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Unique brewing-relevant properties of a strain of *Saccharomyces jurei* isolated from ash (*Fraxinus excelsior*)

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Abstract

The successful application of *Saccharomyces eubayanus* and *Saccharomyces paradoxus* in brewery fermentations has highlighted the potential of wild yeast for brewing, and prompted investigation into the application potential of other members of the genus. Here, we evaluate, for the first time, the brewing potential of *Saccharomyces jurei*. The newly isolated strain from an ash tree (*Fraxinus excelsior*) in Upper Bavaria, Germany, close to the river Isar, was used to ferment a 12°P wort at 15°C. Performance was compared directly with that of a reference lager strain (TUM 34/70) and the *S. eubayanus* type strain. Both wild yeast rapidly depleted simple sugars and thereafter exhibited a lag phase before maltose utilization. This phase lasted for 4 and 10 days for *S. eubayanus* and *S. jurei*, respectively. *S. eubayanus* utilized fully the available maltose but, consistent with previous reports, did not use maltotriose. *S. jurei*, in contrast, utilized approx. 50% of the maltotriose available, making this the first report of maltotriose utilization in a wild *Saccharomyces* species. Maltotriose use was directly related to alcohol yield with 5.5, 4.9, and 4.5 % ABV produced by *S. pastorianus*, *S. jurei* and *S. eubayanus*. Beers also differed with respect to aroma volatiles, with a high level (0.4 mg/L) of the apple/aniseed aroma ethyl hexanoate in *S. jurei* beers, while *S. eubayanus* beers had a high level of phenylethanol (100 mg/L). A trained panel rated all beers as being of high quality, but noted clear differences. A phenolic spice/clove note was prominent in *S. jurei* beer. This was less pronounced in the *S. eubayanus* beers, despite analytical levels of 4-vinylguaiacol being similar. Tropical fruit notes

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37 were pronounced in *S. jurei* beers, possibly resulting from the high level of ethyl hexanoate.
38 Herein, we present the successful results of the first intentional application of S. *jurei* as a yeast
39 for beer fermentation known to us and compare its fermentation performance to other species of
40 the genus. Results indicate considerable potential for *S. jurei* application in brewing, with clear
41 advantages compared to other wild *Saccharomyces* species.

42 **1 Introduction**

43 Yeasts of the genus *Saccharomyces* are by far the most prevalent fermentative microorganisms
44 used in brewing. In addition to the commonly used yeasts *S. pastorianus* and *S. cerevisiae*, wild
45 species of *Saccharomyces* such as *S. paradoxus* and *S. eubayanus* have recently been utilized
46 intentionally for beer fermentations (Gibson, 2015; Osburn et al., 2016; Nikulin et al., 2020). The
47 successful utilization of these non-domesticated species suggests that other species belonging to
48 the genus could be profitably employed in beer production, and could facilitate differentiation of
49 beers, or even creation of novel beer styles (Osburn et al., 2016). Alternative *Saccharomyces*
50 species have the potential to introduce novel flavor profiles to beers and, in particular, to lager
51 beers which are fermented at low temperatures (with the exception of *S. cerevisiae* the genus
52 may be described as psychrophilic) (Magalhães et al., 2021). The potential benefits derived from
53 wild yeasts are off-set by a number of characteristics that may be considered negative in the
54 context of brewing. These include the production of phenolic flavor compounds (which typically
55 lend a spice or smoke note to beer); poor flocculation, which hinders clarification; and inability
56 to use all of the available sugars present in brewers wort, thereby limiting fermentation
57 efficiency. Only *S. paradoxus* and *S. eubayanus* have been fully characterized with respect to
58 brewing potential (Gibson et al. 2013; Nikulin et al. 2020, Mardones et al. 2020) and other
59 species in the genus may prove to be more suitable for efficient production of flavorful beers.

60 Eight *Saccharomyces* species are currently recognized (Naseeb et al., 2017). These have been
61 isolated from a range of habitats, but appear to be most prevalent in woodland environments.
62 Oaks and other species within the *Fagaceae* family, for example, serve as a habitat for both *S.*
63 *paradoxus* and *S. cerevisiae*. Sniegowski et al. have isolated several strains of *S. paradoxus* and
64 *S. cerevisiae* from exudate, soil and bark associated with different trees belonging to the *Quercus*
65 genus, while samples taken from poplar, maple and American beech trees did not yield
66 *Saccharomyces* yeasts (Sniegowski et al., 2002). A clear preference of *Saccharomyces* for oak
67 trees compared to trees outside the *Quercus* genus was also shown by Sampaio et al. (Sampaio
68 and Gonçalves, 2008). Other *Saccharomyces* species, isolated from *Drosophila sp*. in Brazil, and
69 from soil and decayed leaves in Japan, have been classified as *S. cariocanus*, *S. kudriavzevii* and
70 *S. mikatae* respectively (Naumov et al., 2000) (though the former species is now often designated
71 *S. paradoxus* (Liti et al., 2006)). *S. mikatae* has not been isolated from other locations to date
72 suggesting a regional restriction of this species. *S. kudriavzevii* has also been isolated alongside
73 other *Saccharomyces* species from oak bark and soil surrounding oak trees in Europe (Sampaio
74 and Gonçalves, 2008). In 2008, a novel *Saccharomyces* species was isolated from trees in
75 Western China. Three strains of the newly named *S. arboricola* were obtained from the bark of a
76 *Quercus* sp. and a *Castanopsis* sp. tree, both belonging to the *Fagaceae* family (Wang and Bai,
77 2008). *S. eubayanus*, the cryotolerant co-parent of today's widely used lager-brewing yeast *S.*
78 *pastorianus*, was first isolated and identified on southern-beech trees (*Nothofagus spp*.) in
79 Patagonia. Along with *S. eubayanus*, *S. uvarum* was observed in the same habitats indicating that
80 these two cryotolerant species thrive in the cold climate of Patagonia (Libkind et al., 2011).

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81 These two species have later also been isolated in sympatry from trees in China, also including
82 oaks (Bing et al., 2014) and Wisconsin, USA, on American beech and maple trees (Peris et al.,
83 2014).

84 In 2017, Naseeb et al. first isolated and described *S. jurei* (NCYC 3947) from an oak tree
85 (*Quercus robur*) in the French Pyrenees. They found a close relationship between *S. mikatae* and
86 *S. jurei* through phylogenetic analysis and suggest shared evolutionary history of these two
87 species (Naseeb et al., 2018). In a later study, (Alsammar et al., 2019) detected DNA
88 homologous to that of *S. jurei* in natural habitats using high throughput sequencing of the ITS1
89 region specific to *Saccharomyces spp*. By sampling a variety of trees (oak, beech, spruce, larch
90 and pine) at different altitudes in the Italian Alps, the soil surrounding the trees was scanned for
91 evidence of the presence of members of the *Saccharomyces* genus. As expected, *S. cerevisiae*
92 and *S. paradoxus* were abundant, but more interestingly, *S. mikatae* and *S. jurei* were identified
93 in many samples as well as *S. eubayanus* and *S. kudriavzevii* (Alsammar et al., 2019). This did
94 not yield any viable strains as only DNA was extracted from the soil. The apparent paucity of *S.*
95 *jurei* in nature may be simply an artefact of the culturing methods used for isolation and
96 enrichment, which may disadvantage some species relative to others. The use of wooden
97 materials and tools in brewing has been widespread over centuries due to its relative ease of
98 fabrication while metallic materials have only become the major material over the last century
99 (Schnegg, 1921; 1922). The contact between wood harboring yeasts and wort containing
100 fermentable sugars and nutrients may have be the source of yeast being used for brewing in the
101 past or still today.

102 In this study, the potential of a *S. jurei* strain isolated from ash (*Fraxinus excelsior*) in Bavaria
103 was investigated for its brewing potential. Traits studied included wort fermentation efficiency,
104 sugar utilization, and beer flavor profile. Performance was compared to that of *S. eubayanus*,
105 which has already been shown to be a capable brewing yeast, as well as the domesticated lager
106 strain *Saccharomyces pastorianus* TUM 34/70.

107 **2 Materials and Methods**

108 **2.1 Yeast strains**

109 Strains used in this work are listed in Table 1. All strains with VTT codes were obtained from
110 VTT culture collection (http://culturecollection.vtt.fi). The *S. pastorianus* strain TUM 34/70 and
111 *S. jurei* strain TUM 629 were obtained from the TU Munich, Research Center Weihenstephan for
112 Brewing and Food Quality (https://www.blq-weihenstephan.de/en/tum-yeast/yeast-and-
113 bacteria/).

114 **2.2 *Saccharomyces jurei* isolation, maintenance and microscopy**

115 Samples of the bark of an ash tree (*Fraxinus excelsior*) located in the meadows of the Isar-river
116 in Munich (Latitude 48.10931383333333 = 48 ° 6' 33.53'' N, Longitude 11.56397166666667 =
117 11° 33' 50.298'' E, 519.8 m above sea level) with attached moss were taken approximately
118 1 meter above ground with sterilized forceps and placed into sterile bags and stored for 2 days at
119 2 °C until processing. 1 g samples of the collected bark were put into a flask, sealed with a sterile
120 plug, containing 50 mL of autoclaved wort at 12 °P (15 min at 121 °C), pH-value 5.3 (prepared
121 from diluted wort concentrate (Döhler, Darmstadt Germany, original gravity approx. 60°)

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122 through addition of deionized water and tetracycline (tetracycline-hydrochloride, Carl Roth,
123 Karlsruhe, Germany) at an application concentration of 50 mg/L. 0.25 ml of a saturated alcoholic
124 biphenyl solution were pipetted onto the sterile plug to inhibit growth of mold. The flask
125 containing wort and the sample was then incubated aerobically at 20 °C for 2 weeks.

126 An inoculation loop of the incubated medium was streaked on a Wallerstein Nutrient Agar plate
127 (WLN-A, Oxoid GmbH, Wesel, Germany, pH 5,6, 50 mg/L tetracycline) and incubated
128 anaerobically for one week at 20 °C. Single colonies were checked for cell morphology and
129 purity of the colony with a microscope (Nikon, Düsseldorf, Germany) using dark field
130 microscopy with a 1000-fold magnification. The examined colonies were then streaked onto a
131 fresh WLN-A plate as described above. The colonies subsequently identified as *S. jurei* appeared
132 small, round, and umbonate with a pale greenish-white coloration. A pure culture of *S. jurei* was
133 streaked on a wort agar slant which was incubated for 3 days at 28 °C before storage at 2 °C. For
134 strain maintenance recultivation was performed every month. For long term storage cryotubes in
135 cryo-vials (Roti-Store yeast cryo vials, Carl Roth, Karlsruhe, Germany) at -80 °C were prepared
136 according to the manufacturer's directions.

137 2.3 Initial identification using species specific Saccharomyces Real-Time PCR assays and 138 ITS, D1/D2 26S rDNA sequencing

139 Yeast DNA was isolated using a modified InstaGene Matrix (Biorad, Feldkirchen, Germany)
140 protocol (Hutzler, 2009; Meier-Dörnberg et al., 2018). Yeast DNA of the single colony were
141 analyzed using different species-specific *Saccharomyces* Real-Time PCR assays (Hutzler, 2009;
142 Hutzler et al., 2015; J. P. Sampaio, 2017; Meier-Dörnberg et al., 2017b). Sequencing of ITS and
143 D1/D2 26S rDNA loci was performed acccording to White et al., Kurtzman et al using modified
144 protocols according to Hutzler (White, 1990; Kurtzman and Robnett, 1998; Kurtzman et al.,
145 2003; Hutzler, 2009; 2010). Sequences were analyzed using NCBI Blast tool (NCBI) and
146 DNAStar, MegAlign Software (DNASTAR, Inc., Madison, Wisconsin).

147 2.4 Whole-genome sequencing

148 Genomic DNA from strains *S. jurei* C1003 and TUM 629 was isolated using Qiagen 100/G
149 Genomic-tips (Qiagen, The Netherlands). The DNA was sequenced at the Microbial Genome
150 Sequencing Center (Pittsburgh, PA, USA). A 150-bp paired-end Illumina Nextera library was
151 prepared, and sequencing was carried out with a NextSeq 550 instrument. The paired-end reads
152 were trimmed and filtered with Trimmomatic (Bolger et al., 2014). Reads were aligned to the
153 reference genome of *S. jurei* NCYC 3947^{T} (accession number GCA_900290405; (Naseeb et al.,
154 2018)) using BWA-MEM (Li and Durbin, 2009). Variant analysis was performed on aligned
155 reads using FreeBayes (Garrison and Marth, 2012). Prior to variant analysis, alignments were
156 filtered to a minimum MAPQ of 50 with SAMtools (Li et al., 2009). Annotation and effect
157 prediction of the variants were performed with SnpEff (Cingolani et al., 2012). The median
158 coverage over 10,000-bp windows was calculated with mosdepth (Pedersen and Quinlan, 2017).
159 Raw sequence reads have been deposited in the NCBI Short Read Archive under BioProject
160 PRJNA681394.

161 2.5 Wort preparation

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162 The wort for the fermentations was produced in the VTT pilot brewery. Milled Pilsner malt
163 (Viking Malt, Lahti, Finland) was mashed in with local Espoo City water following an infusion
164 mashing procedure (mashing-in at 48 °C; rests: 48 °C 30 min - 63 °C 30 min - 72 °C 30 min - 78
165 °C 10 min), mash was filtered with a Meura (Belgium) mash filter and boiled for 60 minutes
166 with Magnum hop pellets (α-acid content 15%). The wort was hopped to achieve 40 IBU and the
167 strength at knockout was 12 °Plato. The wort was collected hot (over 90 °C) in stainless steel
168 kegs and stored at 0 °C before use. The concentrations of sugars in the wort were 52.5 g/L of
169 maltose, 13.4 g/L of maltotriose, 11.6 g/L of glucose, and 2.7 g/L of fructose.

170 **2.6 Fermentation trials and beer preparation**

171 10 L-scale fermentations were carried out with *S. jurei* TUM 629, *S. eubayanus* C902, and *S.*
172 *pastorianus* TUM 34/70. Yeasts were first propagated by transferring an inoculation loop of
173 yeast from a YPD agar plate to 25 ml liquid YPD culture. The culture was incubated aerobically
174 on a shaker for 24 h, before being transferred to 500 mL YPD. After aerobic incubation with
175 agitation (120 rpm) on an orbital shaker for 3 days, the yeast suspension was centrifuged, a 20%-
176 slurry (200g fresh yeast/L) was prepared in sterile Milli-Q-filtered water and yeast were
177 inoculated into 1.5 L of 12 °P wort in a 2 L Schott-bottle capped with an airlock. After five days
178 of static fermentation, the yeast was removed by centrifugation (4000 rpm; 5 min; 4 °C) and a
179 20% slurry was again prepared. Cell number was determined using the NucleoCounter YC-100
180 (ChemoMetec, Denmark) and cells were inoculated into 8 L of aerated (10 ppm dissolved
181 oxygen) 12 °P wort in 10 L-volume, stainless-steel, cylindroconical vessels, to give a starting
182 cell density of of 1×10^7 cells/mL. Fermentations were conducted at 15 °C, and were monitored
183 through regular sampling for assessment of wort pH, alcohol content and cell mass. After
184 fermentation was complete, i.e. when minimal change in wort density was observed over
185 consecutive days, the fresh beers were transferred from fermenters to kegs, matured for 7 days at
186 10 °C and stabilized seven days at 0 °C before depth filtration (Seitz EK, Pall Corporation, New
187 York, NY, USA). Prior to bottling, the beers were carbonated to 5 g/L, and the bottled beers
188 were stored at 0 °C.

189 **2.7 Screening of *Saccharomyces* type strains for wort fermentation potential.**

190 Wort fermentation screening trials included six wild *Saccharomyces* species and two reference
191 brewing strains: one ale (*S. cerevisiae*) and one lager (*S. pastorianus*) strain (Table 1). Prior to
192 fermentation, an inoculation loop was used to transfer yeast from a stock YPD agar plate to the
193 50 ml liquid YPD medium in a 100 ml Erlenmeyer flask. The cultures were propagated at 20 °C
194 on an orbital shaker ($100 \times g$, Infors AG TR-125). After two days the yeast suspensions were
195 centrifuged (10 min, $9000 \times g$ and 4 °C) and a 20% (200 mg / ml) slurry was prepared for cell
196 counting. The NucleoCounter YC-100 was used to calculate the cell count before the yeasts were
197 transferred to the wort at a pitching rate of $1{\times}10^7$ cells/mL. Cells were pitched according to cell
198 number rather than mass due to the expected differences in cell size amongst the strains.
199 Fermentations were carried out in 100 ml of the 12 °Plato all-malt wort and were conducted in
200 250 ml Erlenmeyer flasks, without agitation, at the typical lager brewing temperature of 15 °C
201 for 40 days. Airlocks containing 2 mL of 85% glycerol were used to seal the flasks. Fermentation
202 progress was monitored by measuring mass loss due to CO_2 release. Fermentations were
203 performed in duplicate. When fermentations were completed, samples were taken to assess
204 alcohol content, yeast mass and viability.

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205 ## 2.8 Analytical methods

206 Alcohol content and pH-value of beer samples were determined using an Alcolyzer Plus with a
207 DMA 5000 density meter and Xsample 122 sample changer (Anton-Paar GmbH, Ostfildern,
208 Germany). Medium chain fatty acids and medium chain fatty acid esters were determined by gas
209 chromatography with a flame ionization detector (GC-FID) with a 50 m 0.32 mm phenomenex-
210 FFAP-0.25 µm column. The temperature protocol was 1 min 60 °C, 3 min 220 °C (5 °C/min), 8
211 min 240 °C (20 °C /min). Detector and injector temperatures were 250 °C and 200 °C,
212 respectively. Fermentation by-products were determined using headspace GC-FID analysis
213 according to Mitteleuropäische Brautechnische Analysenkommision method 2.21.1. Briefly, an
214 INNOWAX cross-linked polyethylene-glycol 60 m × 0.32 mm 0.5 µm column was used.
215 Temperatures of oven, detector and injector were 250 °C, 200 °C and 150 °C, respectively.
216 Injection time was 4 s and analyzing time was 17 min. Turbo-Matrix 40 headspace parameters
217 were: sample temperature, 60 °C; transfer temperature, 130 °C, needle temperature 120 °C. The
218 time for GC-cycle was 22 min, thermosetting was 46 min, pressurization was 1 min and injection
219 time was 0.03 min. Fermentable sugars and glycerol in beer were measured by HPLC. A 1.0 mL
220 sample of wort or beer was filtered through Millipore membrane (pore size of 0.45 µm) filters
221 and frozen (-20 °C). The samples were thawed and prepared for HPLC, which was used to
222 determine concentrations of fermentable sugars (fructose, glucose, maltose and maltotriose) of
223 wort and beers. A Waters 2695 Separation Module and Waters System Interphase Module liquid
224 chromatograph coupled with a Waters 2414 differential refractometer (Waters Co., Milford, MA,
225 USA) were used. An Aminex HPX-87H Organic Acid Analysis Column (300 × 7.8 mm, Bio-
226 Rad, USA) was equilibrated with 5 mM sulphuric acid (H_2SO_4) (Titrisol, Merck, Germany) in
227 water at 55 °C. The samples were eluted with 5 mM H_2SO_4 in water at a 0.3 mL/min flow rate.

228 ## 2.9 Maltotriose transport assays

229 For maltotriose uptake measurement, the yeast strains were grown at 20 °C in liquid YP medium
230 containing maltose (4% w/v) or maltotriose (4% w/v) to an OD600 nm between 4 and 8. The
231 cells were harvested by centrifugation (10 min, 5000 rpm, 0°C), washed with ice-cold water and
232 0.1 M tartrate-Tris (pH 4.2) and re-suspended in the same buffer to a concentration of 200
233 mg/mL fresh yeast. Zero-trans rates of [U-^{14}C]-maltotriose uptake were measured at 20 °C
234 essentially as described by (Lucero et al., 1997). Briefly, aliquots of 40 µl of yeast suspension
235 were added to 20 µl of 15 mM labeled maltotriose (for a final concentration of 5 mM [U-^{14}C]-
236 maltotriose) and incubated for 60 s at 20 °C. The reaction was stopped with the addition of 5 ml
237 ice-cold water. The suspension was quickly filtered and washed with an additional 5 ml of ice-
238 cold water. The filter was submerged in 3.5 ml of Optiphase HiSafe 3 scintillation cocktail
239 (Perkin Elmer, MA, USA) and the radioactivity measured in a Perkin Elmer Tri-carb 2810 TR
240 scintillation counter. [U-^{14}C]-maltotriose (ARC 627) was obtained from American Radiolabeled
241 Chemicals (St. Louis, MO, USA) and re-purified before use as described by (Dietvorst et al.,
242 2005). Maltose (minimum purity, 99%) and maltotriose (minimum purity, 95%) were from
243 Sigma-Aldrich (St. Louis, MO).

244 ## 2.10 Sensory analysis

245 All beer samples were tasted and judged by a trained sensory panel of 10 panelists certified by
246 the Deutsche Landwirtschafts-Gesellschaft (DLG). Single tasting was performed in a dedicated

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247 tasting room (single tasting chambers, white-colored room, no distracting influences, and brown
248 glasses with three-digit number labels) to exclude all external misleading factors. The main
249 flavor impressions were determined at a range from 1 (almost no perception) to 10 (very intense
250 perception). Flavor impressions were chosen according to Meier-Dörnberg et al. (Meier-
251 Dörnberg et al., 2017a). In addition, a tasting was performed under the same circumstances with
252 the DLG scheme, in which the beer is judged by its aroma, taste, carbonation, body and
253 bitterness in a range of 1 to 5, 1 being the lowest value (negative) and 5 being the highest value
254 (positive).

255 3 Results

256 *S. jurei* TUM 629 was isolated from a piece of bark of an ash tree (*Fraxinus excelsior*) close to
257 the river Isar in Munich, Bavaria, Germany. Single colonies of *S. jurei* TUM 629 exhibited a
258 homogenous morphology (Figure 1). On WLN-Agar, colonies appear white with a pale green
259 center, and exhibit a well-defined edge and a distinct umbonate morphology. On wort-agar, the
260 colonies are round and white becoming more transparent towards the edge. The colonies of *S.*
261 *jurei* TUM 629 differed in colony morphology from the colonies of *S. eubayanus* C902 and *S.*
262 *pastorianus* TUM 34/70 (both of which were later used for the brewing trials as reference
263 strains). As both agars are standard tools within brewing microbiology they may serve as useful
264 preliminary differentiation tools for *S. jurei* monitoring.

265 The cell morphology of *S. jurei* in brewer's wort (12 °P pale barley malt wort) is round with
266 single cell-budding of round daughter-cells (dark-field microscopy with scale in Figure S1). Cell
267 diameter is between 4 and 8 µm. Vacuoles and cell organelle structures could be observed in the
268 dark field microscopic picture. Cell morphology of *S. jurei* TUM 629 in wort is different from
269 the cell morphologies of *S. pastorianus* lager strains and from some *S. cerevisiae* brewing strains
270 (e.g. wheat beer strains with larger cell diameter and multilateral budding and star cluster
271 formation).

272 The ITS1-5.8-ITS2 rDNA and D1/D2 units of the 26S rDNA of *S. jurei* TUM 629 were
273 sequenced and NCBI Blast comparison was carried out (sequences in supplementary data).
274 D1/D2 26S rDNA showed 100 % sequence identity and ITS1-5.8-ITS2 rDNA 99.38 % sequence
275 identity to *S. jurei* type strain NCYC 3947^{T} (data not shown). Figure 2 gives a clear indication
276 of species identity of TUM 629 by showing the median coverage of 10 kbp windows of
277 seqeuencing reads to a reference genome of 8 *Saccharomyces* species. A phylogenetic tree based
278 on the Clustal W Alignment of the two *S. jurei* sequences (TUM 629 and NCYC 3947^{T}) along *S.*
279 *pastorianus* and *S. eubayanus* further supporting the species identity can be found in
280 supplementary material (Figures S2 and S3).

281 3.1 Genome sequencing and analysis

282 To confirm the species-level identification of *S. jurei* TUM 629, 150 bp paired-end Illumina
283 whole genome sequencing was carried out. *S. jurei* TUM 629 and VTT C-171003 (= C1003 =
284 NCYC 3947^{T}) were sequenced to an average coverage of 29× and 27×, respectively. A total of
285 9690 single nucleotide polymorphisms (SNPs) were detected in TUM 629 compared to the
286 reference genome of *S. jurei* C1003 (accession number GCA_900290405; (Naseeb et al., 2018)).
287 Of these SNPs, only 303 were heterozygous, suggesting that TUM 629 is a homozygous diploid.

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288 The SNPs in TUM 629 indicate a sequence divergence of about 0.1% relative to the type strain,
289 which was originally isolated in south-eastern France (Naseeb et al., 2017). Sequencing coverage
290 was even across the whole reference genome, suggesting that TUM 629 is euploid (Figure S4,
291 Figure S5 showing the coverage for *S. jurei* C1003). When sequencing reads were aligned to a
292 concatenated reference genome of all *Saccharomyces* species, reads mapped exclusively to *S.*
293 *jurei*, indicating that *S. jurei* TUM 629 is not an interspecies hybrid (Figure 2). As expected, only
294 222 SNPs (218 of which were heterozygous) were detected in the re-sequenced *S. jurei* type
295 strain compared to the same reference genome. Genetic analysis allowed identification of genes
296 potentially encoding maltose and maltotriose transporter proteins (Table S1). Three genes
297 ranging from 82.2% to 84.4% identity with *S. cerevisiae MAL31* and one with 82.6% identity
298 with *S. cerevisiae MAL11* were identified. Additionally, 3 genes were found with 78.9% to 84%
299 identity to *S. cerevisiae IMA5*, encoding an extracellular α-glucosidase.

300 **3.2 Wort fermentation**

301 Wort fermentation was carried out to assess the brewing-relevant properties of *S. jurei* TUM
302 629. Results were compared with those of the type strain of *S. eubayanus* (C902), and the *S.*
303 *pastorianus* lager strain TUM 34/70 (Figure 3). An initially rapid fermentation rate in the first
304 two days was followed by a period of relative inactivity, which lasted for approximately 14 days.
305 After this time alcohol level increased steadily for approx. 30 days, reaching a value of 4.9 %
306 (v/v) at 39 days. *S. eubayanus* likewise exhibited rapid fermentation in the first two days, and
307 this was also followed by a lag phase. In contrast to the *S. jurei* fermentation, this period of
308 inactivity only lasted until 7 days after inoculation before the fermentation rate increased.
309 Fermentation was mostly complete after 18 days, reaching a maximum of 4.4 % alcohol. As
310 expected, fermentation with the lager strain was rapid, with no evidence of a lag phase at any
311 stage. 5.5 % (v/v) alcohol was achieved after 9 days with this strain. Analysis of residual sugars
312 indicated that maltotriose consumption was a determining factor for the extent of fermentation
313 (Figure 4). Approximately 50 % of the available maltotriose was consumed by *S. jurei*. The
314 corresponding depletion in the beers fermented with the lager strain was 85 %. *S. eubayanus*, as
315 expected, had no effect on maltotriose concentration. Maltose was completely consumed by the
316 lager strain and by *S. eubayanus*, though interestingly a small portion (3.6 g/L of initially 52.5
317 g/L) remained in the *S. jurei beer*. An increase in glycerol after fermentation was observed for all
318 strains. This was highest in the wild yeast strains both of which produced 2.9 g/L of glycerol
319 while the corresponding concentration in the lager strain beer was 1.6 g/L.

320

321 **3.3 Maltotriose uptake**

322 To assess the yeast's ability to take up maltotriose directly from the medium, uptake activities
323 were quantified using radiolabeled maltotriose. When using maltose as propagation substrate,
324 both *S. jurei* strains and *S. mikatae* showed maltotriose uptake activities ≤0.5 µmol min^{-1} g^{-1} DY^{-1}
325 indicating the absence of active maltotriose transporters in the plasma membrane (Table 2). An
326 uptake activity ≤0.5 µmol min^{-1} g^{-1} DY is considered negligible. *S. eubayanus* C902 showed an
327 activity level slightly above 0.5 µmol min^{-1} g^{-1} DY, although it is known that this strain lacks
328 any capacity to transport maltotriose (Gibson et al., 2013; Magalhães et al., 2016). *S. pastorianus*
329 was the only species showing enough maltotriose uptake activity to ensure its consumption from

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330 the wort. The fermentation data however showed that *S. jurei* can consume maltotriose
331 (Figure 4). If the maltotriose is taken up by the yeast cells then the transporters are either
332 susceptible to repression by maltose or its expression requires induction by maltotriose. To
333 validate this hypothesis the maltotriose uptake activity was also measured in cells propagated in
334 YP medium with maltotriose as sole carbon source. Growth on maltotriose was slow, and it took
335 6 days to reach an OD600 value greater than 4, however, after this maltotriose exposure, the cells
336 could grow much faster when transferred to fresh medium containing maltotriose as sole carbon
337 source (data not shown). Uptake activity from cells grown on maltotriose confirmed that there is
338 an active transmembrane transport occurring in *S. jurei*. This mechanism appears to require
339 prolonged exposure to maltotriose, or absence of other sugars.

340 **3.4 Analytical and sensory aroma profile**

341 The beers produced by the three different yeast strains *S. pastorianus* TUM 34/70, *S. eubayanus*
342 C902 and the newly isolated *S. jurei* TUM 629 showed significantly different sensory and
343 analytical aroma profiles (Tables 3 and 4). The sample fermented by *S. pastorianus* TUM 34/70
344 had the overall highest concentration of esters (Table 3). Of all esters detected in this sample,
345 ethyl acetate was found at the highest concentration at 42.1 mg/L. The overall highest
346 concentration of 3-methylbutylacetate (iso-amyl acetate) was 3.6 mg/L, and was also found in
347 the *S. pastorianus* beer. Ethyl hexanoate concentration was four times higher in the sample
348 fermented with *S. jurei* TUM 629 (0.40 mg/L) compared to the samples fermented with *S.*
349 *pastorianus* (0.13 mg/L) and *S. eubayanus* CBS 12537 (0.11 mg/L). Ethyl hexanoate (apple,
350 fruity flavor) has a flavor threshold in beer of 0.23 mg/L according to Meilgaard (Meilgaard,
351 1975). The sample fermented by *S. jurei* showed a noticeable apple flavor as shown by the
352 tasting results in Figure 5, which correlates well with the aforementioned analytical findings.
353 Overall, *S. jurei* produced higher amounts of medium chain fatty esters and relatively high
354 amounts of higher alcohols in comparison to the other two yeast strains but was outperformed in
355 the production of acetate esters by the *S. pastorianus* strain, and in higher alcohol production by
356 the *S. eubayanus* strain (Tables 3 and 4). *S. eubayanus* produced a typically high amount of
357 phenyl ethanol (121.4 mg/L) in comparison to the other two yeast strains (Gibson et al., 2013).
358 All samples showed diacetyl levels below 0.1 mg/L and no 2,3-pentanedione (data not shown).

359 The trained panel rated all beers as high quality in the DLG scheme and found no significant
360 difference of the purity of aroma and taste, and the quality of carbonation, body and bitterness
361 among the three samples (data not shown). However, they noted clear differences in the
362 descriptive analysis (Figure 5). A phenolic spice/clove note was prominent in the sample
363 fermented by the *S. jurei* strain TUM 629. Further as mentioned above, a significant apple flavor
364 was detected, which can be related to the considerably higher amount of ethyl hexanoate (Table
365 3). The clove-like flavor was less pronounced in the sample fermented by S. *eubayanus*, despite
366 the analytical levels of 4-VG being similar (*S. eubayanus*: 0.16 mg/L, *S. jurei*: 0.15 mg/L). The
367 sample fermented by *S. pastorianus* TUM 34/70 was described as very fruity and berry like,
368 which can partly be explained by generally higher acetate ester concentration (46.91 mg/L) in the
369 sample in comparison to the other two samples (*S. eubayanus* 26.24 mg/L and *S. jurei* 39.69
370 mg/L) (Table 3). Results indicated considerable potential for *S. jurei* application in brewing, and
371 prompted a comparative study including the type strain of *S. jurei* and a number of other
372 *Saccharomyces* type strains

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373

374 ### 3.5 Screening of *Saccharomyces* type strains for wort fermentation potential.

375 Trial fermentations conducted with 12 °P wort at 15 °C revealed clear differences between the
376 strains in terms of wort fermentation potential (Figure 6). As expected, the two reference strains
377 fermented rapidly, with no evidence of an extended lag phase. Alcohol yield was good for both
378 strains, 5.8 % ABV for *S. pastorianus* and 6.1 % ABV for *S. cerevisiae* (Figure 6). The greater
379 efficiency of the A62 ale strain is due to a strong maltotriose fermentation capacity which has
380 also been observed in previous studies (Krogerus et al., 2018).

381 Fermentation characteristics of the *Saccharomyces* type strains were highly variable (Figure 6).
382 All strains exhibited a relatively rapid fermentation in the first two days after inoculation,
383 presumably due to utilization of the simple sugars present in the wort. *S. mikatae* fermentation
384 appeared to cease after this initial period, while *S. arboricola*, which had an identical
385 fermentation profile until 26 days after inoculation, appeared to start fermenting and was still
386 actively fermenting after 41 days when it had produced an ABV level of 4.5 %. *S. kudriavzevii*
387 fermentations were likewise characterized by an initially slow fermentation rate, which increased
388 over time, giving a final value of 3.6 % ABV. *S. paradoxus* and *S. eubayanus*, did not exhibit
389 any lag phase in fermentation, but overall fermentation efficiency was limited with the strains
390 achieving 3.6 and 3.3 % ABV after 41 days (Figure 7). *S. jurei*, despite an initially low
391 fermentation rate in the first two weeks of fermentation was able to achieve an ABV of 5.3%, a
392 value considerably higher than those found in the other wild yeast beers.

393 ## 4 Discussion

394 With the exception of recent reports on the brewing potential of two wild *Saccharomyces*
395 species, *S. eubayanus* and *S. paradoxus* (Gibson et al., 2013; Cubillos et al., 2019; Nikulin et al.,
396 2020), little is known about the brewing potential of the wild species within the *Saccharomyces*
397 genus. Wild *Saccharomyces* species are not typically isolated from brewery fermentation
398 environments, and when encountered in brewing, or other fermentation environments, typically
399 occur in the form of interspecies hybrids. This has been seen for example with *S. kudriavzevii*,
400 which occurs in a *S. cerevisiae* × *S. kudriavzevii* hybrid form in Belgian ales (Gallone et al.,
401 2019), but is not otherwise isolated from brewing systems. Strains of *S. cerevisiae var.*
402 *diastaticus* are common brewery contaminants (Meier-Dörnberg et al., 2018), but their
403 designation as 'wild' species is questionable considering they group phylogenetically with one of
404 the major ale yeast clades (Krogerus and Gibson, 2020; Pontes et al., 2020). The absence of wild
405 yeast species in fermentation environments is indicative of deficiencies in performance.
406 However, the successful application of *S. eubayanus* in brewing (Gibson et al., 2017) has
407 demonstrated how such strains, when handled appropriately, may act as efficient fermenters, and
408 can support product differentiation. Creative utilization of wild species may help brewers meet
409 the consumer demand for beers with novel flavor profiles and interest in engaging background
410 narratives.

411 The ability of *S. jurei* to utilize wort maltotriose was surprising given that this trait has not been
412 observed previously in wild *Saccharomyces* yeasts and is generally considered to be a trait
413 associated only with domesticated yeasts (Gallone et al., 2018). There are two mechanisms by

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414 which *Saccharomyces* yeast may utilize maltotriose (Krogerus et al., 2019). In the first,
415 maltotriose is taken up directly across the cell membrane and hydrolyzed to glucose monomers
416 through the action of an intracellular maltase. Active uptake is mediated by transporters such as
417 AGT1 and MTT1, which are also responsible for the uptake of maltose (Dietvorst et al., 2005;
418 Vidgren and Londesborough, 2012). Despite the absence of the property in wild species,
419 maltotriose utilization appears to be evolvable, with two separate studies demonstrating the
420 creation of maltotriose transporters from existing maltose transporters via a series of
421 recombination events (Baker and Hittinger, 2019; Brouwers et al., 2019). A second mechanism
422 for maltotriose utilization involves extracellular degradation of maltotriose by 'diastatic' strains
423 of yeast. This is seen in some *S. cerevisiae* strains belonging, in particular, to the Beer 2 group
424 (Gallone et al., 2016). In many of these strains, glucoamylase activity is responsible for the
425 liberation of glucose and maltose from maltotriose, as well as dextrins and soluble starch, and
426 obviates the requirement for transmembrane maltotriose transport (though this can still be
427 present) (Krogerus and Gibson, 2020). Our assessment here of the uptake of radiolabeled
428 maltotriose demonstrates conclusively that transmembrane transport occurs across cell
429 membranes of *S. jurei*. This was only apparent after cells had been propagated on maltotriose,
430 indicating that the expression of the phenotype (either through gene expression or protein
431 configuration or localization) is either limited by the presence of other sugars or requires
432 induction by maltotriose. Known maltotriose transporters such as AGT1 and MTT1 can carry
433 both maltose and maltotriose and do not require specific conditions for their expression
434 (Magalhães et al., 2016). The mechanisms by which maltotriose is consumed by *S. jurei* require
435 further investigation. Nikulin et al., in a study of brewing potential in *S. paradoxus*, noted that
436 the efficient uptake of maltose was influenced by the growth medium, with growth on glucose
437 leading to an extended lag phase prior to maltose use during fermentation (Nikulin et al., 2020).
438 This lag phase was significantly reduced if yeasts were propagated on maltose. These results, and
439 our observation that maltotriose use requires previous exposure to this sugar, suggest that
440 brewing with *S. jurei* would benefit from carefully regulated propagation conditions.

441 The presence of an active trans-membrane-transport system does not exclude the possibility of *S.*
442 *jurei* also hydrolyzing maltotriose extracellularly. Genes encoding both potential
443 maltose/maltotriose transporters and extracellular α-glucosidases were found within the *S. jurei*
444 genome. It is expected that a more thorough knowledge of the maltotriose utilization
445 mechanisms may help to improve the potential brewing performance of *S. jurei*, both in terms of
446 maltotriose utilization efficiency and duration of lag phase.

447 While brewing efficiency is a critically important trait for brewers, the contribution of yeast to
448 beer flavor is more directly relevant for consumers. A new brewing strain should preferably offer
449 a novel sensorial experience to beer drinkers. In this regard, the concentration of yeast-derived
450 volatile aroma compounds is significant. The three strains included in the brewing trials had
451 distinct flavor profiles, with each producing a high concentration of at least one important flavor-
452 active compound. In the case of *S. jurei*, this was ethyl hexanoate, a generally desirable ethyl
453 ester with an apple, cherry or aniseed aroma. The high concentration may have contributed to the
454 tropical fruit notes detected by the sensory panel. In contrast to *S. jurei*, the dominant flavor
455 volatile in the *S. pastorianus* beer was 3-methylbutylacetate. This is a highly desirable flavor
456 compound in commercial brewing and contributes a banana or pear aroma to beer (Meilgaard,
457 1975). In the *S. eubayanus* beer, the typically rose-like phenylethanol was prominent. This has
458 previously been noted for this strain (Gibson et al., 2013). Phenylethanol has previously been

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459 found to mask the perception of other flavor compounds (Bamforth, 2020) and may explain the
460 relatively low perception of phenolic flavor notes in the *S. eubayanus* beer relative to the *S. jurei*
461 beer.

462 The discovery of relatively good fermentation efficiency in *S. jurei* inspired a direct comparison
463 with fermentation performance in other members of the strain. This comparative study included
464 an ale *S. cerevisiae* strain, a lager *S. pastorianus* strain and six type strains of wild
465 *Saccharomyces* species. The only species excluded was *S. uvarum*, the type strain of which was
466 shown previously to be strongly maltose-positive, but maltotriose-negative (Nikulin et al., 2018).
467 The species included were highly variable with respect to fermentation characteristics. Relative
468 to the reference strains, the wild strains tended to have long lag periods following the initial
469 fermentation of monosaccharides, and when fermentation increased, it was at a relatively low
470 rate and often limited in extent. Given these features, it is unsurprising that wild *Saccharomyces*
471 species might be at a competitive disadvantage compared to domesticated yeasts, and thus rarely
472 encountered in wort fermentations. Performance varied however, not just between brewing
473 yeasts and wild yeasts, but also amongst the wild species. As observed previously (Nikulin et al.,
474 2018), *S. mikatae* had only limited fermentation capability, with apparently no ability to
475 metabolize maltose. Other species appeared to be able to utilize this disaccharide, albeit at
476 different rates and after different periods of adaptation following the initial fermentation. Of note
477 was the high level of alcohol production by the type strain of *S. jurei*, suggesting that a superior
478 fermentation efficiency due to maltotriose utilization is not restricted to the Bavarian strain
479 included in the previous fermentation trials.

480 As well as the association between woodland habitats and yeast, there has been traditionally a
481 strong association between wood-based materials and brewing. Wood, in addition to being the
482 main building material for millennia, has also been used for vessels used in food production and
483 storage. The usage of wooden materials in brewing was not only limited to vessels, it also made
484 up tools and additives for beer production. Oak wood was used for vats, casks and barrels; chips
485 or shavings of hornbeam (*Carpinus betulus*) and hazel (*Coryllus avellana*) have been used as
486 clarifying aids; and spruce and birch were commonly used for making barrel bungs (Schnegg,
487 1921; 1922). Schnegg, in his textbook about microscopy for brewers, describes oak wood as
488 being hard but porous with the pores being ideal for harboring microorganisms and residues of
489 beer (Schnegg, 1921). Ash and oak trees have also been described as holy trees in Indo-
490 Germanic culture (Dumont, 1992). Oaks have served as an inoculum for roman wine
491 fermentations and ash trees were often tapped for their tree sap (Feier et al., 2019). Through its
492 unique structure and capability of harboring microorganisms in nature, wood could potentially
493 transfer yeasts from natural habitats to human made fermentations and thereby initiate the
494 process of domestication. Due to the role of both ash and oak and their proven association with
495 yeasts capable of fermenting cereal-based worts it is not unlikely that these trees have served as
496 an inoculum for fermentation throughout history. While many isolates of the *Saccharomyces*
497 species have been associated with oak trees or other members of the order of *Fagales* (containing
498 oak trees, beeches, *Nothofagus* spp. and aforementioned *Castanopsis*), *F. excelsior* does not
499 belong to this order. No yeasts other than *S. jurei* TUM 629 were isolated from the sampled ash
500 tree and little is known about the specific microbiome of ash trees. Other studies have revealed
501 evidence for the presence of *S. jurei* in different habitats (Alsammar et al., 2019). The seemingly
502 low abundance of *S. jurei* in nature may be caused by the disadvantage of culturing methods for
503 isolating species that are present at low numbers in the sample. However, DNA-based methods

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504 of describing complex populations (metagenomics) may also run the risk of biases in this
505 situation (Kebschull and Zador, 2015). Recently, another strain of *S. jurei* has been isolated from
506 the bark of an ash in the black forest, Germany (current study, data not published). More
507 research is needed to assess the role of *S. jurei* in the ecology of yeasts, its preferred habitats and
508 geographical distribution.

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514 **6 Author Contributions**

515 TK: fermentation trials, analysis of fermentation data. FM: transmembrane transport assays. KK:
516 whole-genome analyses. BG: first draft, conceptualization. MM: sensory and aroma analysis on
517 the bottled beers, first draft, conceptualization. MH: first draft, isolation protocol, species
518 identification, physiological characterization, conceptualization. OK: strain isolation, finalization
519 of the manuscript. All authors contributed to the article and approved the submitted version.

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524 **8 References**

525 Alsammar, H.F., Naseeb, S., Brancia, L.B., Gilman, R.T., Wang, P., and Delneri, D. (2019).
526 Targeted metagenomics approach to capture the biodiversity of *Saccharomyces* genus in
527 wild environments. *Environmental Microbiology Reports* 11(2)**,** 206-214. doi:
528 https://doi.org/10.1111/1758-2229.12724.

529 Baker, E.P., and Hittinger, C.T. (2019). Evolution of a novel chimeric maltotriose transporter in
530 *Saccharomyces eubayanus* from parent proteins unable to perform this function. *PLOS*
531 *Genetics* 15(4)**,** e1007786. doi: 10.1371/journal.pgen.1007786.

532 Bamforth, C.W. (2020). The Horace Brown Medal. Forever in focus: researches in malting and
533 brewing sciences. *Journal of the Institute of Brewing* 126(1)**,** 4-13. doi:
534 https://doi.org/10.1002/jib.594.

535 Bing, J., Han, P.J., Liu, W.Q., Wang, Q.M., and Bai, F.Y. (2014). Evidence for a Far East Asian
536 origin of lager beer yeast. *Curr Biol* 24(10)**,** R380-381. doi: 10.1016/j.cub.2014.04.031.

537 Bolger, A.M., Lohse, M., and Usadel, B. (2014). Trimmomatic: a flexible trimmer for Illumina
538 sequence data. *Bioinformatics* 30(15)**,** 2114-2120. doi: 10.1093/bioinformatics/btu170.

539 Brouwers, N., Gorter de Vries, A.R., van den Broek, M., Weening, S.M., Elink Schuurman,
540 T.D., Kuijpers, N.G.A., et al. (2019). In vivo recombination of *Saccharomyces*

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541 *eubayanus* maltose-transporter genes yields a chimeric transporter that enables
542 maltotriose fermentation. *PLOS Genetics* 15(4)**,** e1007853. doi:
543 10.1371/journal.pgen.1007853.

544 Cingolani, P., Platts, A., Wang, L.L., Coon, M., Nguyen, T., Wang, L., et al. (2012). A program
545 for annotating and predicting the effects of single nucleotide polymorphisms, SnpEff. *Fly*
546 6(2)**,** 80-92. doi: 10.4161/fly.19695.

547 Cubillos, F.A., Gibson, B., Grijalva-Vallejos, N., Krogerus, K., and Nikulin, J. (2019).
548 Bioprospecting for brewers: Exploiting natural diversity for naturally diverse beers. *Yeast*
549 36(6)**,** 383-398. doi: https://doi.org/10.1002/yea.3380.

550 Dietvorst, J., Londesborough, J., and Steensma, H.Y. (2005). Maltotriose utilization in lager
551 yeast strains: MTT1 encodes a maltotriose transporter. *Yeast* 22(10)**,** 775-788. doi:
552 10.1002/yea.1279.

553 Dumont, D.J. (1992). The ash tree in Indo-European culture. *Mankind Quarterly* 32(4).

554 Feier, I., Migała, A., Pietruszka, M., and Jackowski, M. (2019). Roman Wine in Barbaricum.
555 Preliminary Studies on Ancient Wine Recreation. *Heritage* 2**,** 331-338. doi:
556 10.3390/heritage2010022.

557 Gallone, B., Mertens, S., Gordon, J.L., Maere, S., Verstrepen, K.J., and Steensels, J. (2018).
558 Origins, evolution, domestication and diversity of *Saccharomyces* beer yeasts. *Current*
559 *Opinion in Biotechnology* 49**,** 148-155. doi: https://doi.org/10.1016/j.copbio.2017.08.005.

560 Gallone, B., Steensels, J., Mertens, S., Dzialo, M.C., Gordon, J.L., Wauters, R., et al. (2019).
561 Interspecific hybridization facilitates niche adaptation in beer yeast. *Nature Ecology &*
562 *Evolution* 3(11)**,** 1562-1575. doi: 10.1038/s41559-019-0997-9.

563 Gallone, B., Steensels, J., Prahl, T., Soriaga, L.B., Saels, V., Herrera-Malaver, B., et al. (2016).
564 Domestication and Divergence of *Saccharomyces cerevisiae* Beer Yeasts. *Cell* 166**,** 1397
565 - 1410.e1316.

566 Garrison, E., and Marth, G. 2012. Haplotype-based variant detection from short-read sequencing.
567 Available: https://ui.adsabs.harvard.edu/abs/2012arXiv1207.3907G [Accessed July 01,
568 2012].

569 Gibson, B. (Year). "Non-conventional yeast as a new tool for beer flavour modification", in:
570 *European Breewry Convention*).

571 Gibson, B., Geertman, J.-M.A., Hittinger, C.T., Krogerus, K., Libkind, D., Louis, E.J., et al.
572 (2017). New yeasts—new brews: modern approaches to brewing yeast design and
573 development. *FEMS Yeast Research* 17(4). doi: 10.1093/femsyr/fox038.

574 Gibson, B.R., Storgards, E., Krogerus, K., and Vidgren, V. (2013). Comparative physiology and
575 fermentation performance of Saaz and Frohberg lager yeast strains and the parental
576 species *Saccharomyces eubayanus*. *Yeast* 30(7)**,** 255-266. doi: 10.1002/yea.2960.

577 Hutzler, M. (2009). *Entwicklung und Optimierung von Methoden zur Identifizierung und*
578 *Differenzierung von getränkerelevanten Hefen.* Dissertation, TU München.

579 Hutzler, M. (2010). *Getränkerelevante Hefen - Identifizierung und Differenzierung*. Saarbrücken:
580 SVH-Verlag.

bioRxiv preprint doi: https://doi.org/10.1101/2021.01.11.426216; this version posted January 11, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

581 Hutzler, M., Koob, J., Riedl, R., Schneiderbanger, H., Mueller-Auffermann, K., and Jacob, F.
582 (2015). "5 - Yeast identification and characterization," in *Brewing Microbiology,* ed. A.E.
583 Hill. (Oxford: Woodhead Publishing), 65-104.

584 J. P. Sampaio, A.P., D. Libkind, M. Hutzler (2017). "Taxonomy, Diversity, and Typing of
585 Brewing Yeasts," in *Brewing Microbiology: Current Research, Omics and Microbial*
586 *Ecology,* ed. C.W.B. N. A. Bokulich. (Norfolk, U.K.: Horizon Press).

587 Kebschull, J.M., and Zador, A.M. (2015). Sources of PCR-induced distortions in high-
588 throughput sequencing data sets. *Nucleic acids research* 43(21)**,** e143-e143. doi:
589 10.1093/nar/gkv717.

590 Krogerus, K., and Gibson, B. (2020). A re-evaluation of diastatic *Saccharomyces cerevisiae*
591 strains and their role in brewing. *Applied Microbiology and Biotechnology* 104. doi:
592 10.1007/s00253-020-10531-0.

593 Krogerus, K., Holmström, S., and Gibson, B. (2018). Enhanced Wort Fermentation with De
594 Novo Lager Hybrids Adapted to High-Ethanol Environments. *Applied and*
595 *Environmental Microbiology* 84(4)**,** e02302-02317. doi: 10.1128/aem.02302-17.

596 Krogerus, K., Magalhães, F., Kuivanen, J., and Gibson, B. (2019). A deletion in the STA1
597 promoter determines maltotriose and starch utilization in STA1+ *Saccharomyces*
598 *cerevisiae* strains. *Applied Microbiology and Biotechnology* 103(18)**,** 7597-7615. doi:
599 10.1007/s00253-019-10021-y.

600 Kurtzman, C.P., Boekhout, T., Robert, V., Fell, J.W., and Deak, T. (2003). "3 - Methods to
601 identify yeasts," in *Yeasts in Food,* eds. T. Boekhout & V. Robert. Woodhead
602 Publishing), 69-121.

603 Kurtzman, C.P., and Robnett, C.J. (1998). Identification and phylogeny of ascomycetous yeasts
604 from analysis of nuclear large subunit (26S) ribosomal DNA partial sequences. *Antonie*
605 *van Leeuwenhoek* 73(4)**,** 331-371. doi: 10.1023/A:1001761008817.

606 Langdon, Q.K., Peris, D., Kyle, B., and Hittinger, C.T. (2018). sppIDer: A Species Identification
607 Tool to Investigate Hybrid Genomes with High-Throughput Sequencing. *Molecular*
608 *biology and evolution* 35(11)**,** 2835-2849. doi: 10.1093/molbev/msy166.

609 Li, H., and Durbin, R. (2009). Fast and accurate short read alignment with Burrows–Wheeler
610 transform. *Bioinformatics* 25(14)**,** 1754-1760. doi: 10.1093/bioinformatics/btp324.

611 Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., et al. (2009). The Sequence
612 Alignment/Map format and SAMtools. *Bioinformatics* 25(16)**,** 2078-2079. doi:
613 10.1093/bioinformatics/btp352.

614 Libkind, D., Hittinger, C.T., Valério, E., Gonçalves, C., Dover, J., Johnston, M., et al. (2011).
615 Microbe domestication and the identification of the wild genetic stock of lager-brewing
616 yeast. *Proc Natl Acad Sci U S A* 108(35)**,** 14539-14544. doi: 10.1073/pnas.1105430108.

617 Liti, G., Barton, D.B., and Louis, E.J. (2006). Sequence diversity, reproductive isolation and
618 species concepts in *Saccharomyces*. *Genetics* 174(2)**,** 839-850. doi:
619 10.1534/genetics.106.062166.

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620 Lucero, P., Peñalver, E., Moreno, E., and Lagunas, R. (1997). Moderate concentrations of
621 ethanol inhibit endocytosis of the yeast maltose transporter. *Applied and environmental*
622 *microbiology* 63(10)**,** 3831-3836. doi: 10.1128/AEM.63.10.3831-3836.1997.

623 Magalhães, F., Calton, A., Heiniö, R.-L., and Gibson, B. (2021). Frozen-dough baking potential
624 of psychrotolerant *Saccharomyces* species and derived hybrids. *Food Microbiology* 94**,**
625 103640. doi: https://doi.org/10.1016/j.fm.2020.103640.

626 Magalhães, F., Vidgren, V., Ruohonen, L., and Gibson, B. (2016). Maltose and maltotriose
627 utilisation by group I strains of the hybrid lager yeast *Saccharomyces pastorianus*. *FEMS*
628 *Yeast Res* 16(5). doi: 10.1093/femsyr/fow053.

629 Meier-Dörnberg, T., Hutzler, M., Michel, M., Methner, F.-J., and Jacob, F. (2017a). The
630 Importance of a Comparative Characterization of *Saccharomyces cerevisiae* and
631 *Saccharomyces pastorianus* Strains for Brewing. *Fermentation* 3(3). doi:
632 10.3390/fermentation3030041.

633 Meier-Dörnberg, T., Kory, O.I., Jacob, F., Michel, M., and Hutzler, M. (2018). *Saccharomyces*
634 *cerevisiae* variety diastaticus friend or foe?-spoilage potential and brewing ability of
635 different *Saccharomyces cerevisiae* variety diastaticus yeast isolates by genetic,
636 phenotypic and physiological characterization. *FEMS Yeast Res* 18(4). doi:
637 10.1093/femsyr/foy023.

638 Meier-Dörnberg, T., Michel, M., Wagner, R., Jacob, F., and Hutzler, M. (2017b). Genetic and
639 Phenotypic Characterization of Different Top-fermenting *Saccharomyces cerevisiae* Ale
640 Yeast Isolates. *BrewingScience* 70**,** 9-25.

641 Meilgaard, M. (1975). Flavor chemistry of beer : Part II : Flavor and threshold of 239 aroma
642 volatiles. *Technical Quarterly MBAA* 12(3).

643 Naseeb, S., Alsammar, H., Burgis, T., Donaldson, I., Knyazev, N., Knight, C., et al. (2018).
644 Whole Genome Sequencing, de Novo Assembly and Phenotypic Profiling for the New
645 Budding Yeast Species *Saccharomyces jurei*. *G3 (Bethesda, Md.)* 8(9)**,** 2967-2977. doi:
646 10.1534/g3.118.200476.

647 Naseeb, S., James, S.A., Alsammar, H., Michaels, C.J., Gini, B., Nueno-Palop, C., et al. (2017).
648 *Saccharomyces jurei* sp. nov., isolation and genetic identification of a novel yeast species
649 from *Quercus robur*. *Int J Syst Evol Microbiol* 67(6)**,** 2046-2052. doi:
650 10.1099/ijsem.0.002013.

651 Naumov, G.I., James, S.A., Naumova, E.S., Louis, E.J., and Roberts, I.N. (2000). Three new
652 species in the *Saccharomyces* sensu stricto complex: *Saccharomyces cariocanus*,
653 *Saccharomyces kudriavzevii* and *Saccharomyces mikatae*. *Int J Syst Evol Microbiol* 50 Pt
654 5**,** 1931-1942. doi: 10.1099/00207713-50-5-1931.

655 Nikulin, J., Krogerus, K., and Gibson, B. (2018). Alternative *Saccharomyces* interspecies hybrid
656 combinations and their potential for low-temperature wort fermentation. *Yeast* 35(1)**,**
657 113-127. doi: https://doi.org/10.1002/yea.3246.

658 Nikulin, J., Vidgren, V., Krogerus, K., Magalhães, F., Valkeemäki, S., Kangas-Heiska, T., et al.
659 (2020). Brewing potential of the wild yeast species *Saccharomyces paradoxus*. *European*
660 *Food Research and Technology* 246**,** 2283 - 2297.

bioRxiv preprint doi: https://doi.org/10.1101/2021.01.11.426216; this version posted January 11, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

661 Osburn, K., Nisaa, N., and Bochman, M. (2016). *Bio-prospecting, selection, and analysis of wild*
662 *yeasts for ethanol fermentation.*

663 Pedersen, B.S., and Quinlan, A.R. (2017). Mosdepth: quick coverage calculation for genomes
664 and exomes. *Bioinformatics* 34(5)**,** 867-868. doi: 10.1093/bioinformatics/btx699.

665 Peris, D., Sylvester, K., Libkind, D., Gonçalves, P., Sampaio, J.P., Alexander, W.G., et al.
666 (2014). Population structure and reticulate evolution of *Saccharomyces eubayanus* and its
667 lager-brewing hybrids. *Mol Ecol* 23(8)**,** 2031-2045. doi: 10.1111/mec.12702.

668 Pontes, A., Hutzler, M., Brito, P.H., and Sampaio, J.P. (2020). Revisiting the Taxonomic
669 Synonyms and Populations of *Saccharomyces cerevisiae*—Phylogeny, Phenotypes,
670 Ecology and Domestication. *Microorganisms* 8(6). doi:
671 10.3390/microorganisms8060903.

672 Sampaio, J.P., and Gonçalves, P. (2008). Natural Populations of *Saccharomyces kudriavzevii* in
673 Portugal Are Associated with Oak Bark and Are Sympatric with *S. cerevisiae* and *S.*
674 *paradoxus*. *Applied and Environmental Microbiology* 74(7)**,** 2144-2152. doi:
675 10.1128/aem.02396-07.

676 Schnegg, H. (1921). *Das mikroskopische Praktikum des Brauers, Anleitung zum eingehenderen*
677 *Studium der Brauereirohstoffe und Gärungsorganismen, 1. Teil: Morphologie und*
678 *Anatomie der Brauereiroh-und Hilfsstoff.* Stuttgart: Verlag von Ferdinand Enke.

679 Schnegg, H. (1922). *Das mikroskopische Praktikum des Brauers, Anleitung zum eingehenderen*
680 *Studium der Brauereirohstoffe und Gärungsorganismen, 2. Teil: Gärungsorganismen.*
681 Stuttgart: Verlag von Ferdinand Enke.

682 Sniegowski, P.D., Dombrowski, P.G., and Fingerman, E. (2002). *Saccharomyces cerevisiae* and
683 *Saccharomyces paradoxus* coexist in a natural woodland site in North America and
684 display different levels of reproductive isolation from European conspecifics. *FEMS*
685 *Yeast Res* 1(4)**,** 299-306. doi: 10.1111/j.1567-1364.2002.tb00048.x.

686 Vidgren, V., and Londesborough, J. (2012). Characterization of the *Saccharomyces bayanus*-
687 type AGT1 transporter of lager yeast. *Journal of the Institute of Brewing* 118(2)**,** 148-
688 151. doi: https://doi.org/10.1002/jib.22.

689 Wang, S.A., and Bai, F.Y. (2008). *Saccharomyces arboricolus* sp. nov., a yeast species from tree
690 bark. *Int J Syst Evol Microbiol* 58(Pt 2)**,** 510-514. doi: 10.1099/ijs.0.65331-0.

691 White, T.J., Bruns, E. Lee, Taylor, J. (1990). "Amplification and direct sequencing of fungal
692 ribosomal RNA genes for phylogenetics," in *PCR protocols: a guide to methods and*
693 *amplifications.* (San Diego, CA: Academic Press), 315-322.

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696

697 **Tables**

698 **Table 1.** Strains used in this study

Species	Strain code and abbreviation	Other culture collection codes	Additional information
S. cerevisiae	VTT A-81062 (A62)		Ale yeast
S. pastorianus	TUM 34/70		Lager yeast
S. arboricola	VTT C-15952^T (C952)	CBS 10644^T	Type strain
S. eubayanus	VTT C-12902^T (C902)	CBS 12357^T	Type strain
S. kudriavzevii	VTT C-15950^T (C950)	CBS 8840^T, ATCC MYA-4449^T, NCYC 2889^T	Type strain
S. jurei	VTT C-171003^T (C1003)	CBS 14759^T, NCYC 3947^T	Type strain
S. jurei	TUM 629		This study
S. mikatae	VTT C-15949^T (C949)	CBS 8839^T, NCYC 2888^T	Type strain
S. paradoxus	VTT C-09850^T (C850)	CBS 432^T, NCYC 2600^T	Type strain

699

700

701

702 **Table 2.** Zero-trans rates of maltotriose uptake activity (µmol min^{-1} g^{-1} DY) of the strains in study,
703 measured at 20°C. Prior to assessment of activity, strains were propagated in YP medium
704 supplemented with either maltose or maltotriose. Values are means of two independent assays (±
705 standard deviation).

	Propagation media

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Strains	YP + Maltose	YP + Maltotriose
S. jurei C1003	0.5 ± 0.11	6.2 ± 0.93
S. jurei TUM 629	0.5 ± 0.10	8.0 ± 0.07
S. eubayanus C902	0.8 ± 0.03	n.q.
S. mikatae C949	0.1 ± 0.05	n.q.
S. pastorianus TUM 34/70	7.4 ± 0.81	n.q.

706 n.q. - not quantified

707

708 **Table 3.** Esters detected in the three different beer samples fermented by *S. pastorianus* (TUM
709 34/70), *S. jurei* (TUM 629) and *S. eubayanus* (CBS 12537) at a fermentation temperature of 15 °C
710 and an original gravity °12 P.

	S. pastorianus	*S. jurei*	*S. eubayanus*
Esters (mg/L)	TUM 34/70	TUM 629	CBS 12537
Ethyl butyrate	0.12	0.21	0.09
Ethyl hexanoate	0.13	0.4	0.11
Ethyl octanoate	0.35	0.38	0.37
Ethyl decanoate	0.13	0.05	0.23
2-Phenylethyl acetate	1.1	1.3	2.7
Isobutyl acetate	0.08	0.08	0.06
Ethyl acetate	42.1	35.7	21.9

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Iso-amyl acetate	3.6	2.6	1.6
Sum of acetate esters	46.91	39.69	26.24
Sum of fatty acid esters	0.61	0.83	0.71

711

712

713 **Table 4.** Higher alcohols detected in the three different beer samples fermented by *S. pastorianus*
714 (TUM 34/70), *S. jurei* (TUM 629) and *S. eubayanus* (CBS 12537) at a fermentation temperature
715 of 15 °C and an original gravity °12 P.

	S. pastorianus	*S. jurei*	*S. eubayanus*
Higher alcohols (mg/L)	TUM 34/70	TUM 629	CBS 12537
n-propanol	7.7	5.5	5.8
Iso-butanol	15	31.6	22.6
Iso-amyl alcohol (2-, 3-methylbutanol)	3.6	127	71.7
Phenyl ethanol	31.7	49.8	121.4
Sum of higher alcohols	58	213.9	221.5

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718

719 **Figures**

720 **Figure 1.** Colony morphologies of *S. jurei* TUM 629, *S. eubayanus* C902, *S. pastorianus* TUM
721 34/70 on WLN-Agar (Wallerstein-Nutrient-Agar) and Wort-Agar.

722 **Figure 2.** The median coverage in 10 kbp windows of sequencing reads from *S. jurei* TUM 629
723 aligned to a concatenated reference genome consisting of 8 species in the *Saccharomyces* genus.
724 Reads align exclusively to *S. jurei,* except for two small regions in the sub-telomeric regions of
725 *S. arboricola* chromosome 4 and *S. mikatae* chromosome 14. Results were visualized in R using
726 modified scripts from sppIDer (Langdon et al., 2018).

727 **Figure 3.** Fermentation progress as monitored by alcohol level (v/v) at 15 °C during 39 days of
728 fermentation of an all-malt 12 °P wort. Strains include one lager reference strain (TUM 34/70), as
729 well as the German strain of *S. jurei* (TUM 629), and the type strain of *S. eubayanus* (C902) Values
730 are means from duplicate fermentations and error bars indicate range.
731 **Figure 4.** Residual sugars and glycerol in all-malt 12 °P wort, and beer after fermentation at 15 °C
732 for up to 39 days. Strains include one lager reference strain (TUM 34/70), as well as the German
733 strain of *S. jurei* (TUM 629), and the type strain of *S. eubayanus* (C902). Values are means from
734 duplicate fermentations and error bars where visible indicate range.

735 **Figure 5.** Tasting results of the beer samples fermented by *S. pastorianus* TUM 34/70, *S. jurei*
736 TUM 629, and *S. eubayanus* at 15 °C fermentation temperature and an original gravity of 12 °P.

737 **Figure 6.** Fermentation performance (measured as loss of mass of the fermentor) in comparison
738 between *Saccharomyces* type strains in 12 °P all-malt wort at 15 °C.

739 **Figure 7.** Final alcohol content of fermentations conducted in 12 °P brewer's wort at 15 °C with
740 *Saccharomyces* type strains.

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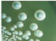
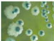
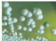
741

WLN-Agar

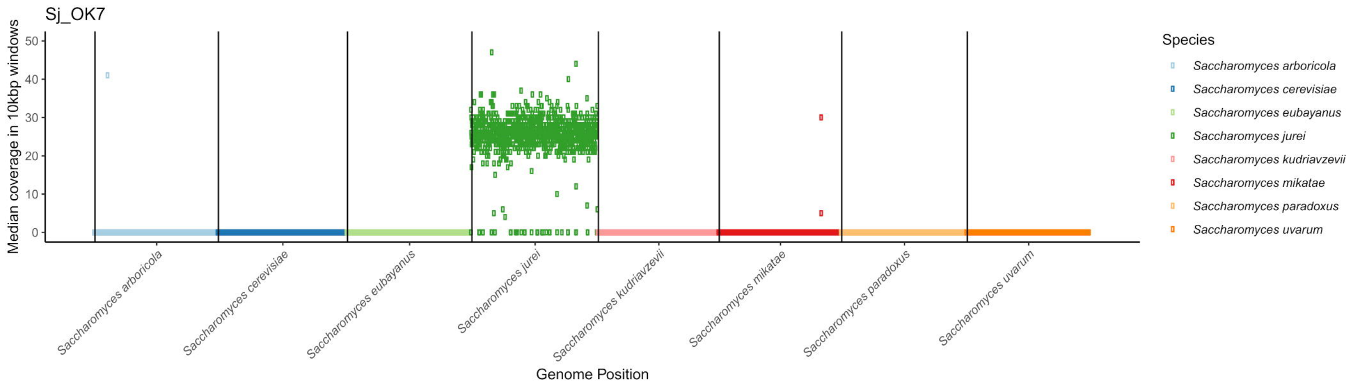


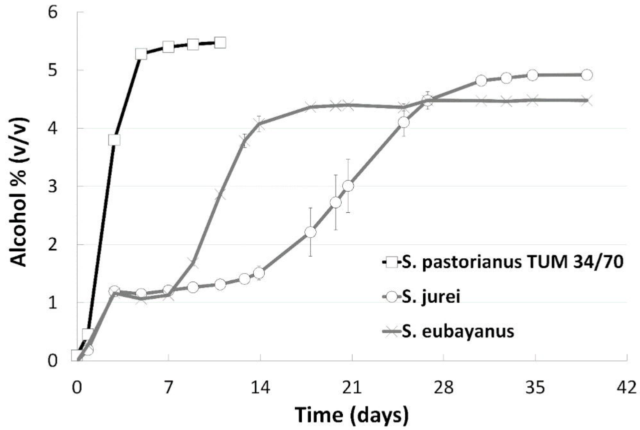


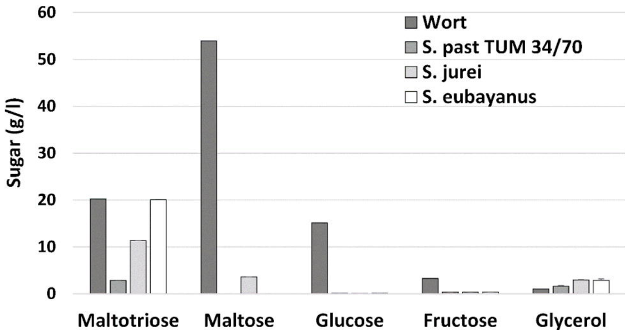


Wort-Agar

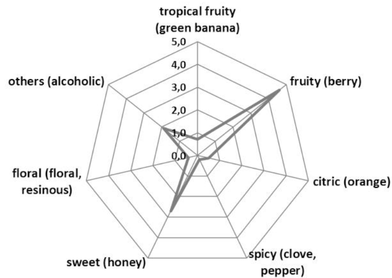
S. jurei TUM 629 *S. eubayanus* CBS 12357 *S. pastorianus* TUM 34/70

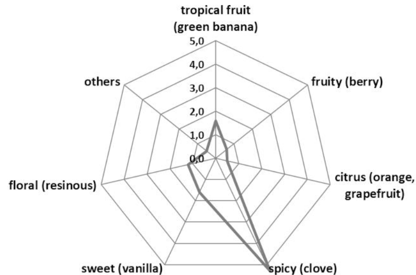

Sj_OK7
Median coverage in 10kbp windows
50
40
30
20
10
0
Species
Saccharomyces arboricola
Saccharomyces cerevisiae
Saccharomyces eubayanus
Saccharomyces jurei
Saccharomyces kudriavzevii
Saccharomyces mikatae
Saccharomyces paradoxus
Saccharomyces uvarum
Genome Position

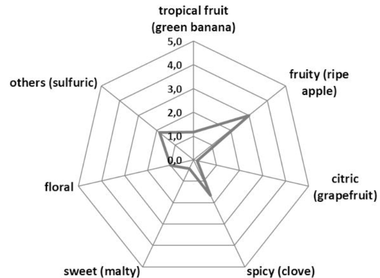

Alcohol % (v/v)
Time (days)
S. pastorianus TUM 34/70
S. jurei
S. eubayanus
0
1
2
3
4
5
6
0
7
14
21
28
35
42

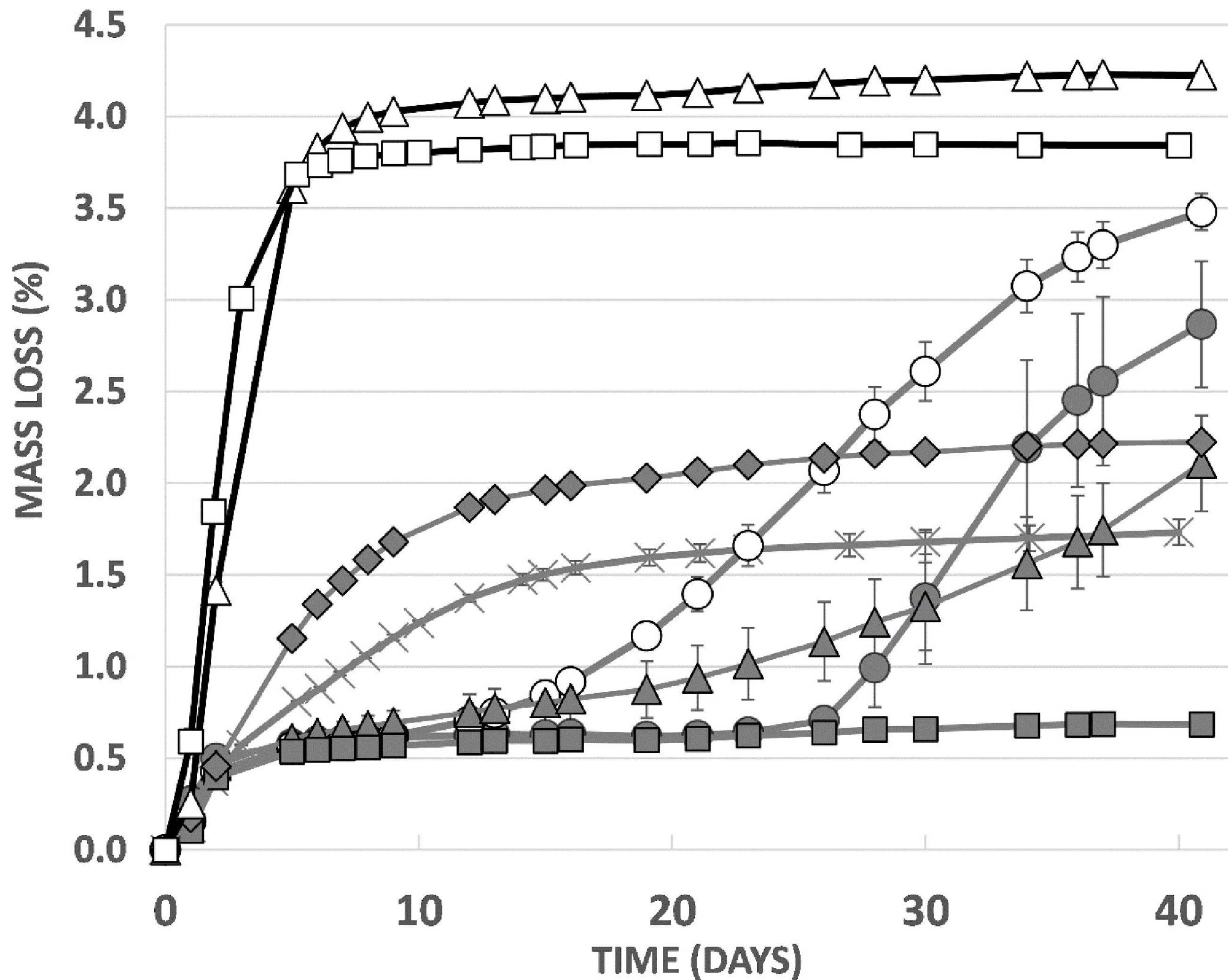


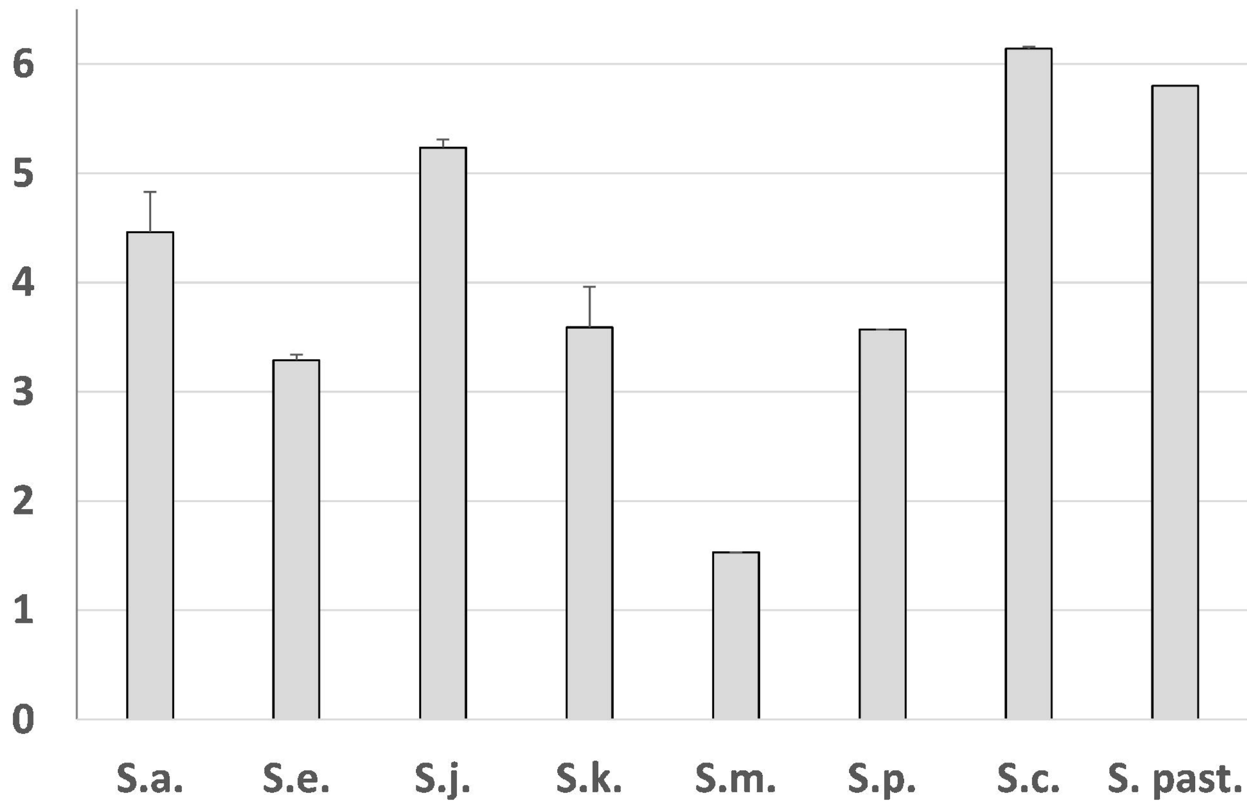
60
50
40
30
20
10
0
Sugar (g/l)
Wort
S. past TUM 34/70
S. jurei
S. eubayanus
Maltotriose
Maltose
Glucose
Fructose
Glycerol


S. pastorianus
tropical fruity
(green banana)
5,0
4,0
3,0
2,0
1,0
0,0
fruity (berry)
citric (orange)
spicy (clove,
pepper)
sweet (honey)
floral (floral,
resinous)
others (alcoholic)


S. jurei
tropical fruit
(green banana)
5,0
4,0
3,0
2,0
1,0
0,0
fruity (berry)
citrus (orange,
grapefruit)
spicy (clove)
sweet (vanilla)
floral (resinous)
others


S. eubayanus
tropical fruit
(green banana)
5,0
4,0
3,0
2,0
1,0
0,0
fruity (ripe
apple)
citric
(grapefruit)
spicy (clove)
sweet (malty)
floral
others (sulfuric)


S.a. C952
S.e. C902
S.j. C1003
S.k. C950
S.m. C949
S.p. C850
S.c. A62
S. past. TUM 34/70
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
MASS LOSS (%)
0
10
20
30
40
TIME (DAYS)


Alcohol % (v/v)
0
1
2
3
4
5
6
S.a.
S.e.
S.j.
S.k.
S.m.
S.p.
S.c.
S. past.