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Chiara Nasuti , Jennifer Ruffini , Laura Sola , Mario Di Bacco , [Stefano Raimondi](#) , [Francesco Candeliere](#) , [Lisa Solieri](#) *

Posted Date: 14 July 2023

doi: 10.20944/preprints202307.0964.v1

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Article

Sour Beer as Bioreservoir of Novel Craft Ale Yeast Cultures

Chiara Nasuti ¹, Jennifer Ruffini ¹, Laura Sola ², Mario Di Bacco ³, Stefano Raimondi ²,
Francesco Candeliere ² and Lisa Solieri ^{1,4,*}

¹ Department of Life Sciences, University of Modena and Reggio Emilia, Via Amendola, 2-Pad. Besta, 42122 Reggio Emilia, Italy; chiara.nasuti@unimore.it (C.N.); 227818@studenti.unimore.it (J.R.)

² Department of Life Sciences, University of Modena and Reggio Emilia, Via Campi 51, 41125, Modena, Italy; laura.sola@unimore.it (L.S.); stefano.raimondi@unimore.it (S.R.); francesco.candeliere@unimore.it (F.C.)

³ Ca' Del Brado Brewery, Via Andrea Costa, 146/2, 40065 Rastignano (BO), Italy; quark@cadelbrado.it

⁴ National Biodiversity Future Center (NBFC), 90133 Palermo, Italy; lisa.solieri@unimore.it

* Correspondence: lisa.solieri@unimore.it; Tel.: +39 0522522026

Abstract: The increasing demand for craft beer drives the search for novel ale yeast cultures from brewing-related wild environments. The focus of bioprospecting for craft cultures is to identify feral yeasts suitable to imprint unique sensorial attributes to the final product. Here, we integrated phylogenetic, genotypic, genetic, and metabolomic techniques to demonstrate that sour beer during aging in wooden barrel is a source of suitable craft ale yeast candidates. Differently from traditional lambic beer maturation phase, during aging of sour-matured production style beer, different biotypes of *Saccharomyces cerevisiae* dominated the cultivable in-house mycobiota, followed by *Pichia membranifaciens*, *Brettanomyces bruxellensis*, and *Brettanomyces anomalus*. In addition, three putative *S. cerevisiae* x *Saccharomyces uvarum* hybrids were identified. *S. cerevisiae* feral strains sporulated, produced viable monosporic progenies, and have *STA1* gene as well as the full-length promoter. During hopped wort fermentation four *S. cerevisiae* strains and the *S. cerevisiae* x *S. uvarum* hybrid WY213 overcame non-*Saccharomyces* strains in fermentative rate and ethanol production, except for *P. membranifaciens* WY122. This strain consumed maltose after a long lag phase, in contrast with the phenotypic profile described for the species. According to *STA1*+ genotype *S. cerevisiae* partially consumed dextrin, but no 4-vinyl guaiacol was detected among VOCs, suggesting that these strains were phenolic off flavor-negative (POF-). In conclusion, the strains characterized here have relevant brewing properties and are exploitable as indigenous craft beer starters.

Keywords: sour beer; craft brewing; *STA1* gene; dextrin; hybrid; *Saccharomyces cerevisiae*; *Saccharomyces uvarum*; *Pichia membranifaciens*

1. Introduction

Craft beer production has gained increasing interest in many countries as an alternative to mainstream beer [1]. Craft brewing refers to relatively small breweries, that aim to produce beers with unique and original sensory characteristics based on quality and diversity [2]. Off-flavors in craft beers increase their perceived quality compared to commercial beer [3,4].

The sensory profile of beer is determined by several factors (choice of malts or adjuncts, hop varieties, water quality, etc) including yeasts, that catalyze metabolic reactions during wort fermentation which impact beer flavor profile. Other than for sugar consumption and ethanol and CO₂ production, yeasts are responsible for releasing aroma-active secondary side-products of alcoholic fermentation that contribute to solvent-like and fruity notes and a sweet taste, such as higher alcohols, ethyl esters, and acetate esters [5]. They also can transform neutral precursors present in wort and hop such as glycosides, polyfunctional thiols, and terpenes, into sensorial active compounds, as reviewed by X et al. [6]. This bio-flavoring aptitude qualitatively and quantitatively varies depending on strain and species employed [7]. However, the pool of commercial *Saccharomyces*

cerevisiae for ale beer and *Saccharomyces pastorianus* for lager beer is restricted and genetically redundant [8], hindering the chance to diversify beer sensorial profile through yeast bio-flavoring.

To expand the number of craft brewing starters and to diversify the portfolio of beer styles, various OGM-free strategies have been proposed. These approaches include hybridization, utilization of non-*S. cerevisiae* strains, and the bioprospecting for novel wild yeasts species and strains from alternative or unusual niches compared to ale and lager brewing [9-11 Krogerus et al., 2017; Cubillos et al., 2019; Iattici et al., 2020]. Recently, genetically modified yeasts were also exploited to boost hop-related flavor active molecules [12-15]. The selection of suitable yeast strains is a crucial step in producing high-quality craft beer affecting both wort fermentation and beer conditioning. Yeast strains have a significant impact on the production of volatile organic compounds (VOCs), which are essential for the beer's flavor and aroma [2, 3], and this is particularly important for craft beers, which are often unfiltered, unpasteurized, and bottle-conditioned [4, 5].

In craft brewing, the interest towards sour beer style is recently growing [16]. Traditionally fermented sour beers, such as Belgian type lambic beers, pass through spontaneous fermentation and wood maturation, which can last up to three years. This process involves conventional and unconventional yeasts, lactic acid bacteria, and, to a lesser extent, acetic acid bacteria [16-20]. These microorganisms are spontaneous beer contaminants arisen from raw materials, air, and brewery environment and equipment, such as the inner surface of barrel [21]. Recently, they have been revised as "drivers" of innovation and diversification of the sensory profiles in sour beer [22]. Other craft brewing strategies mimic Lambic-style beer in complexity, fruity, and sourness, but reduce maturation time as well as increase fermentation control (as reviewed by [16,23]). Among these methods, ale beer can be soured by a primary fermentation with brewery yeast pure cultures, followed by a maturation aging in wooden barrels, where the implantation of a complex house microbiota can lead to acidic and peculiar sensory profiles (the so-called sour-matured production styles).

In this study, we considered in-house microbiota of sour ale beers during ageing as a source for novel brewing cultures. For this purpose, we taxonomically identified and technologically profiled the cultivable microbial fraction present in sour ale beer during maturation step in wooden barrel and identified *Saccharomyces* wild yeast candidates to be exploited as starters in wild ale beer production.

2. Materials and Methods

2.1. Chemicals, Media, and Reference Strains

Chemicals were purchased from Sigma Aldrich (St. Louis, MO, USA), unless otherwise stated. Media and anaerobic systems were purchased from Oxoid (Cambridge, UK), while reagents for molecular biology from Thermo Scientific (Waltham, Massachusetts, United States). Oligonucleotides synthesis and Sanger sequencing were carried out by Bio-Fab Research core facility (Rome, Italy).

Reference strains were listed in Table 1.

Table 1. Reference strains used in this work.

Strain	Species	Features	Source
BY4742	<i>S. cerevisiae</i>	<i>MATα; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0</i>	Euroscarf
BY4743	<i>S. cerevisiae</i>	<i>MATa/α; his3Δ1/ his3Δ1; leu2Δ0/ leu2Δ0; met15Δ0/MET15; LYS2/lys2Δ0; ura3Δ0/ura3Δ0</i>	Euroscarf
3711	<i>S. cerevisiae</i> var. <i>diastaticus</i>	<i>STA1</i> positive French Saison ale yeast	Wyeast

WLP940	Lager	STA1 negative lager yeast	White Lab
Atecrem	<i>S. cerevisiae</i>	Atecrem Belgian Saison	Bioenologia 2.0
WLP565	<i>S. cerevisiae</i>	Belgian Saison I Ale Yeast	White Lab
3724	<i>S. cerevisiae</i> var. <i>diastaticus</i>	Belgian Saison Yeast	Wyeast
PJP9	<i>S. uvarum</i> / <i>S. bayanus</i>	nd	Gift from P. Marullo

Abbreviation: nd, not determined.

2.2. Sour Beer Production and Sampling

The sour beer was produced at a craft brewery located in Bologna, Italy. **Figure 1** illustrates the semi-spontaneous fermentation method employed by the craft brewery.

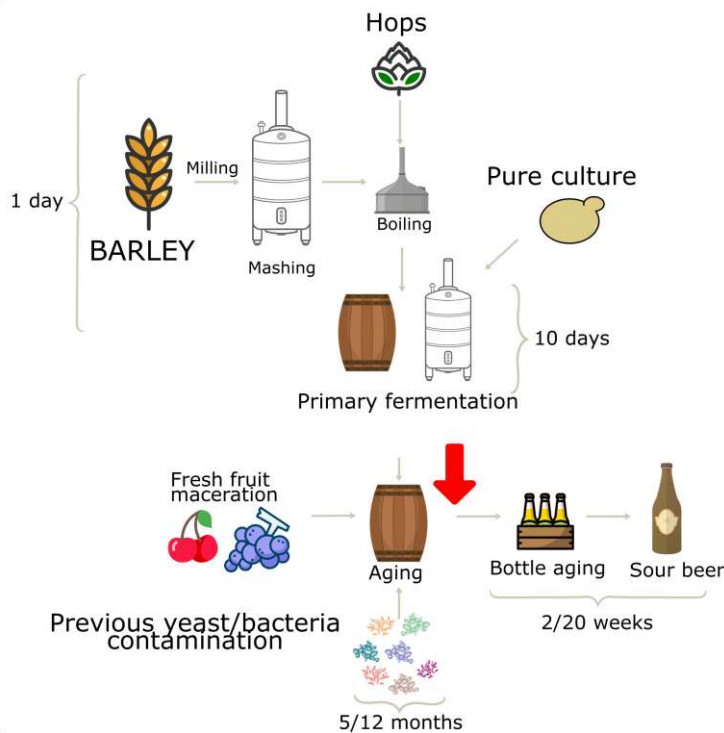


Figure 1. Flow-chart of semi-spontaneous craft beer fermentation. Red arrow indicates point of sampling.

Three distinct beer products labeled as 1, 2, and 3 were blended in equal proportions (1:1:1). In detail, beer 1 was produced by a spontaneous fermentation of a wort (cereal grist: barley malt 72%, unmalted wheat 15%, rye 8%, cara malt 5%) with an adjunct of fresh whole grapes with skins (grapes/wort ratio 1:10) and an ageing of 8 months in barrel; beer 2 was produced through a wort fermentation (same grist as beer 1) with the single strain *Brettanomyces bruxellensis* WLP650 (White Lab), a subsequent aging of 5 months in barrels, and a final maceration (2 months) of fresh whole cherries; beer 3 was produced with a cereal grist of barley malt 62%, unmalted wheat 30%, oat 8% that underwent primary fermentation with a blend of three commercial *S. cerevisiae* strains (WLP565, Atecrem, and 3724; Table 1), followed by a maturation in barrel for 10 months. Samples were shipped to the laboratory under refrigerated conditions and immediately submitted to microbiological and physicochemical determinations.

2.3. Microbial Counts, Bacterial and Yeast Isolation

After ten-fold dilution in saline solution (9 g/L NaCl), appropriate sample dilutions were spread on Petri plates. Yeasts count and isolation was performed on YPDA (1% w/v yeast extract, 1% w/v peptone, 2% w/v dextrose, 2% w/v agar) and Wallerstein Laboratory (WL) media supplemented with chloramphenicol (100 µg/mL), incubated for 24-48 h at 28 °C. Man-Rogosa-Sharpe medium supplemented with maltose (5 g/l) and cycloheximide (20 mg/mL) (mMRS) was used for lactic acid bacteria (LAB) isolation setting the anaerobic incubation at 30 °C for 48-72 h.

Yeast and LAB populations were quantified in triplicate by counting plate colonies ranging from 20 to 200 and expressing the results as mean Log₁₀ CFU/mL. Colonies were purified through at least double streaking on the same isolation medium. Cultures were microscopically examined for cellular morphology, and bacterial isolates were further screened for Gram staining and catalase activity. For long-term preservation, isolates were cryopreserved at -80°C using the corresponding isolation medium supplemented with 25% (v/v) glycerol.

2.4. Yeast and LAB Identification

Yeast genomic DNA was extracted according to Hoffman and Winston [24], while LAB DNA according to Gala et al. [25]. The integrity of DNA was checked by electrophoresis on a 0.8% (w/v) agarose gel in 0.5X TBE buffer (45 mM Tris-HCl, 45 mM boric acid, and 1 mM EDTA, pH 8.0), and DNA quality and quantity were evaluated spectrophotometrically using a NanoDrop ND-132 1000 device (Thermo Scientific, Waltham, USA). All DNA samples were diluted to a concentration of 50 ng/µL with ddH₂O for further analysis.

Primers, reaction mixture, and cycling conditions of PCR amplification reactions were detailed in Supplementary **Table S1**. Concerning yeast identification, the region including 5.8S rRNA gene and the upstream and downstream internal transcribed spacers (globally referred to as ITS region) was PCR amplified in a final volume of 40 µL using the primers ITS1 and ITS4, as described by White et al. [26]. The restriction fragment length polymorphism (RFLP) analysis of ITS amplicons was carried out with the endonucleases *Hae*III and *Hinf*I [27] and the length of PCR amplicons and RFLP fragments were compared to those in the YeastID database (www.yeast-id.org; last accessed on 14 December 2022) using rank parameters at +/-10. Discrimination of *S. cerevisiae*, *S. eubayanus*, and *S. uvarum* species was carried out according to the species-specific PCR method previously described [28-29]. All primers concentrations were set up to 0.4 µM, except for primer SbayR1, which was used at the final concentration of 0.8 µM. Strains representative of each ITS RFLP and species-specific PCR profile were subjected to PCR amplification of D1/D2 region of 26S rRNA (LSU) gene according to Martini et al. [30].

Concerning LAB identification, 16S rRNA gene was PCR amplified with the primers 27f and 1490r [31]. Amplified 16S Ribosomal DNA Restriction Analysis (16S-ARDRA) was performed by the endonucleases *Hha*I, *Hinf*I, and *Tru*II and the resulting 16S-ARDRA profiles were compared to the *in silico* profiles obtained for the most common LAB beer species (Supplementary **Table S2**).

2.5. Phylogenetic Analysis

Phylogenetic analyses of yeast and bacteria strains were performed using D1/D2 LSU and 16S rRNA sequences, respectively. Amplicons were purified using DNA Clean & Concentrator™-5 Kit (Zymo Research, Orange, CA, USA) and sequenced using both amplification primers. Contig sequences were merged using the program SeqMan (DNASTAR, Madison, WI, USA) and the poor-quality ends were edited manually to remove primers. The resulting contig sequences were used as queries in a Blastn search against the NCBI RefSeq database [32] (last asses on 14 February 2023). Cut-offs of 98.7% and 99% gene similarity were used for bacterial and yeast species attribution, respectively [33-34]. The sequences were aligned with Muscle program [35] in MEGA X software [36] and the resulting alignments were subjected to a DNA substitution model analysis to select the best-fitting model. Phylogenetic relationships were inferred using the Neighbor joining method [37]. Confidences for the phylogenetic tree were estimated from bootstrap analysis (1000 replicates). Trees were visualized using Interactive Tree of Life (ITOL) [38] and rooted at outgroup reference species. The sequence data of D1/D2 LSU regions were deposited in the GenBank NCBI database under the

accession numbers OM618137 to OM618146, while the 16S rRNA gene sequences under the accession numbers OM618118 to OM618120.

2.6. Genotyping Analysis

All the LAB and yeasts isolates were genotyped with a repetitive extragenic palindromic-polymerase chain reaction (rep-PCR) fingerprinting analysis based on (GTG)₅ primer (5'-GTG GTG GTG GTG GTG-3') [39], as previously described [40-41].

Commercial ale beer starters used in primary fermentation (**Figure 1**) and listed in **Table 1** were genotyped by three different genotyping methods, such as (GTG)₅ rep-PCR, inter-delta PCR, and R3-RAPD. (GTG)₅ rep-PCR was carried out as reported above. PCR amplification of LTR regions near to TY1 and TY2 retrotransposon (referred to as inter-delta regions) was carried out with primers d12 (5'-TCAACAATGGAATCCCAAC- 3') and d21 (5'-CATCTTAACACCGTATATGA -3') [42], while R3-RAPD PCR amplification was carried out using the primer R3 (5'-ATG-CAGCCAC-3') [43] (**Supplementary Table S1**).

(GTG)₅ rep-PCR profiles were analyzed by electrophoresis on a 1.8% (w/v) agarose gel (25 cm x 27 cm) in 0.5X TBE buffer at the constant voltage of 70 V for 6 h under cold conditions; inter-delta and R3-RAPD profiles on a 1.5% (w/v) agarose gel (15 cm x 25 cm) in 0.5X TBE buffer at a constant voltage of 70 V for 150 min under cold conditions. In all genotyping tests, agarose gels were stained with SYBRTM Safe DNA Gel Stain (Invitrogen, Waltman, MA, USA) and GeneRuler 100 bp Plus DNA LadderTM was used as a molecular weight standard. After visualization under UV light, digitalized images were analyzed using the BioNumerics software v8.10 (Applied Maths, Sint-Martens-Latem, Belgium). Pearson's correlation similarity coefficient was used to convert computed band patterns into similarity matrix. Optimization and curve smoothening parameters were optimized with the specific script present in BioNumerics. The unweighted-pair group method analysis using the arithmetic means (UPGMA) was used for tree construction. Yeasts and LAB isolates with ≥ 92 and 85% similarity values were treated as a single strain, respectively.

2.7. Flocculation, Sporulation Assays and MAT Locus Genotyping

Flocculation assay was performed as reported in [44]. Briefly, yeasts were propagated in YPDA medium until OD_{600nm} > 2.0. Three mL of pure liquid cultures were centrifuged at 2000 g for 5 min, and cells were resuspended in an equal volume of flocculation buffer (50 mmol/L Na acetate/acetic acid, 5 mmol/L CaSO₄; pH 4.5). The OD_{600nm} values were spectrophotometrically measured immediately after resuspension, as well as after 15 and 30 min of incubation at room temperature without agitation. To assess flocculation ability, the following equation was used:

$$F (\%) = (OD_{600nm} \text{ after 15 or 30 min} / OD_{600nm} \text{ at starting time}) \times 100, \quad (1)$$

Yeasts were classified according to F% as follows: score from 0 to 10%, "very flocculant"; from 10 to 30%, "moderately flocculant"; from 70 to 90%, "poorly flocculant"; from 90 to 100%, "non-flocculant".

To induce sporulation, exponentially grown cells were sub-cultured to acetate agar plates (2% w/v agar, 0.6% w/v sodium acetate) [45] and incubated at 28 °C for two weeks. Asci formation was checked and counted with a Bürker chamber after 3 and 14 days using an optical microscope with 400X magnification. Sporulation percentage was calculated as follows:

$$\text{Sporulation } (\%) = [(n^\circ \text{ asci}) / (n^\circ \text{ asci} + n^\circ \text{ cells})] \times 100, \quad (2)$$

Spore viability was determined by asci dissection and monospore clone constitution as previously reported [46], according to the following equation:

$$\text{Spore Viability } \% = (n^\circ \text{ of vital monospore cultures} / n^\circ \text{ of dissected spores}) \times 100, \quad (3)$$

For each strain, at least eight asci were dissected. Both wild strains and their monospore derivatives were genotyped for the MAT locus according to Catallo et al. [46]. Strains BY4742 and BY4743 (**Table 1**) were used as positive MAT α and MAT α /MAT α controls, respectively.

2.8. Maltose Fermentation Tests

Preliminary, maltose fermentation test was carried out according to Kurtzman et al. [45]. Sterile basal medium (0.45% w/v yeast extract, 0.75% w/v peptone and 0.0048% w/v bromothymol blue as pH indicator) (4.5 mL) was supplemented with the filter-sterilized stock sugar solution (either maltose or glucose) at the final concentration of 2% (w/v) and dispensed in test tubes with screw caps with an upside-down inverted Durham tube inserted. Each yeast strain was propagated in YPD medium at 27 °C for 24 h and inoculated at the final concentration of 2×10^7 CFU/mL. Tubes were incubated at 28 °C, and gas production was checked after 3, 7, and 14 days. Scores were attributed as follows: +, strongly positive, insert filled within 7 days; s, slowly positive, insert slowly filled after more than 7 days; –, no gas production. Cells inoculated in basal medium with 2% (w/v) glucose were used as a control.

Maltose consumption tests were carried out in triplicates in 100 mL Erlenmeyer flasks containing 45 mL of YNB (0.67% w/v Yeast Nitrogen Base) medium supplemented with 2% (w/v) of either glucose or maltose and sealed with cotton plugs, as previously reported [46-47]. After 48 h of incubation at 25 °C under shaking conditions (150 rpm), optical density (OD) values at 600 nm (OD_{600nm}) were measured using an UV-Vis photometer.

2.9. Wort Preparation and Micro-Scale Fermentation Trials

For the wort preparation, Pilsen malt (195 kg) (Bestmalz, Heidelberg, Germany), unmalted wheat (130 kg), unmalted oat (*Avena sativa*) (32 kg), and hop pellets Hallertau Perle (Hopsteiner, Mainburg, Germany) were mixed in a final volume of 1600 L. The “Liquor-to-grist” ratio was maintained at 3:1. The mash temperature steps involved 10 min at 45 °C, 20 min at 55 °C, 40 min at 66 °C, and 10 min at 78 °C. The boiling process was carried out for 60 min. The resulting hopped wort (11.5 °Plato original gravity; density 1.07 g/L, pH value of 5.15 ± 0.02) was pasteurized at 100 °C for 10 minutes, and subsequently frozen at -20 °C until use.

Micro-scale wort fermentation trials were conducted in duplicate using 200 mL glassware bottles equipped with airlocks and filled with 100 mL of pasteurized hopped. A single colony of each yeast candidate was propagated at 27 °C for 48 h in YPD medium under orbital shaking (150 rpm). After recovery by centrifugation for 6 minutes at 2500 g, cells were resuspended in pasteurized wort and inoculated in wort at the final concentration of 10^7 cells/mL. Fermentation bottles were maintained at 20 °C for 21 days and daily monitored over time by CO₂ release, weighting the fermenters to track record of weight loss. The un-inoculated beer wort was used as negative control.

The fermentation curves were modeled based on the CO₂ evolution, using the “grofit” package for R [48]. The maximum rate of fermentation (μ), maximum fermentation efficiency (the total amount of CO₂ released at the end of the fermentation) (A), and the time of the lag phase (λ) were determined using parametric fitting methods in “grofit”.

2.10. Physicochemical Analysis

The pH was determined at the sample temperature of 25 °C (Crison Instruments, Barcelona, Spain).

Carbohydrates and organic acids in sour beer, wort, and fermented broths, clarified by centrifugation (13000 rpm for 5 min at 4 °C) and filtration at 0.22 μ m, were analyzed in an HPLC system equipped with a refractive index detector (1200 System, Agilent Technologies, Waldbronn, Germany). Elution in isocratic condition was carried out at 60 °C with 0.6 mL/min of 5 mM H₂SO₄ through an ion exclusion column (Aminex HPX-87 H, Bio-Rad, Hercules, CA, USA).

Volatile organic compounds (VOCs) were traced by solid-phase microextraction (SPME) followed by gas chromatography-mass spectrometry analysis (GC–MS). Two mL of samples were spiked with 100 μ L of 1-chloro-2-fluoro-benzene 5 mg/L, as internal standard, and the headspace was exposed to a divinylbenzene/carboxen/polydimethylsiloxane fiber (DVB/CAR/PDMS Supelco; Sigma-Aldrich, St. Louis, MO, United States) for 1 h at 60 °C. Analytes were desorbed in a GC–MS apparatus (7820–5975; Agilent Technologies, Santa Clara, CA, United States) equipped with a DB-624

column (30 m × 250 mm × 1.4 mm, Agilent Technologies). Separation was achieved with 1.3 mL/min helium flux following a thermal gradient: 2 min isotherm at 50 °C, 6 °C/min increase to 110 °C, 10 °C/min increase to 240 °C, 4 min isotherm at 240 °C. NIST 14 spectral library was utilized to infer the identity of the compounds by comparison of their mass spectra. The peak areas of compounds, normalized by the area of the inner standard, were taken to be proportional to their abundances.

2.11. Screening of *STA1* Gene

The screening for *STA1* gene was conducted by endpoint PCR using the SD-5A (5-CAACTACGACTTCTGTCATA-3) and SD-6B (5-GATGGTGACGCAATCACGA-3) primers [49], while the analysis of the promoter region, particularly the *UAS2* portion, was conducted with the primers STA1_UAS_Fw (5'-CCTGGCTCAAATTAACTTTTCG-3') and STA1_UAS_Rv (5'-ACCACCAATAGGCAATAGAAA-3') [50]. PCR mixtures and cycling conditions were reported in Supplementary **Table S1**. PCR amplicon purification, sequencing and sequence analysis were carried out as reported above.

2.12. Statistical Analysis

The datasets were analyzed using GraphPad Prism 8 software (GraphPad Software Inc., San Diego, CA) through one-way or two-way ANOVA. A *p*-value threshold of 0.05 was set for both analyses. All values presented in this study are the means of three replicates ± standard deviation (SD).

Principal component analysis (PCA) was applied to examine the data matrix of VOCs profiles, presenting a size {20, 53}, consisting of the 53 relative areas of VOCs, determined for the two independent fermentation with the 9 yeast strains in addition to the initial wort. The screen plot was utilized to establish the number of significant principal components (PCs) based on the percentage of variance explained. Scores and loadings of the PCA model were calculated using Past version 4.12 [51], as well as UPGMA hierarchical clustering of samples based on the Bray-Curtis similarity index of VOCs profiles.

3. Results

3.1. Sour Beer Physicochemical Characterization and Microbiological Analysis

The samples were collected during the late maturation phase. Sour beers are distinguished from ales and lagers by their elevated levels of organic acids, specifically lactic acid and acetic acid. These acids contribute to a lower pH and a more pronounced acidic taste. The beer was characterized by a pH of 3.37±0.01 and by the abundance of lactic acid (13.0 g/L), which was the major metabolite, after ethanol (51.8 g/L), likely resulting from the catabolic conversion of wort sugars operated by LAB. Other primary catabolites observed in the beer were glycerol (3.3 g/L) and acetic acid (1.0 g/L). Microbiological counts on YPDA and WL media resulted in values of 4.39±0.10 and 4.28±0.3 Log₁₀ CFU/mL, respectively. mMRS count was 6.00±0.03 Log₁₀ CFU/mL confirming the primary role of LAB in the production process and their predominance on the yeast population.

3.2. LAB Identification and Genotyping

All LAB isolates were cocci-shaped, Gram positive, and catalase negative. They were submitted to 16S-ARDRA with three endonucleases, namely *Hha*I, *Hinf*I, and *Tru*I. These restriction enzymes were proved to be diagnostic for most LAB species frequently encountered in sour beer fermentation (Supplementary **Table S2**). 16S-ARDRA patterns supported the attribution of LAB isolates to *Pediococcus parvulus*/*Pediococcus damnosus*, which are recognized as beer spoilage species and frequently found as dominant in wood aging phase of sour beer process (as reviewed by [52]).

Phylogenetic analysis of 16S rRNA gene sequences was carried out on 7 randomly selected LAB isolates. The strains formed a monophyletic group with *P. damnosus* strain DSM 20331^T (GenBank accession number: NR_042087.1) (**Figure 2**, panel A). This species is critical in sour beer the

production, as it is more hop-tolerant and low pH-tolerant than lactobacilli [23] and it is very persistent in wood barrels [53]. *Pediococcus* spp. produce diacetyl, that is responsible for a rancid and buttery flavor. However, in craft beer this effect is attenuated by some non-*Saccharomyces* yeasts which consume diacetyl. On the other hand, *Pediococcus* spp. decrease pH more slowly than lactobacilli, assuring yeasts to complete primary fermentation [55].

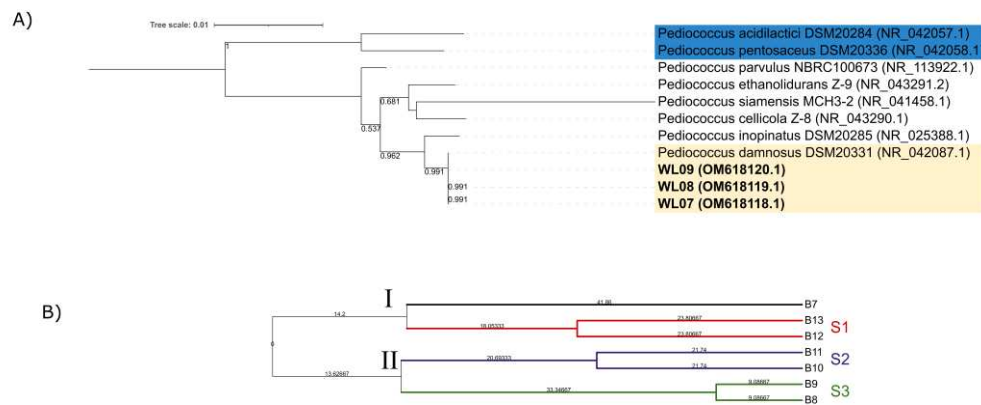


Figure 2. Phylogenetic and genotyping analysis of LAB isolates. (A) Neighbor-joining tree based on 16S rDNA sequences showing genetic relatedness between *Pediococcus damnosus* isolates and related species. The evolutionary distances were computed using the Kimura 2-parameter method and the rate variation among sites was modeled with a gamma distribution. Bootstrap values are indicated beside branches (> 50%). The tree was rooted with *P. acidilactici* and *P. pentosaceus* (blue). (B) UPGMA clustering of LAB isolates based on (GTG)₅ rep-PCR fingerprinting analysis. Similarities were calculated as Pearson correlation coefficient with Bionumerics software. The tree data (Newick) were exported and visualized using ITOL [38].

(GTG)₅ rep-PCR was applied to analyze the genetic diversity within the group of LAB isolates. The patterns consisted of a minimum of 10 bands to a maximum of 13 bands with dimensions ranging from 150 to 2270 bp. The UPGMA dendrogram identified 5 singleton strains and one subcluster at 85% of rep-PCR profile similarity, suggesting high diversity of LAB strains isolated from sour beer (Figure 2, panel B).

3.3. Yeast Identification

A total of 50 yeast isolates were randomly selected from countable plates, 20 from YPDA and 30 from WL, respectively. Raw data about PCR-RFLP analysis of ITS regions were reported in Supplementary Table S3. Search in Yeast-ID database allowed to match five different patterns (A to E) to the following species: pattern A to *S. cerevisiae* (100%)/*Saccharomyces cariocanus* (100%)/*Saccharomyces paradoxus* (100%); pattern B to *Pichia membranifaciens* (73%); pattern C to *Saccharomyces bayanus* (89%)/*Saccharomyces kudriavzevii* (89%)/*Saccharomyces pastorianus* (89%)/*Saccharomyces mikatae* (89%); pattern D to *Kluyveromyces blattae* (67%); and pattern E to *Brettanomyces bruxellensis* (83%).

Representative strains from each ITS PCR-RFLP pattern were submitted to sequencing of variable domain D1/D2 at the 5' end of the 26S rRNA gene. In details, strains WY104, WY115, and WY203 (pattern A) grouped monophyletically with *S. cerevisiae* NRRL Y-12632 (97% bootstrapping) (Figure 3). Strains WY122 and WY102 (pattern B) shared the highest D1/D2 26S rRNA sequence similarity with *Pichia membranifaciens* (Figure 3). However, three substitutions (60T>C, 167C>T, and 179C>T) did not allow the monophyletic clustering of these strains with *P. membranifaciens* strain CBS107^T. This species has been identified in lambic and gueuze beers [17,55], wine [56-58], and other beverages [59,60]. *Pichia membranifaciens* is one of the most frequently yeast associated to mycobiome of sour beer during wood aging [20,61] due to the capability to form biofilm which accounts for the long persistence of this species on the wooden surface [62]. While it has been described that *Pichia*

spp. can be involved in the biotransformation of hop terpenes (incl. geraniol) [63], this species also produces unpleasant compounds, such as 4-ethylphenol and 4-ethylguaiacol [57].

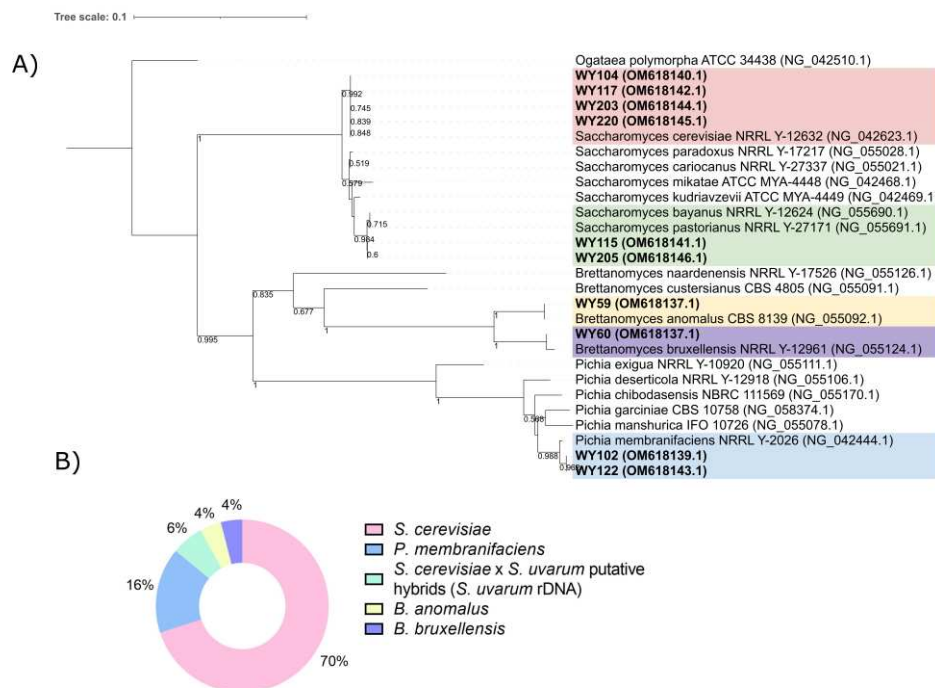


Figure 3. Species attribution of yeast isolates (in bold). (A) Phylogenetic tree obtained by neighbor joining (NJ) method applied to a dataset of 28 D1/D2 26S rDNA sequences. The evolutionary distances were calculated by the Tajima-Nei method. The gamma distribution was used to model the rate of change between sites. The lengths of the branches are proportional to the number of nucleotide substitutions, and they have been measured using the divergence scale shown at the top left. Bootstrap values (1000 replicates) are indicated beside branches (> 50%). The tree was rooted with *Ogataea polymorpha* as outgroup. The tree data (Newick) were exported and visualized using ITOL [38]. (B) Pie-chart representing species abundance in sour beer. Pink, *S. cerevisiae*; blue, *P. membranifaciens*; green, *S. cerevisiae* x *S. uvarum* hybrids; yellow, *D. anomala*; purple, *D. bruxellensis*.

Strains WY115, WY205, and WY213 (pattern C) were phylogenetically related to the complex taxon *S. bayanus*, which includes two well-differentiated groups of strains, such as *S. bayanus* var. *bayanus* (*S. eubayanus* × *S. uvarum*) and *S. bayanus* var. *uvarum* (Figure 3) [64,65]. Libkind et al. [66] proposed the use of *S. eubayanus* and *S. uvarum* as descriptors of species but restricted the name *S. bayanus* to the hybrid lineages between these pure species. *S. uvarum* was isolated in natural European wine [67–69] and cider fermentations [70–71], as well as in South American chicha fermentations [72]. *S. bayanus*/*S. uvarum* and the hybrid species *S. pastorianus* (synonym *Saccharomyces carlsbergensis*) are extensively used to produce lager beer through bottom fermentation at low temperature [73]. Occurrence of *S. bayanus*/*S. uvarum* was previously documented in the early stages of lambic beer fermentation; after that this species should disappear due to high ethanol content and low pH [20].

Finally strains WY59 (pattern D) and WY60 (pattern E) clustered together with *Brettanomyces anomalus* (homotypic synonym: *Dekkera anomala*) and *B. bruxellensis* with highest bootstrapping values, respectively (Figure 3). For strain WY59, phylogenetic results partially disagreed with ITS PCR-RFLP analysis. This strain showed an amplified ITS product of 550 bp (Supplementary Figure S1, panel A) instead of the expected 800 bp-long ITS product reported for *B. anomalus* in Yeast-ID database (last access on 2nd May 2023). Phylogenetic analysis of ITS sequences confirmed that strain WY59 formed a monophyletic cluster with *B. anomalus* CBS 77^T (Supplementary Figure S1, panel B).

The presence of *S. bayanus*/*S. uvarum* is unusual in old-style sour beers which are produced at room temperature. Furthermore, hybrid strains frequently retain only one parental type of rDNA

arrays in their genome, resulting in ITS restriction profiles either of *S. cerevisiae* type or *S. eubayanus*/*S. uvarum* type [74] (**Figure 4**, panel A). This loss of heterozygosity in rDNA marker makes ITS-RFLP analysis unable to discriminate hybrids from their parental lineages. Based on these considerations, we performed three species-specific PCR assays targeting *S. eubayanus*, *S. cerevisiae*, and *S. uvarum* species, respectively, to confirm species attribution of strains WY115, WY205, and WY213 to *S. uvarum*/*S. bayanus* and of 35 strains to *S. cerevisiae*. As expected, *S. cerevisiae* reference strains BY4743 and BY4742, as well as sour beer strains with pattern A were positive to *S. cerevisiae*-specific PCR and negative to *S. bayanus*/*S. uvarum* and *S. eubayanus* species-specific PCR assays (**Figure 4**, panel B). Remarkable, *S. uvarum*/*S. bayanus* sour beer strains WY115, WY205, and WY 213 resulted positive to both *S. cerevisiae* and *S. bayanus*/*S. uvarum* species-specific PCRs but gave no PCR amplicons with *S. eubayanus*-specific primer pair (**Figure 3**, panel B). Overall, this evidence supported that the strains WY115, WY205, and WY213 could be *S. cerevisiae* x *S. uvarum* natural hybrids which only retained the *S. uvarum*/*S. bayanus* rDNA copy in their genomes.

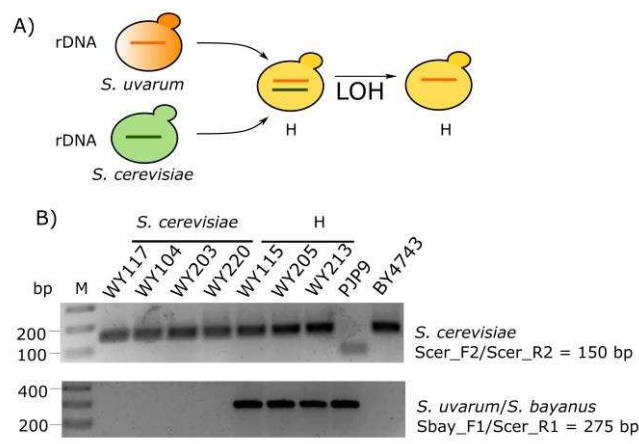


Figure 4. Characterization of *Saccharomyces* strains by species-specific PCR assays. (A) Illustration of loss of heterozygosity (LOH) occurring in rDNA arrays of hybrid lineages. (B) *S. cerevisiae*-specific and *S. bayanus*/*S. uvarum*-specific PCR assays with Scer_F2/Scer_R2 primers targeting *MEX67* gene (amplicon size 150 bp) and primers Sbay_F1/Sbay_R1 targeting *DBP6* gene (amplicon size 275 bp), respectively. All tested strains were negative to PCRs targeting *S. eubayanus*-specific *FSY1* gene (amplicon size 228 bp) (data not shown). *S. cerevisiae* BY4743 and *S. uvarum* PJP9 were used as internal control. *S. cerevisiae* strains WY117, WY104, WY203, and WY220 were selected as representative of pattern A. Abbreviations: M, molecular weight marker; S. cer, *S. cerevisiae*; Sbay, *S. uvarum*/*S. bayanus*; H, hybrid; LOH, loss of heterozygosity.

As a whole, the culturable yeast fraction of natural sour beer considered in this study consisted of *S. cerevisiae* (70%), followed by *P. membranifaciens* (16%), *S. cerevisiae* x *S. uvarum* hybrids (6%), *D. anomala* (4%), and *B./D. bruxellensis* (4%) (**Figure 3**, panel B).

3.4. Yeast Genotyping

The genetic diversity of the 50 yeast isolates was estimated using the (GTG)₅ rep-PCR method. The resulting patterns comprised from 4 to 23 bands with dimensions between 200 and 2830 bp. A UPGMA dendrogram, based on Pearson's similarity coefficient, was grouped the 50 isolates into two major clusters (I and II) (**Figure 5**). Applying a reproducibility cutoff of 92% led to the identification of 14 biotypes, consisting of 9 subclusters (S1 to S9) and 5 singletons.

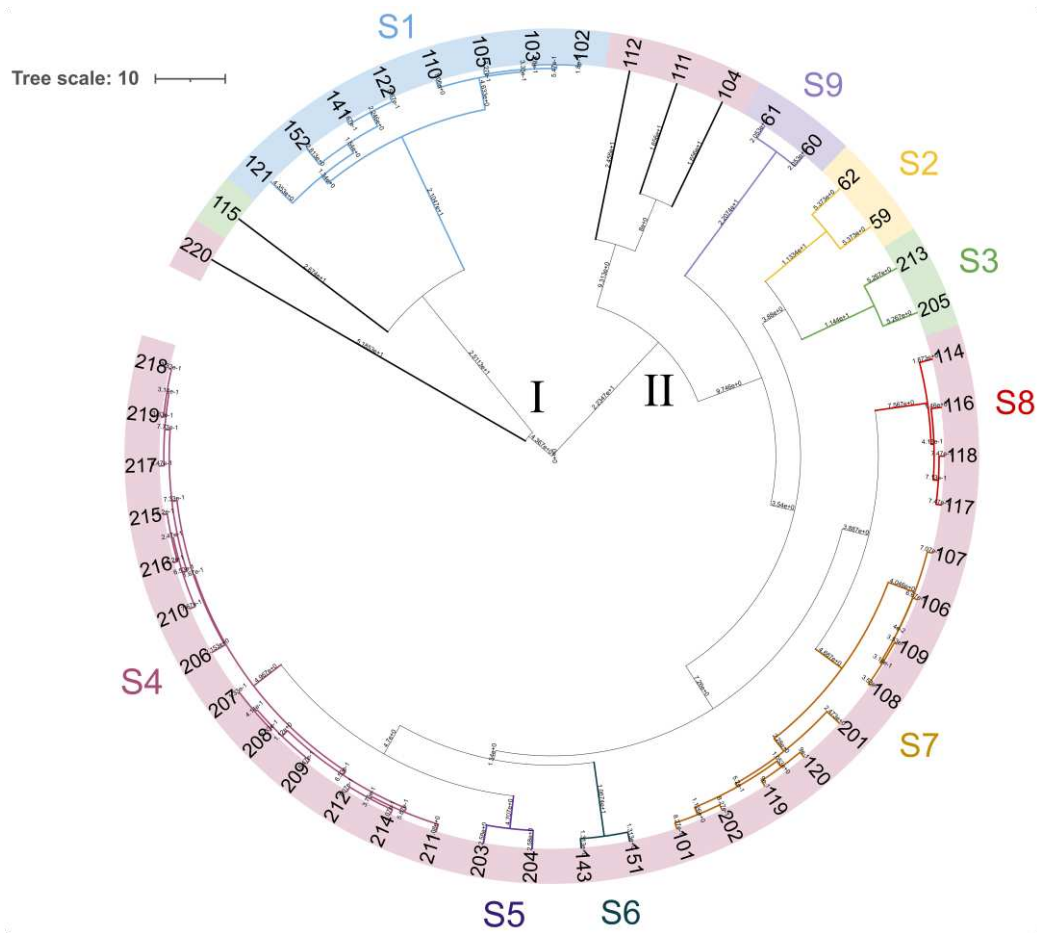


Figure 5. Dendrogram generated from (GTG)₅ rep-PCR fingerprints of 50 sour beer wild yeasts. Similarity percentages were calculated using Pearson correlation coefficient, while hierarchical clustering analysis was carried out using the UPGMA (unweighted pair-group method with arithmetic mean) method with Bionumerics software. The tree data (Newick) were exported and visualized using ITOL [38]. The similarity value above 92% was used to discriminate biotypes numbered from S1 to S9 and singletons. The color codes were attributed as follows: pink, *S. cerevisiae*; blue, *P. membranifaciens*; green, *S. cerevisiae* x *S. uvarum* hybrids; yellow, *D. anomala*; purple, *D. bruxellensis*.

Cluster I comprised 8 yeast isolates belonging to the species *P. membranifaciens* (subclusters S1 and 2 singletons), as well as the *S. cerevisiae* x *S. uvarum* hybrid strain WY115 and *S. cerevisiae* strain WY220. Within cluster II, 30 *S. cerevisiae* isolates were divided into 5 subclusters (S4 to S8), while *S. cerevisiae* isolates WY112, WY111, and WY104 were singletons. *S. cerevisiae* x *S. uvarum* hybrids WY213 and WY205 clustered together in minor cluster S3, while *B. anomalus* and *B. bruxellensis* isolates divided into two subclusters, S2 and S9, respectively.

We investigated whether the *S. cerevisiae* isolates, one belonging to each (GTG)₅ REP-PCR subcluster, were genetically different from the commercial *S. cerevisiae* pure cultures used in primary fermentation, namely the strains Atecrem Belgian Saison, 3724, and WLP565 (Table 1). The (GTG)₅ rep-PCR technique was not discriminatory. Conversely, two alternative techniques, the inter-delta PCR assay, which amplifies LTR regions among retrotransposable elements Ty1 and Ty2, and the R3-RAPD technique, which randomly amplifies non-coding and coding DNA using R3 sequence as a primer showed that the *S. cerevisiae* wild isolates were genetically distinct from the commercial starter ones (Supplementary Figure S2, panels A and B).

3.5. Maltose Consumption and Flocculation

A pool of 9 selected sour beer wild strains were screened for their ability to consume maltose and flocculate. Maltose is the primary fermentable sugar in brewer's wort, while flocculation can be desirable in removing yeasts after fermentation. As shown in **Figure 6**, *S. cerevisiae* strains WY117, WY203, and WY104 consumed maltose at the same extent of glucose ($p>0.05$), while *B. anomalus* WY59, and *B. bruxellensis* WY60 did not grow on maltose as unique carbon source after 48 h of incubation. This result was further confirmed by conventional fermentation test (data not shown). Differently from the conspecific strain WY102, *P. membranifaciens* WY122 was able to consume maltose ($p<0.05$). Conventional fermentation test confirmed this result (Supplementary **Figure S3**), which contradicted the maltose-negative phenotype described for this species [75]. Concerning flocculation assay, none of the strains showed significant flocculent phenotype (Supplementary **Table S3**).

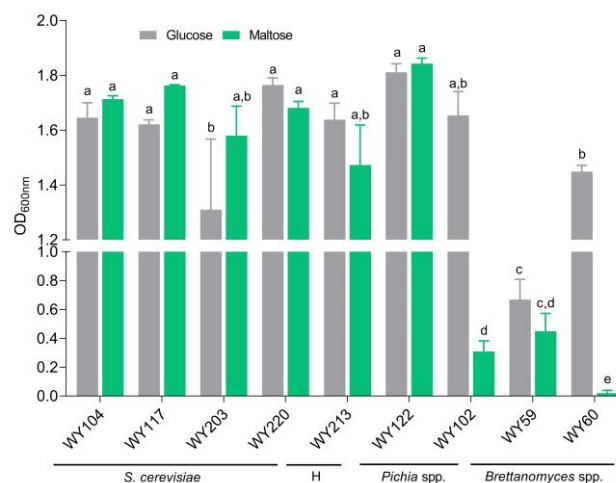


Figure 6. Maltose (green) and glucose (grey) consumption tests. Statistical differences are indicated with different letters (two-way ANOVA; $p<0.05$). Abbreviation: H, *S. cerevisiae* x *S. uvarum* hybrid.

3.6. Sporulation and MAT Genotyping

Four *S. cerevisiae* strains and 2 *S. cerevisiae* x *S. uvarum* hybrids were evaluated for their exploitability as parental lineages in hybrids construction. For this purpose, we determined sporulation efficiency and spore viability. Sporulation efficiency of *S. cerevisiae* strains varied from 22 to 23% after 3 days of incubation at 28 °C on agar acetate plates and slightly increased after 14 days (Table 2). *S. cerevisiae* x *S. uvarum* hybrids were not able to sporulate.

Table 2. Sporulation efficiency and spore viability of *Saccharomyces* wild sour beer strains. Abbreviation: nd, not determined.

Species	Strains	Sporulation efficiency (%)		Spore viability (%)	MAT loci
		3 d	14 d		
<i>S. cerevisiae</i>	WY104	22.96	24.56	75	MATa/MATα
	WY117	23.84	32.72	75	MATa/MATα
	WY203	22.70	27.85	81	MATa/MATα
	WY220	22.78	32.13	56	MATa/MATα
hybrids	WY205	1.22	0.89	nd	nd
	WY213	2.48	0.41	nd	nd

Spore viability can be defined as the ability to growth on a reach medium at 28 °C for 2 to 5 days and to produce a viable monospore colony after tetrad dissection. Despite *S. cerevisiae* strains were moderately sporulating, the spore viability was higher than 50%, with *S. cerevisiae* WY203 showing the highest spore viability (Table 2).

Since ploidy status and homothallic/heterothallic lifestyle strongly affect hybridization yield of *Saccharomyces* strains [46], we determined the *MAT* locus in each *S. cerevisiae* strain and their monospore progeny. All strains exhibited a *MATa/MAT α* genotype (**Table 2**) and produced *MATa/MAT α* monosporic progenies, suggesting that they are diploid and homothallic (*HO/HO*) strains (Supplementary **Figure S4**).

3.7. Micro-Scale Wort Fermentation

We performed test fermentations using both maltose consuming (*S. cerevisiae* WY104, WY117, WY203, and WY220; *P. membranifaciens* WY122; *S. cerevisiae* x *S. uvarum* WY213) and maltose non-consuming strains (*P. membranifaciens* WY102, *D. anomala* WY59, and *D. bruxellensis* WY60). To assess the kinetics variables during wort fermentation, we monitored the CO₂ loss in the fermentations by weighing. *S. cerevisiae* strains exhibited the shortest lag phase and the highest fermentation efficiency values ($p < 0.05$) (**Figure 7**) and did not differ significantly from *S. cerevisiae* x *S. uvarum* hybrid WY213 ($p > 0.05$). After a long lag phase, maltose-positive *P. membranifaciens* WY122 showed fermentation efficiency and fermentation rate similar to *S. cerevisiae*. As expected, maltose-negative *P. membranifaciens* strain WY102 was slow to adapt to wort environment and very low in fermentation efficiency but, after a long lag phase, it exhibited high fermentative rate. *B. bruxellensis* WY60 was better than *B. anomalus* WY59 to ferment wort ($p < 0.05$).

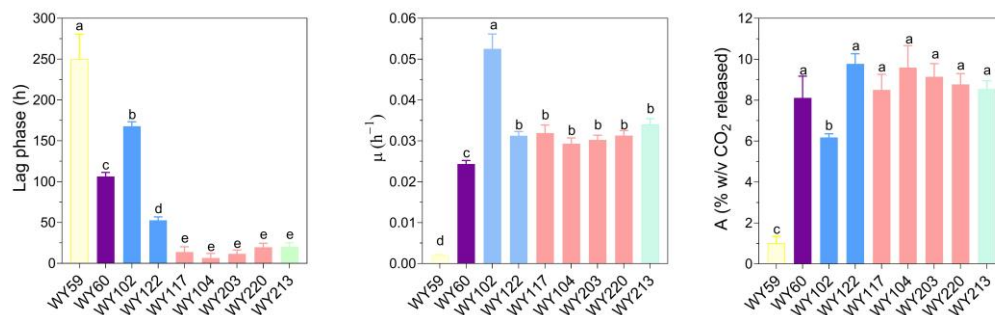


Figure 7. Kinetics parameters (lag phase λ , fermentation rate μ , and maximum fermentation efficiency) of yeast candidates in micro-scale wort fermentation (11.5 Plato; 20 °C). Species was represented as follows: pink, *S. cerevisiae*; blue, *P. membranifaciens*; green, *S. cerevisiae* x *S. uvarum* hybrids; yellow, *B. anomalus*; purple, *B. bruxellensis*.

The consumption of carbohydrates and ethanol content were measured at the end of fermentation and were consistent with the fermentation curves (**Figure 8**). Specifically, *Saccharomyces* strains outperformed non-*Saccharomyces* ones in maltose and maltotriose utilization, except for *P. membranifaciens* WY122. Coherently, *Saccharomyces* strains and *P. membranifaciens* WY122 produced higher amounts of ethanol (ranging from 20.8 to 34.1g/L). Notably, *S. cerevisiae* x *S. uvarum* hybrid WY213 demonstrated not only the highest ethanol production, but also the highest rate of carbohydrate-to-ethanol conversion (40%w/w), considering that the maximum theoretical value is 51%.

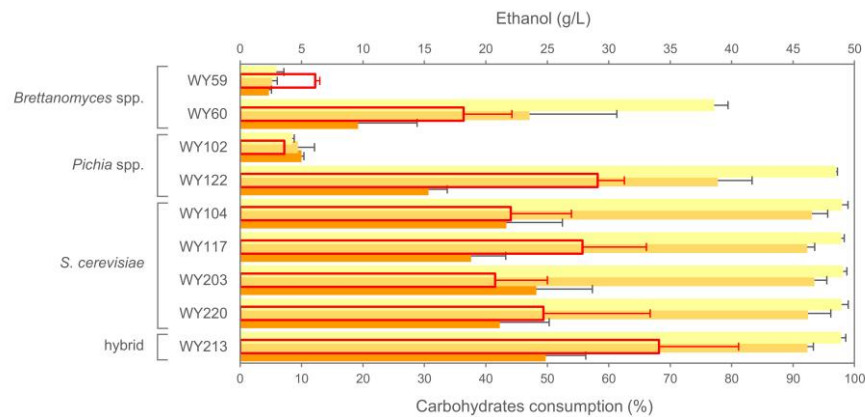


Figure 8. HPLC analysis of microscale fermentation broths inoculated with selected yeast strains. % consumption of maltose (yellow), maltotriose (light orange) and maltodextrins (orange); ethanol formation (g/L; red). Hybrid: *S. cerevisiae* x *S. uvarum* hybrid.

HPLC analysis revealed a partial consumption of dextrins, which generally are unfermentable sugars for commercial (domesticated) ale yeasts (**Figure 8**). *STA1* gene encodes extracellular glucoamylase which mostly contributes to dextrin consumption [50-76]. Recent studies showed that the mere presence of gene *STA1* is not enough to be diagnostic for a “diastatic” phenotype. Krogerus et al. [50] proved that a full-length promoter assures *STA1* gene expression in truly diastatic strains, while the UAS2 region of promoter is deleted in poorly diastatic *STA1*+ strains. Therefore, we investigated the presence of *STA1* gene and the related functional promoter in *S. cerevisiae* strains. As shown in **Figure 9**, *STA1* gene was present in our *S. cerevisiae* sour beer strains, but the promoter-specific PCR gave a PCR product longer than that expected (630 bp instead of 599 bp). Sequencing of promoter PCR amplicon showed two indels and 20 substitutions in UAS2 promoter region of *S. cerevisiae* WY117 compared to the truly diastatic strain *S. cerevisiae* 3711.

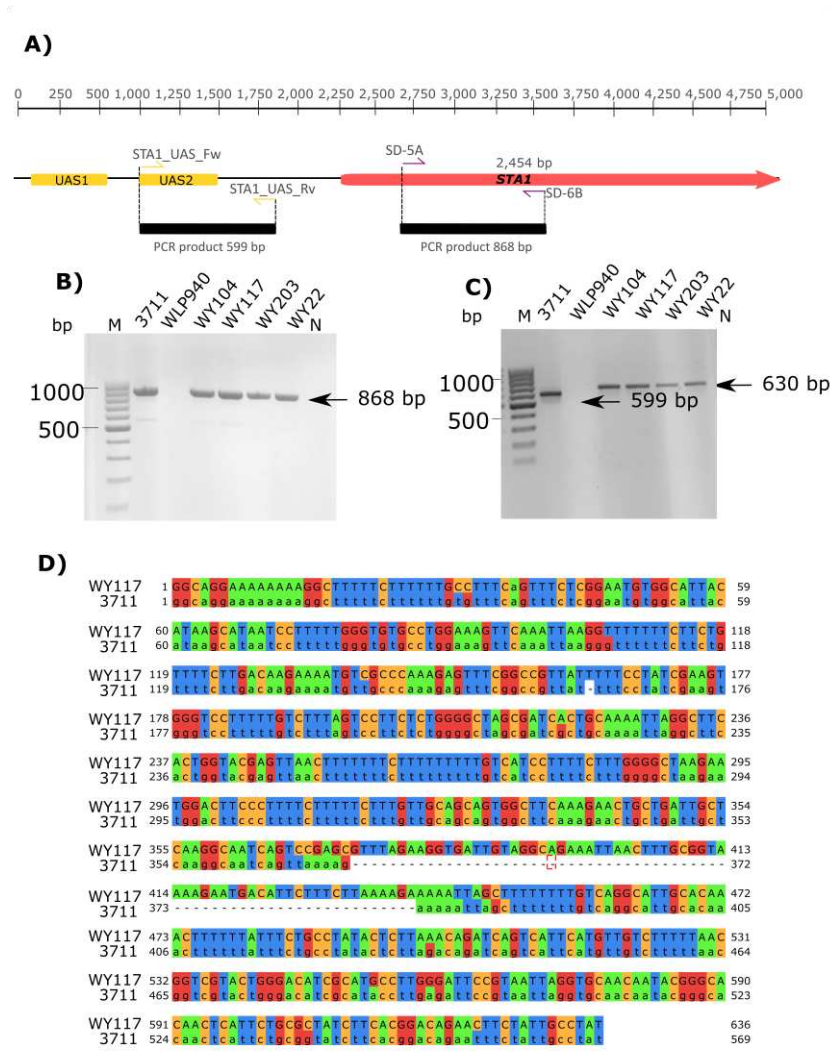


Figure 9. Screening for *STA1* gene and the related promoter in *S. cerevisiae* sour beer strains. (A) Strategy to PCR amplify *STA1* open reading frame and the promoter region with UAS_1 and UAS_2 located upstream the activation sequence [50]. (B) Individual PCR reactions using primers SD-5A/SD-6B with 3711 as reference strain with “diastatic” phenotype (*STA1*+, no deletion in *STA1* promoter) and WLP940 (*STA1*–) as reference strain with no “diastatic” phenotype. (C) Individual PCR reactions using primers STA1_UAS_Fw/STA1_UAS_Rv with 3711 as “diastatic” reference strain (*STA1*+, no deletion in *STA1* promoter) and WLP940 reference strain with no “diastatic” phenotype (*STA1*–) as no “diastatic” control. (D) Sequence alignment of UAS2-2 region of WY117 and 3711. Promoter region of diastatic control strain 3711 was retrieved from [50].

3.8. VOCs Determination

In the headspace of wort and fermented samples, SPME-GC analysis revealed a total of 53 compounds detected in at least two samples (Supplementary **Figure S5**). The VOCs occurring most frequently and abundantly were ethanol and other alcohols, esters, organic acids, aromatic compounds, furans, aliphatic hydrocarbons, and terpenoids. The PCA model revealed that the 2 highly informative dimensions, PC1 and PC2, accounted for 61.5% and 21.1% of data variability (**Figure 10**, panel A). Ethanol, 3-methyl-1-butanol, and phenylethyl alcohol presented positive loadings to PC1, resulting the major contributors to differentiate fermented samples (**Figure 10**, panel B). Conversely, terpenoids like humulene and beta-myrcene negatively contributed to PC1 distinguishing the profile of unfermented worth. A group of ethyl esters (i.e., hexanoic-, decanoic-, octanoic-, and ethanoic- acid ethyl esters) also contributed to characterize fermented samples but with positive loadings along PC2. Fermented samples appeared spread in two groups: samples

inoculated with strains *B. anomalus* WY59, *B. bruxellensis* WY60, and *P. membranifaciens* WY102 were mainly located in the second quarter of the graph, at negative values of PC1 and positive PC2. This agrees with previous observation that *Dekkera/Brettanomyces* spp. produce fatty acid ethyl esters, such as octanoate and ethyl decanoate, responsible for fruity and grape odor, respectively, better than *S. cerevisiae* [77]. Samples obtained by fermentation with *Saccharomyces* strains and *P. membranifaciens* WY122 were located in the first and fourth quarters, at positive values of PC1. WY122 exhibited a VOCs profile intermediate between *Saccharomyces* and non-*Saccharomyces* strains, characterized by concomitant presence of a more complex ethyl esters profile and high amount of ethanol. Among the aroma-active esters, ethyl esters, which play a crucial role in determining the aroma of beer [78], were the second-most abundant VOCs after acetate esters. Examples of ethyl esters include ethyl hexanoate (aroma like anise seed or apple), ethyl octanoate (sour apple aroma), and ethyl decanoate (floral odor). Ethyl esters are formed intracellularly by fermenting yeast cells, the rate of formation is highly strain-dependent and is mainly affected by the concentrations of the two precursors, ethanol and acyl coenzyme A, where the acid group is a medium-chain fatty acid (MCFA) [79]. During alcoholic fermentation, MCFA intermediates are prematurely released from the cytoplasmic fatty acid synthase (FAS) complex. The key enzyme involved in the regulation of fatty acid biosynthesis can promote the release of MCFAs, which dramatically affects the formation of ethyl esters and contribute to differentiates the yeast strain's profile.

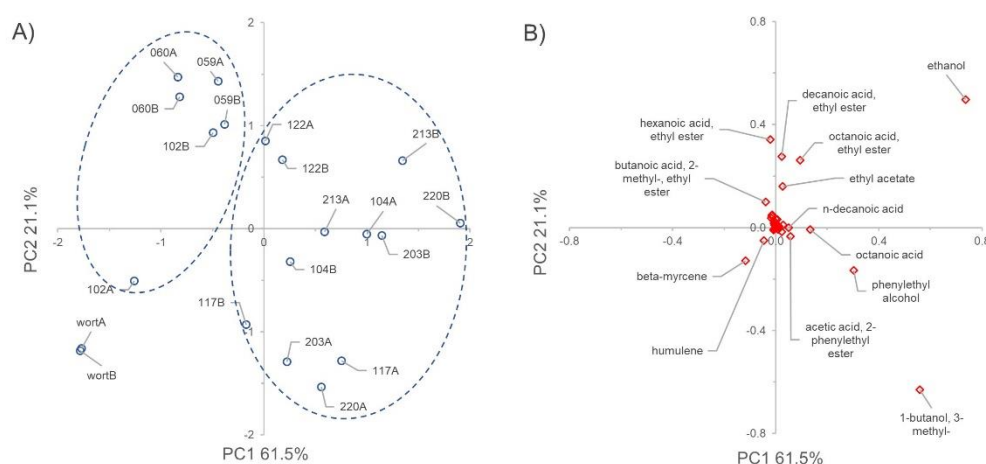


Figure 10. VOCs profiles of wort and micro-fermented samples reconstructed by headspace SPME-GC-MS analysis, followed by principal component analysis (PCA). (A) Scores plot (PC1 vs. PC2) accounting for 61.5% and 21.1% of variability, respectively. (B) Loading plot of the volatile molecules. The compounds with the highest loading value in PC1 and PC2 are labeled. A and B were replicated samples.

4. Discussion

Bioprospecting for native strains isolated from environments either alternative or related to brewery can contribute to diversify the portfolio of craft beer cultures. For instance, sourdough strains were proposed as *S. cerevisiae* parental lineage in synthetic hybrid construction [46] and as brewing cultures to produce low ethanol beer [80]. Low alcohol beer was also successfully produced with *S. cerevisiae* inhabiting brewery environment [81]. In this study, we applied a culturable approach to characterize the yeast fraction present in sour beer during aging in wooden barrel and proved that these indigenous yeasts can have promising brewing attributes. DNA barcoding techniques and PCR fingerprinting assured the constitution of a pool of assorted yeast candidates for subsequent phenotypic assays.

Previous studies on microbial succession during traditional lambic beer process showed that *Saccharomyces* species dominate the early stages, when they sequentially consume mono-, di-, and trisaccharides, releasing yeast-associated metabolites, such as ethanol, succinic acid, and methyl-1-

butanol [82,83]. Depletion of maltose and maltotriose caused starvation of *Saccharomyces* species and was followed by the acidification phase [18]. In late stage of maturation, oxidative species such as *Dekkera* spp., *B. bruxellensis*, and *P. membranifaciens* should dominate the mycobiota as they possess intra- and extracellular α -glucosidases responsible for maltooligosaccharide degradation. The sour-matured production style considered here entails the inoculation with *S. cerevisiae* commercial starters to obtain a base beer that is then submitted to the maturation step in wood barrels. This method shortens the time of brewing and increases predictability of the process compared to traditional lambic beer process, without forgoing the crucial role of the wooden barrels as an additional microbial inoculation source. To the best of our knowledge, no studies have characterized the cultivable yeast fraction from microbrewery plants based on a sour-matured production style process. Unlike the sequential succession of yeast species documented in traditional lambic beer process, in this study *S. cerevisiae* dominated the cultivable mycobiota at the maturation stage in barrels, followed by *P. membranifaciens*, *B. anomalus*, and *B. bruxellensis*. These yeast species co-exist with LAB species *P. damnosus*. Using ITS1 metabarcoding approach, Shayevitz et al. [84] also demonstrated that *S. cerevisiae* is highly abundant in aged sour ale beer samples. As *S. cerevisiae* is frequently isolated from oak wood [85], it is reasonable that this species is a common resident in wood barrels used for beer souring. Furthermore, *S. cerevisiae* wild strains were genetically distinct from commercial cultures used for base beer fermentation, supporting that the strains are feral yeasts inhabiting the craft brewery plan. Further investigations on domestication signatures in the genome could elucidate whether they are truly wild strains or brewing strains that have evaded the domestication niche and were able to colonize natural environments [86].

Another relevant question is how these *S. cerevisiae* strains can survive in depletion of maltose and maltotriose. During co-fermentation experiments, Coehlo et al. [87] reported that both *S. cerevisiae* and *B. bruxellensis* partially consume ethanol when glucose was depleted, according to the make-accumulate-consume strategy [88]. However, *B. bruxellensis* can outperform *S. cerevisiae* in ethanol consumption, the latter being able to metabolize a wider range of carbon sources and turn to ethanol as a last resort. All our strains possess *STA1* gene under the control of a promoter slightly longer than that found in “diastatic” reference strain 3711. This gene encodes an extra-cellular glucoamylase (EC 3.2.1.3) which cleaves α -1,4-glycosidic bonds from the non-reducing ends of soluble maltodextrin chains releasing glucose, subsequently fermented. Deletion of *STA1* gene reduced maltotriose consumption in *STA1*-positive beer strains belonging to lineage Beer 2 [50,76], suggesting that *Sta1p* contributes to maltotriose consumption in these strains. The partial degradation of dextrin detected during microscale wort fermentation support the “diastatic” nature of our *S. cerevisiae* strains. Although we did not investigate the *STA1* gene expression, we can hypothesize that the ability to survive during beer aging can be related to a “diastatic” phenotype. “Diastatic” is generally considered a negative trait in brewery as being responsible for over-attenuation and beer quality deterioration. Furthermore, “diastatic” strains generally have functional *PAD1* and *FDC1* genes responsible for the decarboxylation of ferulic acid into 4-vinyl guaiacol, a glove-like aroma [89]. However, in some kinds of craft beers, such as sour and Saison-style beers, 4-vinyl guaiacol and other phenolic derivative molecules, such as 4-ethyl phenol, contribute to the ‘funky’ character of these beers by imparting spicy and barnyard flavor note [81]. We did not specifically investigate POF (phenolic off-flavor) phenotype, but wort fermentation with *S. cerevisiae* strains did not reveal 4-vinyl guaiacol while highlighted the presence of phenylethyl alcohol which contributes to fruity-like aroma. These data indicate that these strains could contribute to formation of desirable aroma-active esters.

The intermediate sporulation efficiency associated to high viability of the spores supported that the novel isolates, as well as their monosporic derivatives, could be exploitable in synthetic hybrid construction. Interestingly, we isolated three strains positive to both *S. cerevisiae* and *S. uvarum*-specific PCR assays targeting housekeeping genes. Both ITS-RFLP analysis and D1/D2 26S sequencing indicated that these strains only retained the *S. uvarum* rDNA array. These results suggested that these strains could be *S. cerevisiae* x *S. uvarum* hybrids. According to this hypothesis, no sporulation was observed under the conditions used in this study, suggesting that either pre-

zygotic barriers or polyploidy/aneuploidy prevent meiosis in these strains. *S. cerevisiae* × *S. eubayanus* and *S. cerevisiae* × *S. kudriavzevii* interspecies hybrids were frequently documented in craft brewing. *S. pasteurianus* was one of the most abundant species at the end of alcoholic fermentation phase in traditional lambic sour beer production [17,55], while many Trappist-style beers from Belgium are brewed with hybrids of *S. cerevisiae* × *S. kudriavzevii* [90]. This might be a consequence of winter season temperature which favors yeasts that are more cryotolerant, such as *S. kudriavzevii* and *S. pasteurianus*. *S. cerevisiae* × *S. uvarum* hybrids were documented in brewing less frequently than *S. pasteurianus* and *S. cerevisiae* × *S. kudriavzevii* interspecies hybrids. Recently, a *S. cerevisiae* × *S. uvarum* hybrid, the so-called Muri isolate, was characterized from a “kveik” culture, a traditional Norwegian farmhouse brewing yeast culture [91,92]. Dextrin consumption in microscale wort fermentation suggested that, like Muri isolate, WY213 could be *STA1*-positive.

Pichia membranifaciens is described as a typical yeast species colonizing sour beer in late maturation step mainly due to this ability to adhere wooden surface [62]. As having a non-fermentative metabolism, it is generally considered a beer contaminant and poorly attractive as brewing cultures. Here the *P. membranifaciens* strain WY122 consumed maltose and exhibited an intermediate phenotype between non-*S. cerevisiae* species, such as the conspecific strain WY102, *B. anomalus* and *B. bruxellensis*, and the *Saccaromyces* strains. To the best of our knowledge only a *P. membranifaciens* strain from Chinese steamed bread was reported to use maltose as carbon source [93]. Genomic characterization of strain WY122 will contribute to better elucidate its taxonomic position.

5. Conclusions

During aging phase of sour-matured beer production style in a microbrewery, *STA1*-positive *S. cerevisiae* feral strains dominate the cultivable mycobiota fraction. Sour beer at the maturation stage can serve as reservoir of novel yeast cultures with interesting brewing attributes. A variety of yeast strains, including *S. cerevisiae* isolates, *S. cerevisiae* × *S. uvarum* putative hybrids, and *P. membranifaciens* WY122, possess industrially desirable phenotypic traits making them attractive candidates for both monocultures and cocultures exploitation in craft beer production.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Figure S1: Analysis of ITS region in *D. anomala* isolates. (A) Electrophoretic gel of ITS amplicons. *D. bruxellensis* strain WY60 was used as reference strain. (B) Phylogenetic tree obtained by neighbor joining (NJ) method [1] applied to a dataset of 7 rDNA sequences. The evolutionary distances were calculated by the Tamura 3-parameter method [2] considering the number of nucleotide substitutions per site. The gamma distribution was used to model the rate of change between sites. All positions containing gaps and missing data were eliminated (complete deletion option). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test [3] (1000 replicates) are shown next to the branches. The strain collected in this study is shown in bold. The lengths of the branches are proportional to the number of nucleotide substitutions, and they have been measured using the divergence scale shown at the top left. The tree was rooted using *Yarrowia lipolytica* as outgroup. The tree data (Newick) were generated with MegaX [4] and exported and visualized using ITOL [5]. Abbreviation: M: molecular size marker; Bb: *Brettanomyces bruxellensis*; Figure S2: Dendrograms generated using inter-delta PCR (A) and R3-RAPD PCR (B) fingerprints of 11 *S. cerevisiae* strains (7 indigenous wild strains isolated in this study from sour beer, 3 commercial starter cultures commonly used in the brewery plant, and BY4743 as reference strain). Commercial starters (in red) were detailed in Table 1. Similarity percentages were calculated using Pearson correlation coefficient, while hierarchical clustering analysis was carried out using the UPGMA (unweighted pair-group method with arithmetic mean) method with Bionumerics software. Numbers near the branches represent branch lengths. The tree data (Newick) were generated with MegaX [4] and exported and visualized using ITOL [5]; Figure S3: Glucose and maltose fermentation test. Tubes containing Durham inverted tubes were photographed after 3 days of incubation at 27°C. Tests were carried out according to Kurtzamn et al. [6]. Abbreviations: G, glucose; M, maltose; Figure S4: Mating type genotyping of four *S. cerevisiae* sour

beer wild strains and their monosporic derivatives. For each strain at least 8 meiotic events (asci) were dissected; Figure S5. Distribution of the 53 volatile organic compounds (VOCs) identified in the headspace of the fermented wort samples. The area of each VOC was mean centered and normalized by the standard deviation. Values are reported as colors ranging from the lowest (grey) to the highest (deepest red); Table S1: Primers, compound concentrations, and thermal conditions used this study. Table S2. *In silico* 16S-ARDRA profiles of the main beer LAB species. Table S3. ITS RFLP-PCR analysis of yeast isolates from natural sour beer.

Author Contributions: For research articles with several authors, a short paragraph specifying their individual contributions must be provided. The following statements should be used “Conceptualization, L.S. (Lisa Solieri); methodology, L.S. (Lisa Solieri); formal analysis, L.S. (Lisa Solieri), F.C., and S.R.; investigation, C.N., L.S. (Laura Sola), and J.R.; resource, M.D.B.; data curation, L.S. (Lisa Solieri) and S.R.; writing—original draft preparation, L.S. (Lisa Solieri); writing—review and editing, L.S. (Lisa Solieri), C.N., J.R., L.S. (Laura Sola), S.R., and F.C.; visualization, L.S. (Lisa Solieri) and L.S. (Laura Sola); supervision: L.S. (Lisa Solieri); funding acquisition, L.S. (Lisa Solieri). All authors have read and agreed to the published version of the manuscript.

Funding: L.S. (Lisa Solieri) acknowledge the support of the NBFC to the University of Modena and Reggio Emilia, funded by the Italian Ministry of University and Research, PNRR, Missione 4 Componente 2, “Dalla ricerca all’impresa”, Investimento 1.4, Project CN00000033.

Data Availability Statement: The sequence data generated in this study have been submitted to NCBI database under accession numbers OM618137 to OM618146 and OM618118 to OM618120.

Conflicts of Interest: M.D.B. was employed by Ca’ Del Brado Brewery. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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