growth rate and performed better than the ale parent Sc-500. In YP + 2% maltotriose, however, all hybrids had medium/low growth rate indicating moderate maltotriose assimilation. H4 was the best among them, in that sugar source, although it showed a significantly lower performance than both the parental strains. In YPD, hybrids H5, H7 and H8 had the highest growth rate and greater than ale strain Sc-500.

Overall, from this family, H4 was the best performing hybrid in maltose and maltotriose whilst, H6 was the least good.

Next, we evaluated the resistance of the hybrid strains to the hyperosmotic stress derived by the high sugar concentration present in wort and their performance in a mixed sugar medium through microfermentation in malt extract using the Biolector (Fig. 1B). All the strains derived from the Sj-95 x Sc-200 cross, were able to grow and could cope with the changing environment of sugars, pH and ethanol production. H2 had the faster growth among the hybrids.

For the cross Sj-88 x Sc 500, the hybrid progeny H4, H5 and H6 showed higher growth rate than the parent strains. H8 was the worst performing strain in unhopped wort.

Next, we examined the fitness of all strains in solid media contaning glucose or maltose at different temperatures. As *S. jurei* is cold tolerant (Naseeb et al., 2017), we tested the phenopyes of the hybrids at 22 °C, 16 °C and 8 °C. Strains from crossings between Sj-95 x Sc-200 in YPD at 22 °C and 16 °C showed a similar growth pattern (Fig. 2A and C), while at 8 °C Sj-95, H1 and H2 grew better compared to the other strains, indicating that traits linked to a higher fitness at cold have been inherited from *S. jurei* (Fig. 2E). In YP-maltose at 8 °C, H1 grew much better than the other strains (Fig. 2F). In this medium at 22 °C and 16 °C all the strains grew marginally better than Sj-95 (Fig. 2B and D).

As for the Sc-500 x Sj-88 hybrids (H4-H8), we observed good growth for all strains in YPD at 22 °C and 16 °C (Fig. 2A and C). However, at 8 °C H4 and H8 had the best growth among all strains (Fig. 2E). Similarly in YP-Maltose, H4 and H8 had the best growth and colony formation among all strains in 8 °C (Fig. 2F). Whilst at 22 °C all strains grew well and at 16 °C H4 and H8 grew better (Fig. 2B and D).

H1 (crossing Sj-95 x Sc-200) and H8 (crossing Sj-88 x Sc-500) displayed a good maltose assimilation, inherited from the *S. cerevisiae* ale strain, and a good performance in colder temperatures too as derived
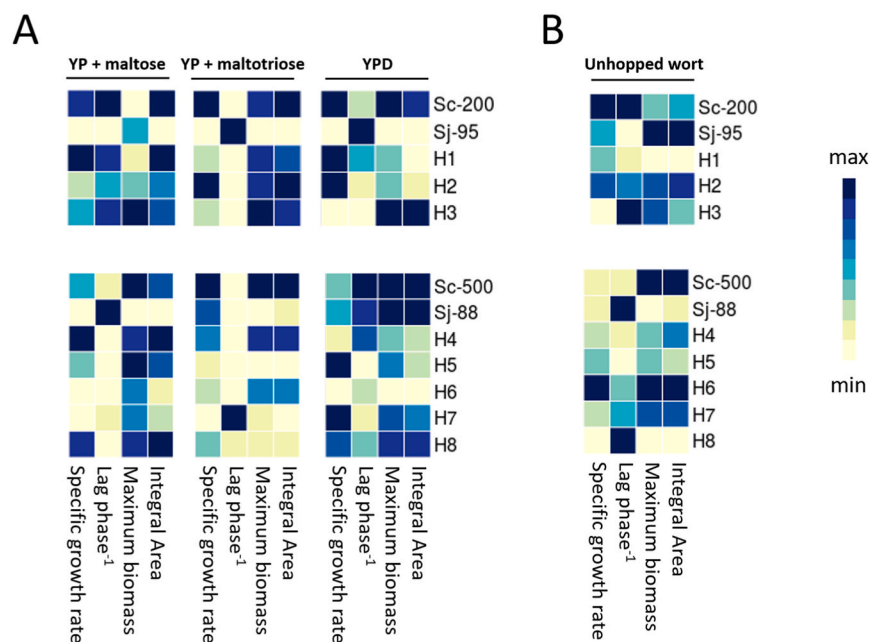

Fig. 1. Heat map of growth parameters of Sj-95, Sj-88, Sc-200, Sc-500 and H1-8 hybrids. Growth parameters were assessed in YPD, YP + 2% maltose an YP + 2% maltotriose at 16 °C (A) and in unhopped wort at 20 °C (B). For each parameter, min and max represent the lowest and highest value reported for each hybrid cross in a specific medium. Lag phase is expressed as (lag phase)$^{-1}$ so the highest value correspond to the lowest lag phase observed.
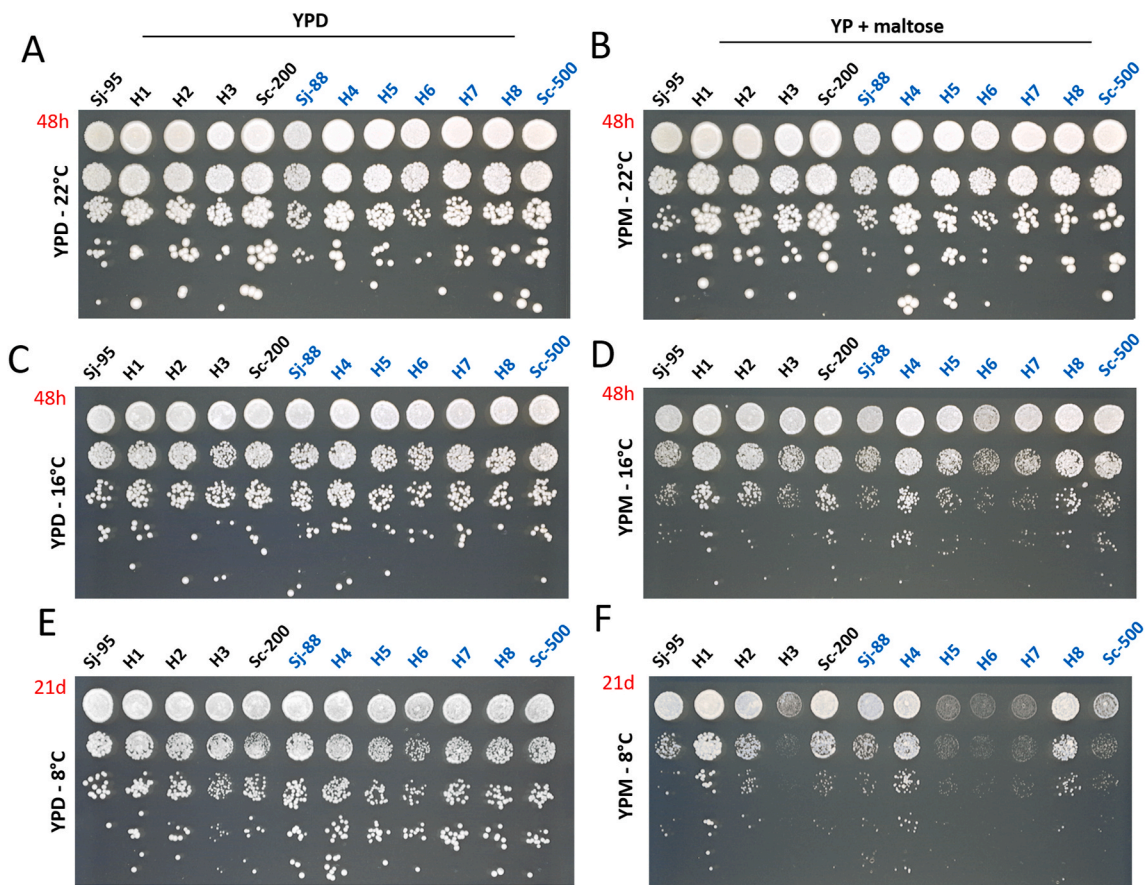
Fig. 2. Spot test assay of Sj-95, Sj-88, Sc-200, Sc-500 and H1-8 hybrids at different temperatures. Each strain was spotted in YPD at 22 °C (A), 16 °C (C) and 8 °C (E) and YP + 2% maltose at 22 °C (B), 16 °C (D) and 8 °C (F). Plates were incubated for 48h (A, B, C, D) and 21 days (E, F).

from *S. jurei* (Fig. 2). In fact, in maltose at 16 °C and 8 °C degree H1, H4 and H8 were showing heterosis.

Ale beer fermentation is commonly between 16 and 22 °C and overall most of the hybrids grew well in this temperature range, with H1, H2, H4 and H8 showing a better grow in maltose at 16 °C.

Beer fermentation experiments were then carried out with the hybrid progenies of the cross Sj-95 x Sc-200 (H1-3) based on the performance in unhopped wort of the specific *S. jurei* parent (Sj-95 is growing better than Sj-88 as shown in Fig. S1) and the relative hybrids.

3.2. Pilot beer fermentation in 10L vessels

In beer fermentation, sugar attenuation and cell viability are important at the end of fermentation (Sanchez et al., 2012). In brewing terms, attenuation describes the level of wort carbohydrates that are converted into ethanol during fermentation (Vidgren et al., 2009) and it is often desired to a level of 70–80% as the leftover sugars contribute a desirable sweetness and body composition to the final beer products. Certain *S. cerevisiae* strains are characterized by their ability to secrete a glucoamylase (*STA1* gene), an enzyme that catalyses the digestion of dextrins. This amylolytic activity can lead to hyper-attenuation, meaning that almost all the sugars (fermentable and non-fermentable) are consumed during beer fermentation, and/or secondary fermentation which can cause excess carbon dioxide formation in bottles, cans or kegs (Meier-Dörnberg et al., 2018) (Yamashita et al., 1985). Results from 10L beer fermentation experiment evaluated the sugar attenuation and cell viability of all strains at pilot scale (Fig. 3). Sc-200 was found to over-attenuate the beer, with a decrease of 92% of the original gravity. The poor performance of Sj-95 observed in microfermentations was mirrored in pilot scale fermentations, with a total decrease in gravity of
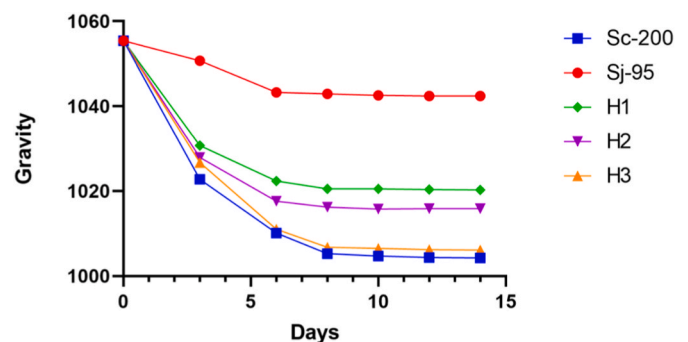

Fig. 3. Fermentation kinetics of strains Sj-95, Sc-200 and the generated hybrids H1-3. Beer fermentation was done with pale wort of initial OG 1055.4. The plot contains reduction of sugars as gravity points measured with density meter.

only 23% across 14 days. All the hybrids showed improved fermentation ability compared to Sj-95, having inherited from Sc-200 the necessary traits to sustain the stressors of industrial fermentation and efficiently utilized the wort sugars. H3 behaved similarly to Sc-200, over attenuating the beer (89%), while H1 and H2 attenuated the beer to 70% of the original gravity, a desirable characteristic for different beer styles. The gravity remained stable after the 14th day indicating that no further fermentation took place. All the strains were stained with methylene blue to estimate cell viability which is important for re-pitching. No significant difference in viability across strains was detected (*i.e.* approximately 50% viable cells at the end of the fermentation process).

Based on the hyper-attenuation characteristic that was observed in

the fermentation profiles, we also tested for the presence of the *STA1* gene in the newly made hybrids via PCR (Yamauchi et al., 1998). Our genome data (Naseeb et al., 2018) indicates that the *S. jurei* strains do not have the *STA1* gene. The PCR results showed that only the ale parent strain Sc-200 and H3 possess the *STA1* gene, while H1, H2 and Sj-95 do not (Fig. S5). Therefore, it is likely that Sc-200 is heterozygotic for the *STA1* gene, and only H3 inherited that allele. Hence, in H1 and H2 the undesirable trait for hyper-attenuation was eliminated.

End point fermentation samples were analysed via HPLC to estimate the sugars consumed and ethanol produced by the different strains (Fig. 4). The consumed sugars detected at the end of the fermentation confirmed the attenuation observed in the gravity data (Fig. 3). Sc-95 showed the highest amount of residual sugars and the lowest production of ethanol compared to the Sc-200 and the generated hybrids. The ethanol produced (% ABV) varied between all strains, with levels of 6.6% (Sc-200), 6.4% (H3), 5.2% (H2), 4.5% (H1) and 1.8% (Sj-95).

3.3. Aroma profiling of generated hybrids reveals different aroma compounds from both S. jurei and S. cerevisiae parents

Identification of volatile aromatic compounds for all strains was carried out at the end of the beer fermentation using GCxGC-MS. In total, 18 esters, 11 alcohols and 33 volatile compounds including acids, terpenes, aldehydes, ketones and phenolic compounds were identified (Table 1). Peak area was used as an indication of relative compound concentration between beer samples.

As expected, the levels of volatile compounds varied between the parent strains. A strong tropical fruit character was detected in Sc-200 due to the greater production of esters and other volatiles (Table 1 and Fig. 5). Sj-95 had instead a lower production of esters, but produced a significant amount of spice/clove and alcoholic aromas deriving from phenolic compounds and fusel alcohols, respectively. The hybrid strains inherited different combined flavour profiles from the parental strains. Ethyl hexanoate, a compound with a sweet fruity/apple aroma, derived from strain Sc-200 was only inherited in H1 and H2 hybrids. Compared to the parents, a higher production of specific compounds, such as ethyl propionate and α-terpinyl-acetate, which intensify some aroma characteristics, were also present in the hybrids. 4-vinyl-guaiacol, a spicy phenolic aroma was detected in Sj-95 and inherited in lower concentrations in H1 and H2, while not detectable in H3. A low production of propyl acetate and ethyl isobutyrate, both compounds with fruity aroma, was detected in both parental strains. Similar moderate production of the same metabolite is recorded in hybrids H1 and H2 but the beer made by H3 did show a higher concentration of this specific compound out-performing both parental strains. Different parental allele inheritance in the hybrids is responsible for the diversification of the aroma characteristics in the beer by strengthening or weakening specific

features. Clustering of the samples according to the amount of compounds detected showed H1 and H2 aroma profiles resembled those of Sc-200 and Sj-95, respectively (Fig. 5). Interestingly, H3 does not clearly cluster with any of the two parents and shows a more diverse flavour profile.

Transcription levels of *PAD1* and *FDC1* alleles were quantified via RT-PCR since these two genes encode for enzymes involved in the decarboxylation of ferulic acid to 4-vinyl guaiacol (Mukai et al., 2010). For the *PAD1* gene, all hybrids expressed the *S. cerevisiae* allele in lower levels than the parent Sc-200; while the *S. jurei* allele was only significantly different from the parent Sj-95 in the hybrid H3 (Fig. 6A). For the *FDC1* gene, a similar expression of the *S. jurei* allele was detected in H1, H2, H3 and Sj-95, but a lower expression of the *S. cerevisiae* allele was observed in all hybrids when compared to the Sc-200 parent (Fig. 6B). Therefore, the undetectable levels of 4-vinyl-guaiacol in H3 could be due to the lower expression of *PAD1* and *FDC1* for both *S. cerevisiae* and *S. jurei* alleles in that strain.

A sensory evaluation of the final beers was carried out at Cloudwater Brew Co (Fig. 7; Table S6). Finished beers made with H1 and H2 possessed a nice estery character with predominant notes of apple, banana and pear. Finished beer made with strain Sc-200 was characterized by a predominant estery profile contributing to fruity characteristics but with an astringent character and dry after taste. Metabolites related with these aromas were in fact detected in high concentrations through GC – MS in those beer samples (Table 1). A clove/phenolic character present in different intensities was tasted in Sj-95, H1 and H2 beers and can be linked to the presence in low level of 4-vinyl-guaiacol. Beer made from H3 was described as fruity and tropical, with notes of peach and berries, with a more astringent and light sour character than H1 and H2, and with very low phenolic content. H2 aroma and flavour profile was considered as the most balanced one, with a nice sweetness and soft body characteristics. H1 and H2 were selected for larger scale beer fermentation in 200L barrels to assess the character in a more complex environment with the addition of hops and other adjuncts.

4. Conclusions

Recent years have brought a remarkable increase on the demand of craft beer. Consumers request unique recipes and flavours beyond the traditional and well known beer styles. Aromatic diversity and combination of different flavours is a way to create new products. Through interspecific hybridisation, complex aromatic profiles can be generated from the different parental species.

In this study, we evaluated the beer fermentation performance and aroma profile of *S. jurei* and non GMO generated brewing hybrids. *S. jurei* is able to utilize maltose and it can grow in low temperatures and this ability gives the potential of industrial applications and breeding with other brewing strains. We also showed that *S. jurei* can assimilate maltotriose, another brewing related sugar. It is our understanding that maltotriose uptake mechanisms and brewing potential of a novel *S. jurei* strain isolated from *Fraxinus excelsior*, Bavaria, Germany, have been the topic of a collaborative study involving VTT Technical Research Centre of Finland, TU Berlin, and TU Munich, and that this work will be reported shortly in *Frontiers in Microbiology* (https://doi.org/10.1101/2021.01.08.425916). An intriguing outcome of this work has also been the observation that *S. jurei* has the ability to utilize maltotriose and may, under the right conditions, or with appropriate evolutionary engineering, serve as a brewing strain in its own right. Our study, however shows that, *S. jurei* fermentation ability is limited when compared with a *S. cerevisiae* ale strain, hence we constructed hybrids between those 2 different species through spore to spore mating. In that way, we can see a combination of inherited traits in the hybrids contributing to different phenotypes and growth characteristics. The good performance of the hybrids could result from an additive interaction between the alleles from the parental species (heterosis; Bernardes et al., 2017). The ability of both parents to ferment maltose and maltotriose efficiently provides a
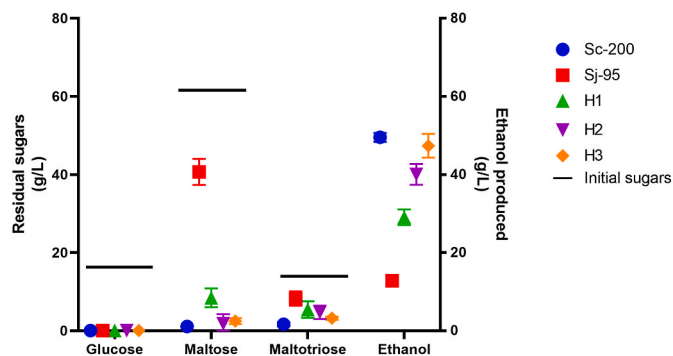

Fig. 4. Sugar content and production of ethanol in final beer samples analysed via HPLC. Graph shows the residual wort sugars and the ethanol production at the end of fermentation from strains Sc-200, Sj-95, H1, H2, and H3.

Table 1
Target volatile metabolites of beer samples after 14 days of fermentation with Sj-95, Sc-200, H1, H2 and H3.

Compound name	Flavour description	Peak Area x 10^6				
		Sj-95	H1	H2	H3	Sc-200
Esters						
Ethyl Acetate	fruity, pear	0.10	14.07	n.d	2.17	20.55
Isoamyl acetate	fruity, banana	113.09	118.07	113.51	33.91	195.70
Isobutyl acetate	fruity	1.34	3.08	0.80	1.24	2.47
Phenylethyl Acetate	floral, roses	n.d	n.d	49.89	73.73	204.76
Ethyl hexanoate	fruity, apple	n.d	139.98	0.63	n.d	182.91
Ethyl benzoate	medicinal	0.10	n.d	0.83	0.13	1.31
Propyl acetate	fruity, pear	9.16	30.06	17.27	46.10	13.12
Ethyl Propionate	fruity, pineapple	5.91	12.57	5.93	16.99	5.79
Ethyl heptanoate	fruity, grape	9.52	14.08	23.08	8.11	16.54
Ethyl octanoate	fruity, wine, sweet	122.15	384.92	341.67	91.09	256.04
Isopentyl isobutyrate	fruity, apricot, banana	7.12	10.87	8.37	7.30	n.d
Ethyl decanoate	sweet, apple, grape	n.d	135.00	20.73	2.73	9.26
Ethyl isobutyrate	fruity	0.65	0.82	0.55	1.46	0.79
Methyl Phenylacetate	honey	3.28	3.75	3.31	3.60	3.13
cis-Carvyl acetate	minty	n.d	n.d	0.91	0.75	0.75
Propyl octanoate	coconut, cacao	0.12	5.47	0.50	n.d	0.10
Alcohols						
Isoamyl alcohol	fusel, alcoholic, fruity	775.86	523.41	615.71	0.58	1.67
Phenylethyl alcohol	floral, rose, honey	n.d	141.54	102.47	n.d	199.83
Butanol	solvent, fusel	4.22	4.79	4.55	5.62	17.69
Propanol	solvent, fusel	58.09	113.88	0.27	84.11	40.09
Isobutanol	etheral, wine	31.05	35.44	18.22	27.29	31.40
Furfuryl alcohol	sweet	n.d	3.13	1.44	2.83	1.67
Hexanol	pungent, fusel, fruity	19.07	23.32	17.57	22.85	19.38
Methionol	meaty, onion	0.59	0.63	n.d	0.81	0.98
Acids						
Hexanoic Acid	sour, cheesy	114.68	191.37	110.19	171.14	126.50
Butanoic Acid	acidic, unpleasant	11.08	3.88	7.04	5.46	5.81
Isovaleric Acid	cheesy	12.92	10.76	9.12	17.20	9.96
Isohexanoic acid	fruity	n.d	0.08	0.03	0.69	0.42
Propanoic acid	pungent, acidic	2.07	5.02	3.94	8.40	1.64
Acetic Acid	sour, acidic	32.58	0.68	19.70	n.d	0.68
Octanoic Acid	rancid, cheesy	n.d	315.80	173.67	186.57	116.58
Terpenes and other volatile compounds						
1-alpha-Terpinyl acetate	herbal	3.70	4.44	2.16	4.61	2.91
Hop ether	floral, woody	11.40	10.06	10.93	14.16	8.13
Myrcene	woody, spicy	3.79	14.06	13.58	9.78	10.76
Linalool	citrus, floral, waxy	97.57	n.d	n.d	107.32	n.d
4-vinyl guaiacol	clove, spicy, smoke	1.95	2.30	1.36	n.d	n.d
2-4-di-tert-butylphenol	phenolic	3.16	3.84	2.94	3.52	3.46
Alpha-Phellandrene	citrusy, peppery	0.56	0.63	0.58	0.58	0.68
o-Cymene	spicy	9.48	9.30	8.54	10.72	7.83
Dehydro-p-cymene	fresh, citrus	0.46	0.56	0.32	n.d	0.39
Nonanol	citrus	17.61	14.38	48.42	23.02	n.d
Limonene	citrus, fresh, sweet	0.61	0.16	0.90	n.d	0.27
2-Acetylfuran	sweet, nutty	n.d	1.31	n.d	1.54	1.24
2-Hexanone	acetone like	0.03	0.06	0.04	n.d	0.06
Terpinolene	pine	0.27	n.d	0.29	0.41	0.42
Camphene	fresh, herbal, woody	0.04	0.04	0.02	0.05	n.d
(E)-2,6-Dimethylocta-1,5,7-trien-3-ol	camphoreous, lime	1.73	2.29	1.17	2.01	2.44
Nonanal	citrus	0.31	0.54	0.55	n.d	0.54
Ocimene	fruity	13.09	11.71	9.43	n.d	9.77
Pinene	woody, pine	n.d	2.16	n.d	17.93	2.16
1-Decene	sweet	0.29	5.06	7.55	n.d	5.79

fertile ground for hybrids possessing an allele combination which improves on the parental phenotype.

This is shown for several industrial relevant traits such as growth in maltose and maltotriose, which contribute to the good performance in brewing wort, and in growth at cold temperature. Also, the superior fermentation performance is observed in generated hybrids, overcoming *S. jurei* inability to complete the fermentation. This suggests that efficient wort sugar utilization is not essential to be derived from both parental strains to create successful hybrids for the brewing industry and opens the potential on usage of alternative *Saccharomyces* species in the production of fermented beverages.

Different allele inheritance in the generated hybrids resulted in the elimination of the unwanted hyper-attenuation character. The *S. cerevisiae* ale parent is likley to be heterogyzous for the particular gene, *STA1*, conferring this characteristic. Therefore, through spore to spore mating the character is not present in all the hybrids. Similar results regarding the phenotypic influence of specific allele inheritance in the generated hybrids is also observed on the 4-vinyl guaiacol production from the *PAD1/FDC1* genes. In H3, the lower expression of the inherited genes resulted in undetectable levels of the clove like aroma. Breeding techniques and meiotic segregation can be useful for the elimination of *STA1+/POF + S. cerevisiae* strains with a great potential of applications in brewing industry (Krogerus et al., 2017) (Nikulin et al., 2018)(Tubb et al., 1981).

Also, in this study we performed aroma analysis on fermented beers from a selection of strains generated in this project. This is the first time the aroma profile of *S. jurei* in beer products is described. A combination of tropical and floral character was inherited in the hybrid strains
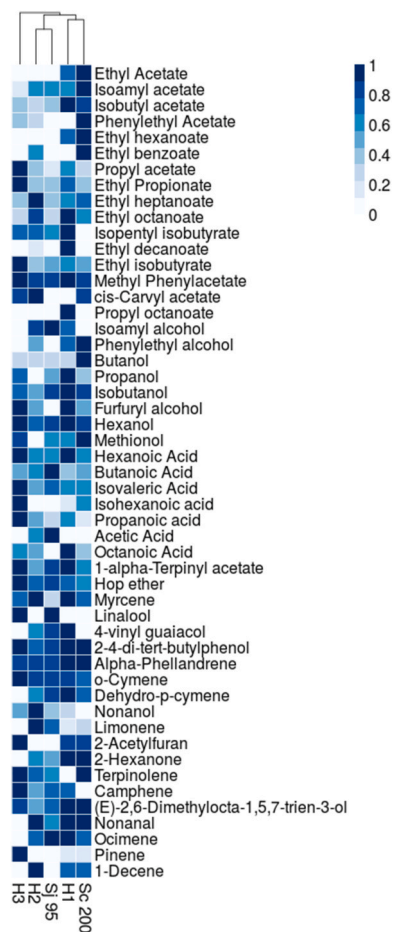
Fig. 5. Clustering and heatmap visualization of aroma profiling. Normalized values per detected volatile compound across 14 days fermentation beer samples with Sj-95, Sc-200, H1, H2 and H3.

deriving from complementary aroma profiles of both *S. jurei* D5095 and *S. cerevisiae* OYL200. The 3 hybrids present different concentrations and intensities of various compounds and complete absence or presence of some volatiles related with their parents. Loss of 4-vinyl guaiacol production is a signature of domestication in brewing yeast (Gonçalves et al., 2016) (Gallone et al., 2016) and it is indicated that the POF character can be eliminated through spore clone selection. Mixed or

intermediary flavour profiles are achievable and highly recommended to facilitate yeast selection in specific beer styles or new recipes.

Applying classical breeding to ale strains can be challenging as strains often suffer from poor sporulation ability and efficiency (Codon et al., 1995) but interspecific hybridisation can be a way of creating new yeast strains conferring characteristics from different species. Various studies demonstrated hybridisation as a mechanism of domestication in beer and wine environments (Gallone et al., 2018) (Gallone et al., 2019) (Giannakou et al., 2020) (González et al., 2008) and also hybrids within the *Saccharomyces* genus have been reported for their usage in fermented beverages (Krogerus et al., 2018) (García-Ríos et al., 2019) (Mertens et al., 2015). Selection of parental strains for breeding or hybridisation experiments possessing desirable characteristics will improve the phenotypic landscape of industrial candidates.

Author contributions

DD conceived the study; DD and UV supervised the genetic and phenotypic experiments, and the GC-GC-MS analysis, respectively. MC
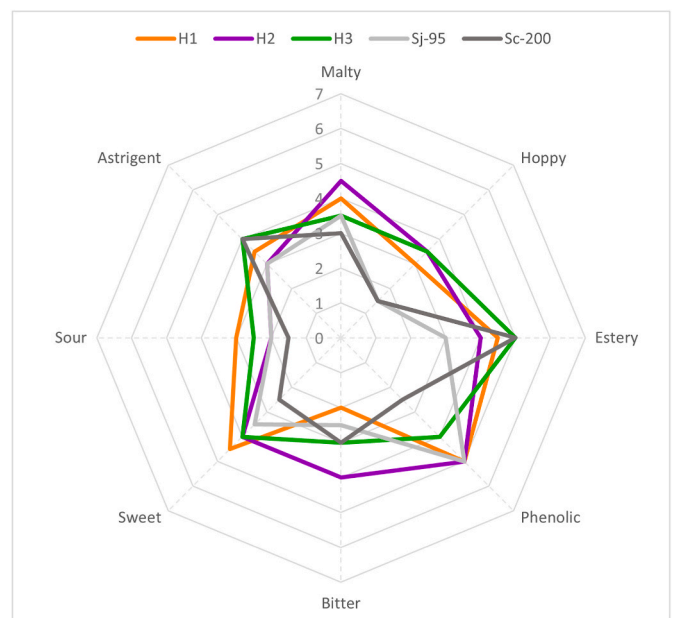

Fig. 7. Descriptive sensory analysis. Final samples from 10L beer fermentation with Sc-200, Sj-95 and hybrids 1–3 for flavour assesment.
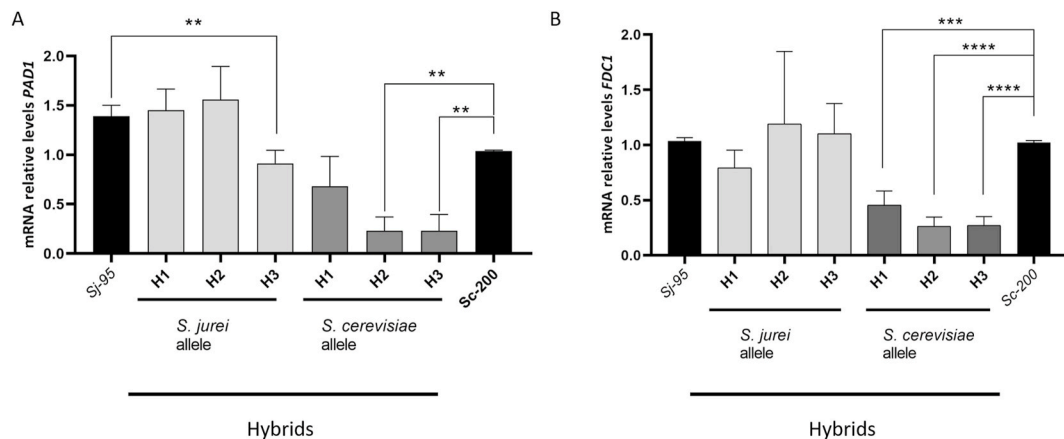

Fig. 6. Relative *PAD1* and *FDC1* expression in all strains. The mRNA levels of *PAD1* (A) and *FDC1* (B) normalized to *ACT1* were determined by RT-qPCR. Sj-alleles in H1-3 (light gray) are compared with Sj-95. Sc-allele in H1-3 (dark gray) are compared with Sc-200. ** indicate $p < 0.005$, *** indicate $p < 0.0005$, **** indicate $p < 0.0001$.

and PJ supervised the pilot-scale fermentation. KG carried out the experimental work with the inputs of FV. NN contributed to the construction of five hybrids. KG and FV analysed the genetic and physiological data and KG, FV and PZ performed and analysed the GCxGC-MS volatile compounds spectra. KG, FV and DD wrote the manuscript with input of MC, PZ, UV. All authors contributed to the article and approved the submitted version.

Declaration of competing interest

All authors declare that there is no conflict of interest related to this article.

PJ is the co-founder and general director of Cloudwater Brew Co. and MC is employed by the company Cloudwater Brew Co. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2021.103838.

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