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Profiling potential brewing yeast from forest and vineyard ecosystems

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ABSTRACT

The brewing ability of wild yeast strains obtained from forest and vineyards ecosystems was analysed and compared with commercial yeast strains. The selection of new yeast strains as a way to create new beer aromas and flavours and to use local strains to promote the proximity ingredients in brewing is a topic of interest in the craft beer sector. Seventy-six wild *Saccharomyces* and non-*Saccharomyces* isolates and eighteen control strains were evaluated for their enzymatic activity and brewing capacity. The early screening system was set up to profile their enzymatic activity, utilisation of wort sugars and the effect of hop acids and ethanol on yeast growth. The microvolume screening method allows a large number of samples to be studied at the same time, permitting an affordable and rapid characterization in a relatively short period of time. Twenty-eight strains were selected using this method and tested in small-scale fermentations. Finally, three of these strains, all belonging to the species *Lachancea thermotolerans*, showed great potential and adaptability to ferment different wort styles, although further studies will be necessary to test their possibilities as beer starters. Understanding yeast enzymatic profiles and the influence of beer ingredients on their fermentation activity provides a platform to select strains for further consideration in brewing research.

1. Introduction

The systematisation of the brewing process due to the use of a genetically reduced set of commercial yeast strains has limited the diversity of beers. *Saccharomyces cerevisiae* (ale yeast) and *Saccharomyces pastorianus* (lager yeast) have dominated the brewing industries (Walther et al., 2014). However, in recent years, non-*Saccharomyces* yeasts have attracted more attention from homebrewers, craft brewers and industry, looking for innovative ingredients, aromas and flavours. Non-*Saccharomyces* yeasts have great potential in the development of beers with different alcohol contents and a broad range of brewing properties such as *Meyerozyma guilliermondii*, *Pichia membranefaciens*, *Candida friedrichii*, *Naumovia castellii*, *Dekkera anomala*, *Priceomyces* spp., *Cryptococcus keutzingii*, *Rhodotorula mucilaginosa*, *Candida krusei*, *Pichia fermentans*, *Pichia opuntiae*, *Metschnikowia pulcherrima*, *Zygosaccharomyces bailii*, *Torulaspora delbrueckii* in spontaneous fermentation (Bokulich et al., 2012; Michel et al., 2016; Postigo et al., 2022; Spitaels et al., 2014).

In addition, the ability to identify and propagate isolated native (or indigenous) yeasts has an additional marketing angle, the potential to provide a differentiated product from the competition, and a beer elaborated completely from local ingredients that creates a strong identity that favours and allows its insertion in the market. The brewing market is going through a period of change led by consumer demand for product diversity (Kellershohn and Russell, 2015). Increasing requests for traditional beer styles, alternative flavours and low-alcohol beers has encouraged research on the potential benefits of alternative yeasts (Gibson et al., 2017; Postigo et al., 2022; Saerens and Swiegers, 2014).

Previous projects related to the isolation and characterization of wild brewer's yeasts were mainly addressed in fruit due to the sugar content (Daenen et al., 2008; Lee et al., 2011; Lentz et al., 2014). However, several investigations have shown that tree bark, especially from oak species, is a natural reservoir for *Saccharomyces* species (Kowallik and Greig, 2016; Nikulin et al., 2020; Sampaio and Gonçalves, 2008; Wang et al., 2012). Furthermore, yeast isolates are found in diverse habitats, namely in oak exudates (Naumov et al., 1998; Sniegowski et al., 2002), insect gut (Suh et al., 2005), plant leaves and grape berries (Martini et al., 1996). Because of the potential of new yeasts for commercial purposes, chemical, enzymatic and genetic techniques have recently been employed to search for new yeasts in different ecosystems (Michel

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et al., 2016; Molinet and Cubillos, 2020). In fact, the lack of diversity in the yeasts used for the production of beers limits the obtaining of alternative or new products, stressing the need to explore new genetic resources to differentiate each fermentation product. In this sense, wild strains harbor a higher genetic and phenotypic diversity than previously thought, representing a feasible option to generate new fermented beverages.

Therefore, the main objective of this research was to develop an efficient selection method to isolate yeasts from environmental samples with desirable characteristics for brewing, such as ethanol and hop tolerance, and metabolism of maltose and maltotriose, which represent >80 % of the carbohydrates in beer wort (Stewart, 2009). In addition, the presence of interesting enzyme activities for brewing yeasts was also included in the screening. The most promising strains based on the results of this microplate screening were selected to evaluate their fermentative performance in small-scale processes. The properties of non-conventional yeast (wild *Saccharomyces* and non-*Saccharomyces*) were evaluated with the main aim of generating new sources of yeasts with the ability to create new flavours, flavourful non-alcoholic beers and improve brewing efficiency.

2. Materials and methods

2.1. Sample collection

Sampling was carried out in two periods. The first sampling was carried out in March 2020 in the forest ecosystems of northern Spain, in 11 locations (Arkaia, Arkaute, Deva, Eskalmendi, Landa, Izki, Munain, Murgia, Narea, Salinas de Leniz, Ullivarri Arrazua) (Supplementary Table S1). The autochthonous species of oak sampled were *Quercus faginea*, *Q. ilex*, *Q. pyrenaica*, *Q. petraea* and *Q. robur* and, as a representative species of the exotic species of *Quercus* in the plantations of northern Spain, *Q. rubra*. The second sampling was carried out in October 2020, in *Vitis vinifera* cv. 'Tempranillo' in 20 commercial vineyards located in Rioja, Spain (Supplementary Table S1).

Pieces of bark were taken from the base of the sampled tree, using a sterile knife; soil and acorn samples were collected around the sampled tree, at a distance not exceeding 1 m from the tree, with a spatula. Cross contamination was avoided by cleaning and disinfecting tools between samples with a disinfectant solution containing 76 % (*v*/*v*) ethanol. Oak samples were cut into 0.5 cm square pieces, mixed and transferred to 50 mL conical tubes. All selected samples were kept at 4 °C until processing.

Grapes samples were taken from 20 vineyards. In each vineyard, a total of 10 trees were sampled, collecting one bunch of ripe grapes per tree in a sterile bag (1 bag per vineyard), using gloves and disinfecting them between samplings with the disinfectant solution. Once in the laboratory, under aseptic conditions, the grapes of each bag were crushed and representative aliquotes of 50 mL were collected in conical tubes as a working sample.

2.2. Microorganism culturing

4 mL of stored samples were deposited in sterile 12-mL vials and filled with sterile Sniegowski enrichment medium (3 g/L yeast extract, 3 g/L malt extract, 5 g/L peptone, 10 g/L sucrose, 7.6 % (*v*/v) EtOH, 1 mg/L chloramphenicol, 0.1 % (v/v) 1 M HCl) (Sniegowski et al., 2002). Then, vials were capped tightly and incubated for 10 days at 25 °C (this period was extended up to 15 days in those samples in which no signs of microbial growth were observed) without shaking. Cultures were daily monitored by evaluation of gas and yeast biomass formation (Boynton et al., 2019; Sniegowski et al., 2002). This selective enrichment was used because we wanted to isolate yeasts, from these two natural niches (oak forests and vineyards), that were tolerant to ethanol and then study their ability for brewing.

After this incubation period, serial dilutions of the samples were prepared and 10 μL of these dilutions were plated by the spot plating technique in Wallerstein Laboratory Nutrient Agar (WLN) (Pallmann et al., 2001), using 15-mm square plates with 13-mm grids (Becton Dickinson Labware, Bedford, MA, USA). Plates were incubated for 5 days at 25 °C and the growth of yeast colonies was examined. Then, each colony type isolated on WLN medium was re-cultured on YPD-Cm agar (yeast extract 1 % (*w*/*v*), peptone 2 % (w/v), glucose 2 % (w/v), agar 2 % (w/v), chloramphenicol 10 μg/mL) to obtain isolated clones and stored in 15 % (*v*/v) glycerol at −20 °C until species identification (Rose et al., 1991; Treco et al., 2001).

2.3. Species identification

Genomic DNA from the different clones was isolated using the Thermo Scientific Yeast DNA Extraction Kit, (Jena, Germany) following the manufacturer's protocol. DNA concentrations were diluted to 20 ng/μL working stock for polymerase chain reaction (PCR) amplifications and stored at −20 °C until further use. Then, the D1/D2 domain of the 26S rDNA gene was amplified using the primers NL1 (5′-GCA TAT CAA TAA GCG GAG GAA AAG-3′) and NL4 (3′-GGT CCG TGT TTC AAG ACG G-5′) (Kurtzman and Robnett, 1998). PCR reactions contained 20 ng DNA, 2.5 μL 10× PCR reaction buffer, 2.5 mM $MgCl_2$, 400 nM of each primer, 200 μM of each dNTP and 1 U Faststart *Taq* DNA Polymerase (Roche Diagnostics, Indianapolis, IN, USA). Reaction volumes were adjusted to 25 μL with sterile PCR water (AppliChem GmbH, Darmstadt, Germany). PCR reactions were carried out on QIAamplifier 96 well Thermal cycler (QIAGEN, Madrid, Spain). The cycling conditions included an initial denaturation at 95 °C for 5 min, followed by 35 cycles at 94 °C for 2 min, 52 °C for 1 min, 72 °C for 2 min, and a final extension at 72 °C for 10 min (Dabassa et al., 2019). Amplicons were purified using the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel, Düren, Germany) and sequenced by Eurofins (Genomics, Germany). Sequences were edited and aligned using Finch TV and MEGA X software (Kumar et al., 2018). A minimum of 99 % of sequence identity with the type strain of a yeast species was required for species identification (Fell et al., 2000; Kurtzman and Robnett, 1998). DNA sequences characteristics of each species were deposited in the NCBI database (https://www.ncbi.nlm.nih.gov/).

2.4. Species diversity

The diversity of yeast species was determined in the selective enrichment medium (Sniegowski medium) to evaluate which ecosystem and sample type presented the greatest diversity of yeasts with high tolerance to ethanol. In all the samples, species richness and evenness of abundance distribution were assessed and similarity of yeast species composition between samples and hosts was also examined.

For the diversity measures, the Shannon Index (Shannon and Weaver, 1963), the Dominance Index (Simpson, 1949) and the Equitability or Evenness (Margalef, 1991) were estimated. Moreover, the Margalef index was also determined, which measures species richness and is highly sensitive to sample size (Magurran, 2004). This index is usually used in conjunction with indices such as the Shannon and Simpson (Gamito, 2010). This analysis was implemented with Past 4.03 software. Finally, rarefaction was also calculated to assess species richness from the results of sampling (Gotelli and Colwell, 2001; Walker et al., 2008).

2.5. Microplate screening of isolate technological properties

After the identification of the isolates, a first selection was made based on previous scientific publications, discarding the isolates belonging to species associated with human diseases, and those presenting cultivation or recovery problems. After that, a total of 76 wild yeast isolates (*Saccharomyces* and non-*Saccharomyces*) were tested for their brewing abilities compared to 18 control strains. Twelve commercial strains were used as positive controls (cp) and six strains isolated in brewing environments, belonging to *S. cerevisiae* var. *diastaticus* species, were included as negative controls (cn) (Table 1). This screening was performed using two miniaturised tests. In the first one, the growth in the main wort carbohydrates (glucose, fructose, sucrose, maltose, melibiose and maltotriose) and the tolerance to ethanol (5, 6, 8 and 10 % (*v*/v)) and iso-α-acid (5 and 10 IBU) were tested to evaluate the strain potential for the brewing fermentation process (Supplementary Table S2) (Methner et al., 2019). In these assays, Phenol Red Broth Base (Condalab, Spain) was used as a base medium. Since different species and strains of yeast may show different sedimentation behavior, this effect may impact on the turbidity measurement (Methner et al., 2019). To minimize this effect, this medium contains the Phenol Red dye, a pH indicator, which serves to detect the production of carbon dioxide that dissolves in the medium, forming carbonic acid, and therefore, lowering the pH of the medium (Held, 2018). The quantitative estimation was implemented by measuring the absorbance at 560 nm in an EMax® Plus spectrophotometry microplate reader (Molecular Devices, CA, USA). Based on the absorbance values of blank (medium without yeast inoculation), the absorbance limit to consider that the test is positive and therefore, there is activity is 0.4.

Miniaturised fermentation tests were carried out in triplicate, including three blank samples for each medium, in sterile polystyrene 96-well plates (Sigma-Aldrich, Germany). Isolates grown in YPD plates were resuspended in 1 mL of phosphate-buffered saline (PBS, Fisher bioreagents, Belgium) and a final concentration of 500 cells/μL was inoculated in each well (Methner et al., 2019). Microtiter plates were then sealed with a cover sheet SealPlate® 100-SEAL-PLT (Excel Scientific, Victorville, CA, USA) and incubated at 27 °C for 5 days (Kali et al., 2015). Before each measurement, the plate was automatically shaken for 30 s.

In the second miniaturised test, a broad enzymatic profile of the isolates was determined using an API ZYM system (BioMérieux, Marcy l'Etoile, France) in duplicate, according to the manufacturer's guidelines. This system allows a semi-quantitative analysis of 19 enzymatic reactions (Supplementary Table S3). Yeast suspension and inoculation were implemented following the protocols described in the previous miniaturised assay. Enzyme activity was graded from 0 to 5 by comparing the colour obtained in the samples with the API-ZYM colour reaction chart, being 0, no activity, 1, low intensity, 2–3, moderate intensity and 4–5, high intensity (Tiquia-Arashiro, 2002). Enzyme substrate reactions were consistent upon two replicates of the test per studied yeast isolates.

Principal component analysis (PCA) was applied to discriminate yeast strains according to their desirable and undesirable technological properties based on their proximity to control strains included in the assays and to group isolates that have similar patterns of sugar utilisation, tolerance to ethanol and isoα-acids and enzyme activities. To do that, the online ClustVis tools (http://biit.cs.ut.ee/clustvis; accessed on 05 March 2022) were used. Heat maps and dendrograms were depicted to retain the most contrasting patterns, Euclidean distance measures and Ward's clustering algorithm were used for rows (isolates) and columns (studied substrates) (Golub and Van Loan, 1996; Metsalu and Vilo, 2015).

2.6. *Small-scale brewing fermentations*

Twenty-eight wild yeasts were selected for the fermentation tests based on the results obtained in the previous sections and their proximity (Euclidean distance) to control strains. The preparation of the inoculum was performed from fresh colonies cultured in 10-mL tubes of YPD Broth in a shaking incubator at 350 rpm for 48 h at 25 °C.

The hopped worts were prepared using canned-hopped malt extract purchased from DIY Beer Coopers (Regency Park, South Australia, Australia) or BrewFerm (Belgium) according to the manufacturer's instructions and autoclaved for 10 min at 105 °C to avoid Maillard reactions between carbohydrates and amino-compounds (Thesseling et al., 2019). Fermentations were carried out in duplicate in 250-mL Erlenmeyer flasks with 150 mL of wort at 20 °C without stirring, each

Table 1
Control strains used in this study.

Yeast type	Sample ID	Commercial name (company)	Yeast species	Control type	Assay[a]
Top fermenting_ale yeasts	vlb1	OK3 (VLB)	*Saccharomyces cerevisiae*	Positive controls	Mp
	vlb9	68obg (VLB)			Mp
	vlb10	Nr94 (VLB)			Mp
	CP1	SafAle™ BE-256 (Fermentis)			Fe
	CP2	SafAle™ T-58 (Fermentis)			Fe
	CP3	Mauribrew Weiss (Mauri)			Fe
	CP4	Coopers yeast (Diybeer)			Fe
	CP5	SafAle™ S-04 (Fermentis)			Fe
	CP6	SafAle™ US-05 (Fermentis)			Fe
Bottom fermenting_lager yeasts	vlb3	He-Bru (VLB)	*Saccharomyces pastorianus*		Mp
	vlb4	1901 (VLB)			Mp
	vlb7	Nr221 (VLB)			Mp
	S-189	S-189 (Fermentis)			Fe
Yeasts for low alcohol beer	vlb2	Srosei (VLB)	*Saccharomyces rosei*		Mp
	vlb5	Sdairensis (VLB)	*Saccharomyces dairensis*		Mp
	vlb6	Sludwigii (VLB)	*Saccharomyces ludwigii*		Mp
Special yeasts	vlb8	ET1/6 (VLB)	*S. cerevisiae var. diastaticus*		Mp
	daire	DAir (VLB)	*S. dairensis*		Mp, Fe
	Levan	Frest yeast (Levanoba)	*S. cerevisiae*		Mp
	dias1	–[b]	*S. cerevisiae var. diastaticus*	Negative controls	Mp, Fe
	dias2				Mp, Fe
	dias3				Mp, Fe
	dias4				Mp, Fe
	dias5				Mp, Fe
	vlbdias	Sdiastaticus (VLB)			Mp

VLB: Versuchs- und Lehranstalt für Brauerei in Berlin (Germany); Fermentis: Lassafre, Lille (France); Levanoba: Lessafre Ibérica, Valladolid (Spain); Diybeer: Diybeer Coopers, Adelaide (Australia); Mauri: AB Mauri, Toowoomba (Australia).

[a] Mp: microplate assays; Fe: fermentation assays.

[b] Non-commercial strains, isolated from brewery environment.

with the rubber stoppers perforated with an airlock, filled with 15 mL of a pH indicator buffer instead of water, and monitored daily for 7 days. The pitch rate was 1×10^6 viable cells mL^{-1}. This pH indicator solution in the airlock allows visual detection of CO_2 dilution, showing a wide colour variation from pH 9 (blue) to pH 4 (red) (Supplementary Fig. S1). This pH indicator was prepared by mixing 100 mg of phenolphthalein, 50 mg of bromothymol blue, 12.5 mg of methyl red and 5 mg of thymol blue in 100 mL of ethanol and 100 mL of water, and then 52.63 mL of this solution was made up to 1 L with a standard pH buffer solution (pH 9.2)(Sugai et al., 2015).

In the first test, a low-density beer wort (density 1025 g/L and pH 5.67) prepared with a European Lager (Coopers DIY) was fermented with the 28 wild isolates and 13 control strains (Table 1) to evaluate their brewing capacity. The second test was carried out with a set of six isolates, selected from the first small-scale fermentation, in 3 different beer worts to evaluate their brewability under different osmotic and ethanol conditions: (i) European Lager (Coopers), with a density of 1052.6 g/L and pH 5.43, (ii) English IPA (India Pale Ale, BrewFerm), with a density of 1074.2 g/L and pH 5.47 and (iii) Belgian Dubbel (BrewFerm), with a density of 1103.8 g/L and pH 5.39.

Small-scale wort fermentations were monitored by density, weight loss and pH variation. Weight loss and colour change in the airlock were monitored daily. The pH, density and the refraction index of the wort were measured at the beginning and at the end of the brewing process with a portable pH meter (Hanna HI-9125, Eibar, Spain), a portable density meter (Densito 30PX Portable Density Meter, Barcelona, Spain) and a refractometer (Hanna HI 96816), respectively.

3. Results

3.1. Yeast isolation using a selective enrichment medium

In this study, 134 yeast isolates were obtained from 203 samples of tree barks, leaves, acorns, rhizosphere soil and grapes, using a selective enrichment medium as a tool to obtain brewer's yeast candidates (Table 2). Among the isolates, 20 (14.9 %), 30 (22.4 %), 29 (21.6 %), 33 (24.6 %) and 22 (16.4 %) were isolated from grapes, leaves, acorns, rhizosphere soil and bark, respectively (Fig. 1a).

Taking into account the host species from which they have been isolated, 48 (35.8 %), 6 (4.5 %), 24 (17.9 %), 10 (7.5 %), 13 (9.7 %), 13 (9.7 %) and 20 (14.9 %) were isolated from *Q. robur*, *Q. rubra*, *Q. faginea*, *Q. ilex*, *Q. petraea*, *Q. pirenaica* and *V. vinifera*, respectively (Fig. 1b).

3.2. Species identification and diversity

After isolation in this selective enrichment medium containing a high percentage of ethanol (7.6 % *v*/v), 19 genera and 35 species were identified, according to the sequence analysis of the 26S rDNA D1/D2 domain (Table 2). The species *Saccharomyces paradoxus* (32), *Kluyveromyces dobzhanskii* (22), *Lachancea thermotolerans* (18) and *Torulaspora delbrueckii* (12) were the most frequently isolated yeasts. *L. thermotolerans* was the only yeast species associated with all sample sources (including barks, acorns, leaves, rhizosphere soils and grapes) and with all the ecosystems, except *Q. petraea* (Fig. 2 and Table 2). *S. paradoxus* and *K. dobzhanskii* were the species most frequently detected and only isolated in *Quercus* species, from all kind of samples (bark, soil, acorn and leaf samples), while *L. thermotolerans* and *T. delbrueckii* were the most isolated species in vineyard ecosystems. Moreover, *Saccharomyces cerevisiae*, *Naganishia albida*, *Starmerella bacillaris*, *Hanseniaspora uvarum*, *Hyphopichia pseudoburtonii* and *Metschnikowia pulcherrima* were isolated exclusively in samples from *V. vinifera* (grapes), with *L. thermotolerans* the species most frequently isolated (Fig. 2 and Table 2).

Q. robur, *Q. faginea*, *V. vinifera* and *Q ilex* were the host species with the highest number of isolates and taxa, resulting in the niches with the highest Simpson, Shannon and Margalef indices and consequently with the lowest dominance and evenness (Table 3). On the other hand, *Q. pyrenaica*, *Q. rubra* and *Q. petraea* were the ecosystems in which the isolates obtained were less diverse. In relation to the sample type, the bark and the leaves were the samples that presented the highest and the lowest diversity of isolates, respectively. The data was confirmed by the rarefaction curves (Supplementary Fig. S2).

To visually represent the similarities and differences between the studied ecosystems at the species composition level, Venn diagrams were constructed (Supplementary Fig. S3). All ecosystems shared some species but also exhibited some exclusive species. However, there were two exceptions, *Q. rubra* and *Q. petraea*, which shared all the species with the rest of the hosts, and *Candida norvegica*, which was exclusively isolated in *Q. pyrenaica*. Moreover, *Q. robur* was the ecosystem with the highest number of species shared with the rest of the ecosystems, and together with *Q. farigea*, also the one with the highest number of exclusive species (Supplementary Fig. S3a). On the other hand, all the samples types presented specific species but, at the same time, they shared some with the other types of samples, being grapes the sample type with the highest number of exclusive species (Supplementary Fig. S3b).

3.3. Microplate screening of isolate technological properties

After the identification of the isolates and the first selection made according to previous information and healthiness (those presenting cultivation or recovery problems) of the cultures, 76 isolates were tested for their brewing abilities together with 18 control strains.

The ability of the different isolates to grow in different wort sugars and their tolerance to ethanol and hops compared to control strains was analysed by measuring maximal OD in all these conditions (Supplementary Table S4). Similarities between these wild strains and the controls (positive and negative controls) based on these features were represented in Fig. 3 and Supplementary Fig. S4a. The growth of most tested yeasts was little affected by isoα-acid concentrations (IBU 50 and 100) or 5 % (*v*/v) of ethanol (Supplementary Fig. S5a). In fact, the IBU50 condition completely inhibited the growth (maximal OD < 0.4) of only one isolate among the 94 screened yeasts and it was a control strain, specifically vlb6. Consequently, all yeast isolates can tolerate the typical level of ethanol and isoα-acids found in standard beer, although growth was limited in some of them. The percentage of yeast strains whose growth was inhibited by the presence of ethanol in the medium increased when higher concentrations of this compound were applied, from 0, 6, 8 and 11 isolates in 5, 6, 8 and 10 % (v/v) of ethanol, respectively. However, there were some exceptions, such as isolate 6g, whose growth seemed to be more affected at 5 % than at 6 % of ethanol (Supplementary Table S4). In relation to the use of different wort carbohydrates, the majority of the selected yeasts (>82 %) were able to grow in the presence of all the tested carbohydrates (melibiose, glucose, fructose, sucrose, maltose and maltotriose), but the activity average of fructose, glucose and sucrose was almost double the activity shown by the isolates in the presence of maltose, melibiose and maltotriose. However, a group of strains, which clustered together in the heatmap, showed a good ability to use maltose and maltotriose. These strains were 129q and 115q of *C. tropicalis*, 116q and 101q of *Meyerozyma caribbica* and 119q of *S. paradoxus* (Fig. 3 and Supplementary Table S4). This group of yeast, according to the growth on these sugars, behaved in a similar way to the controls classified as special yeasts (Table 1). Moreover, these strains are the ones that grew best in all the sugars tested. On the other hand, 5g (*Naganishia albida*), 6g (*Hyphopichia pseudoburtonii*), 8g (*Lachancea thermotolerans*), 113q (*Zygotorulaspora florentina*), 10g and 4g (*Starmerella bacillaris*) and 1381q (*Pichia kudriavzevii*) are the isolates that presented the lowest growth on the different tested carbohydrates. In general, a remarkably varied behavior against these traits was observed both between species and between wild and commercial yeasts. Among the control strains, three clusters were observed, lager yeast, ale yeast and special yeast (Fig. 3), although this separation was

Table 2
Isolates identification at species level.

Species identification	ID Code	Location	Sample	Host	Species identification	ID Code	Location	Sample	Host
C. baotianensis	152q	Munain	Acorn	*Q. faginea*	*Ph. inflatum*	120q	Eskalmendi	Soil	*Q. faginea*
C. molendinolei	30q	U. Arrazua	Acorn	*Q. faginea*	*P. fermentans*	121q	Eskalmendi	Leave	*Q. faginea*
C. norvegica	164q	Izki	Acorn	*Q. pyrenaica*		117q	Eskalmendi	Bark	*Q. ilex*
	157q	Izki	Soil	*Q. pyrenaica*	*P. kudriavzevii*	142q	Eskalmendi	Bark	*Q. ilex*
C. parapsilosis	13q	Deva	Acorn	*Q. robur*		138q	Eskalmendi	Acorn	*Q. robur*
C. subhashii	102q	Eskalmendi	Soil	*Q. robur*		146q	Eskalmendi	Soil	*Q. robur*
C. tropicalis	129q	Eskalmendi	Soil	*Q. ilex*		107q	Eskalmendi	Bark	*Q. ilex*
	115q	Eskalmendi	Bark	*Q. robur*		7g	Rioja	Grapes	*V. vinifera*
C. tsuchiyae	147q	Munain	Bark	*Q. faginea*	*P. manshurica*	139q	Eskalmendi	Soil	*Q. petraea*
H. osmophila	47q	Izarra	Bark	*Q. robur*		137q	Eskalmendi	Acorn	*Q. robur*
	66q	Landa	Soil	*Q. robur*		136q	Eskalmendi	Soil	*Q. robur*
H. uvarum	11g	Rioja	Grapes	*V. vinifera*	*P. membranifaciens*	148q	Munain	Leave	*Q. faginea*
Hy. pseudoburtonii	6g	Rioja	Grapes	*V. vinifera*	*S. cariocanus*	56q	Izarra	Bark	*Q. robur*
Ka. Servazzii	17q	U. Arrazua	Acorn	*Q. faginea*	*S. cerevisiae*	20g	Rioja	Grapes	*V. vinifera*
	16q	U. Arrazua	Leave	*Q. faginea*	*S. jurei*	156q	Izki	Soil	*Q. pyrenaica*
	48q	Izarra	Acorn	*Q. robur*		12q	Deva	Acorn	*Q. robur*
	5q	Arkaute	Bark	*Q. robur*	*S. paradoxus*	27q	U. Arrazua	Bark	*Q. faginea*
	39q	Murgia	Soil	*Q. robur*		28q	U. Arrazua	Soil	*Q. faginea*
K. dobzhanskii	161q	Munain	Soil	*Q. faginea*		119q	Eskalmendi	Soil	*Q. ilex*
	109q	Eskalmendi	Acorn	*Q. ilex*		83q	S. de Leniz	Leave	*Q. petraea*
	87q	S. de Leniz	Acorn	*Q. petraea*		96q	Eskalmendi	Soil	*Q. petraea*
	78q	S. de Leniz	Leave	*Q. petraea*		166q	Izki	Soil	*Q. pyrenaica*
	88q	S. de Leniz	Leave	*Q. petraea*		40q	Murgia	Leave	*Q. robur*
	77q	S. de Leniz	Soil	*Q. petraea*		46q	Murgia	Soil	*Q. robur*
	150q	Izki	Acorn	*Q. pyrenaica*		26q	U. Arrazua	Acorn	*Q. faginea*
	159q	Izki	Acorn	*Q. pyrenaica*		20q	U. Arrazua	Leave	*Q. faginea*
	151q	Izki	Bark	*Q. pyrenaica*		29q	U. Arrazua	Leave	*Q. faginea*
	165q	Izki	Leave	*Q. pyrenaica*		21q	U. Arrazua	Soil	*Q. faginea*
	71q	Landa	Acorn	*Q. robur*		25q	U. Arrazua	Soil	*Q. faginea*
	72q	Landa	Bark	*Q. robur*		76q	S. de Leniz	Acorn	*Q. petraea*
	67q	Landa	Leave	*Q. robur*		86q	S. de Leniz	Bark	*Q. petraea*
	179q	Narea	Leave	*Q. rubra*		84q	S. de Leniz	Soil	*Q. petraea*
	168q	Izki	Leave	*Q. pyrenaica*		84q	S. de Leniz	Soil	*Q. petraea*
	45q	Murgia	Acorn	*Q. robur*		90q	S. de Leniz	Soil	*Q. petraea*
	63q	Landa	Acorn	*Q. robur*		158q	Izki	Leave	*Q. pyrenaica*
	69q	Landa	Acorn	*Q. robur*		34q	Murgia	Acorn	*Q. robur*
	58q	Izarra	Leave	*Q. robur*		59q	Landa	Acorn	*Q. robur*
	33q	Murgia	Soil	*Q. robur*		43q	Murgia	Bark	*Q. robur*
	178q	Narea	Acorn	*Q. rubra*		60q	Landa	Bark	*Q. robur*
	172q	Narea	Leave	*Q. rubra*		70q	Landa	Bark	*Q. robur*
K. marxianus	23q	U. Arrazua	Bark	*Q. faginea*		36q	Murgia	Leave	*Q. robur*
L. thermotolerans	162q	Munain	Acorn	*Q. faginea*		49q	Izarra	Leave	*Q. robur*
	104q	Eskalmendi	Leave	*Q. faginea*		53q	Izarra	Leave	*Q. robur*
	118q	Eskalmendi	Acorn	*Q. robur*		62q	Landa	Leave	*Q. robur*
	51q	Izarra	Soil	*Q. robur*		50q	Izarra	Soil	*Q. robur*
	182q	Narea	Bark	*Q. rubra*		68q	Landa	Soil	*Q. robur*
	8g	Rioja	Grapes	*V. vinifera*		2q	Arkaute	Soil	*Q. robur*
	160q	Munain	Soil	*Q. faginea*		169q	Narea	Soil	*Q. rubra*
	103q	Eskalmendi	Acorn	*Q. ilex*	*Saturnispora sp.*	73q	Landa	Soil	*Q. robur*
	163q	Izki	Bark	*Q. pyrenaica*	*St. bacillaris*	4g	Rioja	Grapes	*V. vinifera*
	153q	Izki	Leave	*Q. pyrenaica*		10g	Rioja	Grapes	*V. vinifera*
	170q	Izki	Soil	*Q. pyrenaica*	*T. delbrueckii*	22q	U. Arrazua	Bark	*Q. faginea*
	42q	Murgia	Acorn	*Q. robur*		79q	S. de Leniz	Leave	*Q. petraea*
	12g	Rioja	Grapes	*V. vinifera*		31q	Murgia	Bark	*Q. robur*
	13g	Rioja	Grapes	*V. vinifera*		44q	Murgia	Leave	*Q. robur*
	14g	Rioja	Grapes	*V. vinifera*		74q	Landa	Leave	*Q. robur*
	15g	Rioja	Grapes	*V. vinifera*		181q	Narea	Leave	*Q. rubra*
	16g	Rioja	Grapes	*V. vinifera*		24q	U. Arrazua	Leave	*Q. faginea*
	19g	Rioja	Grapes	*V. vinifera*		24xq	U. Arrazua	Leave	*Q. faginea*
M. fructicola	14q	Deva	Acorn	*Q. robur*		41q	Murgia	Bark	*Q. robur*
	18g	Rioja	Grapes	*V. vinifera*		2g	Rioja	Grapes	*V. vinifera*
M. pulcherrima	9g	Rioja	Grapes	*V. vinifera*		3g	Rioja	Grapes	*V. vinifera*

(*continued on next page*)

Table 2 (*continued*)

Species identification	ID Code	Location	Sample	Host	Species identification	ID Code	Location	Sample	Host
M. sinensis	134q	Eskalmendi	Soil	*Q. ilex*		17g	Rioja	Grapes	*V. vinifera*
Me. Caribbica	101q	Eskalmendi	Leave	*Q. ilex*	*Tr. vanleenenianus*	145q	Eskalmendi	Soil	*Q. robur*
	116q	Eskalmendi	Bark	*Q. ilex*	*W. anomalus*	18q	Deva	Acorn	*Q. robur*
N. albida	5g	Rioja	Grapes	*V. vinifera*		1g	Rioja	Grapes	*V. vinifera*
O. dorogensis	1q	Arkaute	Leave	*Q. robur*	*Z. florentina*	113q	Eskalmendi	Acorn	*Q. faginea*

Q.: *Quercus*; *V.*: *Vitis*; *C.*: *Candida*; *H.*: *Hanseniaspora*; *Hy.*: *Hyphopichia*; *Ka.*: *Kazachstania*; *K.*: *Kluyveromyces*; *L.*: *Lachancea*; *M.*: *Metschnikowia*; *Me.*: *Meyerozyma*; *N.*: *Naganishia*; *O.*: *Ogataea*; *Ph.*: *Phialemonium*; *P.*: *Pichia*; *S.*: *Saccharomyces*; *St.*: *Starmerella*; *T.*: *Torulaspora*; *Tr.*: *Trichomonascus*; *W.*: *Wickerhamomyces*; *Z.*: *Zygotorulaspora.*

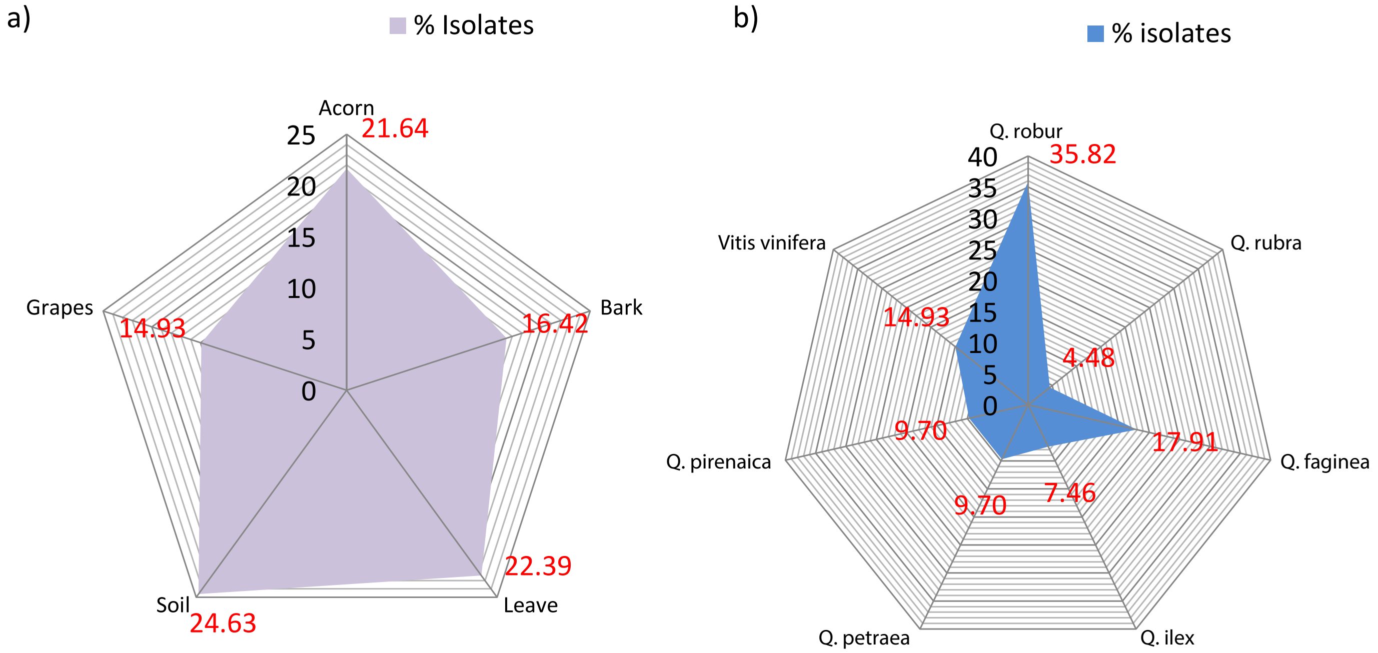


Fig. 1. Frequency of yeast isolation among the sample types (a) and the host species (b).

not so clear in the PCA, where control and wild isolates were mixed (Supplementary Fig. S4a).

Subsequently, the presence of different enzyme activities using an API ZYM system were investigated in the same isolates to deepen in their potential brewing abilities (Fig. 4 and Supplementary Figs. S4b and S5b and Supplementary Table S5). Leucine arylamidase, acid phosphatase, valine arylamidase and naphthol-AS-BI-phosphohydrolase activities were detected in all strains, except for the acid phosphatase in controls vlb5 and dias1 and in valine arylamidase for control vlbdias. Leucine arylamidase was the enzyme with the highest activity, practically all strains exhibiting a high intensity (4 or 5), followed by the acid phosphatase and valine arylamidase. On the other hand, alkaline phosphatase, esterase and esterase lipase activities were frequently detected, although in the case of the esterase and esterase lipase, the activity was remarkably low in commercial and wild yeast, with the exception of some isolates obtained from grapes, especially 8g (*L. thermotolerans*) and 1g (*Wickerhamomyces anomalus*). Activity of α-glucosidase was only detected in half of the strains, but in general, exhibited a high intensity. This activity was present in the 92 % of *L. thermotolerans* isolates, being in 77 %, moreover, with the highest intensity (5), and in half of *S. paradoxus* isolates. On the other hand, no isolate belonging to *Pichia* genus displayed this activity. Finally, β-galactosidase, β-glucuronidase, trypsin, α-galactosidase and *N*-acetyl-β-glucosaminidase activities were present in 3 or less isolates, while α-fucosidase and α-chymotrypsin were totally absent in the studied isolates.

There was more heterogeneity between the control strains according to their enzymatic profile, which was reflected in the heatmaps and PCAs, since in this case, unlike the previous heatmap, the control strains did not cluster, but were scattered and mixed with the wild isolates (Fig. 4), which was corroborated by the PCA (Supplementary Fig. S4b). No clusters based on the origin (wild vs. commercial) were formed, evidencing the wide diversity of enzymatic profiles in both groups.

In fact, most of these enzymatic activities were isolate-dependent, this is to say, there was a great diversity in the results obtained in these activities tests at the level of wild strains but also between the control strains. Some strains were grouped due to a specific activity, such as the cluster composed of 18q, 23q, 5g and 83q isolates, because of the high activity of β-glucosidase, or the cluster composed of 21q, 107q, 117q, 1171q, 121q due to the high activity of acid phosphatase and naphthol-AS-BI-phosphohydrolase.

3.4. Small-scale brewing fermentations

Based on previous results and isolates proximity to commercial brewing yeasts included as reference controls (calculated by Euclidean distance), a set of 28 wild yeast, 13 isolated from oaks (25q, 29q, 49q, 53q, 70q, 86q, 90q, 104q, 118q, 138q, 142q, 146q, 163q) and 15 from grapes (2, 4g, 5g, 7g, 8g, 9g, 10g, 11g, 12g, 13g, 14g, 15g, 16g, 17g and 20g) were selected to be tested in small-scale brewing fermentations, initially with a low-density wort (Fig. 5a). Differences in behavior among individuals of the same and different species were observed as expected from previous results. Among the wild isolates, five strains

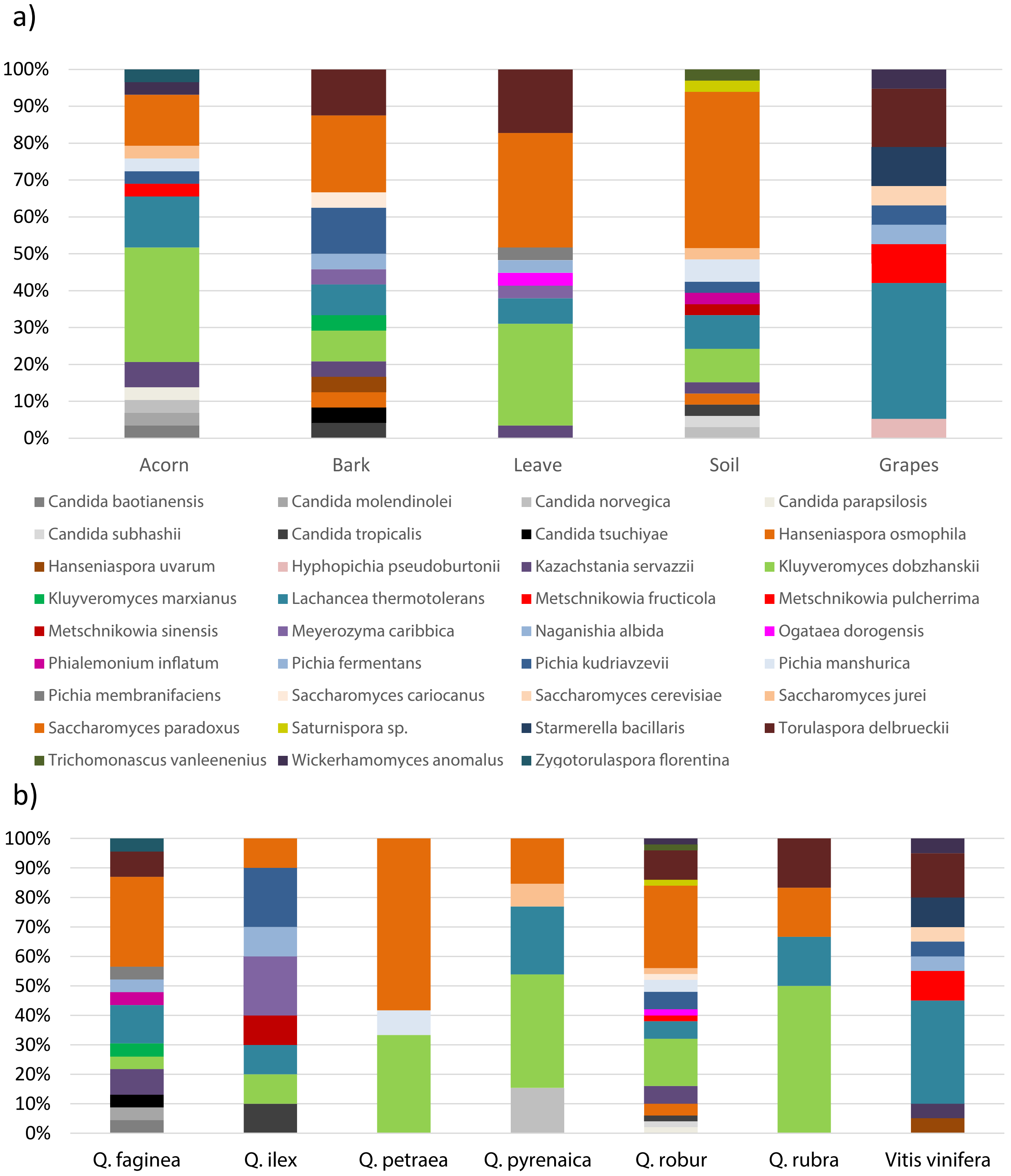


Fig. 2. Yeast species distribution according to the sample type (a) and the host species (b).

(25q, 86q, 138q, 146q, 14g) practically did not ferment, while six strains (104q, 118q, 163q, 12g, 13g, 16g) showed a good fermentative capacity, similar to the commercial strains and, in some cases, even better. Interestingly, all these isolates belonged to the same species, *L. thermotolerans*, three isolated from grapes (12g, 13g and 16g), and three from oaks (104q, 118q and 163q). With these six isolates, a new set of fermentations were carried out but using 3 different worts (European Lager, English IPA and Belgian Dubbel) to evaluate their brewability, using three commercial strains as controls, one strain characteristic of each style of beer tested (Fig. 5b–g). The strains that presented the best fermentation capacity in all the tested worts were 13g, 118q and 163q, although the attenuation achieved was lower than that obtained with the commercial strains, especially in the English IPA style. On the other hand, surprisingly, the strain 16g, which was the best strain in the first screening, even better than some commercial strains, exhibited the worst fermentative profile in all the wort styles tested (Fig. 5). Moreover,

Table 3
Biodiversity indexes according the host and the sample type.

		Taxa	Individuals	Dominance	Simpson Index	Shannon index	Evenness	Margalef richness index
Host	*Q. faginea*	13	24	0.14	0.86	2.28	0.75	3.83
	Q. ilex	8	10	0.14	0.86	2.03	0.95	3.04
	Q. petraea	4	13	0.40	0.60	1.91	0.74	0.80
	Q. pyrenaica	6	13	0.25	0.75	1.58	0.81	1.56
	Q. robur	18	48	0.13	0.87	2.43	0.63	4.35
	Q. rubra	4	6	0.33	0.67	1.24	0.87	1.67
	V. vinifera	11	20	0.18	0.83	2.08	0.73	3.34
Sample type	Acorn	14	29	0.15	0.85	2.26	0.68	3.86
	Bark	14	22	0.11	0.88	2.38	0.83	4.09
	Leave	9	30	0.21	0.79	1.79	0.66	2.38
	Soil	15	33	0.21	0.79	2.14	0.56	4.00
	Grapes	10	20	0.19	0.82	2.08	0.73	3.06

there was a correlation between the fermentative capacity of the strains and the colour change of the pH indicator in the airlock. This pH indicator can be an early detector of failure in fermentation activity, since if there is no colour change in 1 day, the strain will be not a good strain to be selected.

4. Discussion

Diversification is becoming increasingly important in the beer market. The emergence of craft brewing and new consumer demands have influenced the importance of reinforcing the role of the yeast in beer flavour and aroma profile, promoting innovation in the fermentation process field (Capece et al., 2018). Therefore, this study aimed to show potential local sources of wild yeasts such as forest and vineyard ecosystems using a selective enrichment medium to favor the growth of ethanol tolerant yeasts and to define a preliminary screening method to be used as a tool to study a great number of isolates according to their diverse brewing abilities in a short period of time.

The study was focused on oak forest and vineyard ecosystems, as both were reported as relevant sources of yeast species with brewing potential (Callejo et al., 2017; Molinet and Cubillos, 2020; Naumov et al., 1998; Nikulin et al., 2020). Due to the difficulty of isolating certain yeast species, such as some of the *Saccharomyces* genus, from natural sources, sampling was performed using a selective enrichment medium (Robinson et al., 2016; Sniegowski et al., 2002; Sweeney et al., 2004). Isolation methods may select some genotypes since enrichment culturing will favor the isolation of individuals with good fitness in these specific media; in our case, yeasts with high tolerance to ethanol. Such potential biases in sampled yeast phenotypes are likely to lead to biases in sampled genotypes, masking part of the diversity present in the studied ecosystems (Boynton et al., 2019). In our study, this bias was sought since we wanted to rule out yeasts with low or no ethanol tolerance from the study, since they would not have applicability in brewing, using tolerance to high concentrations of ethanol (7,6 % *v*/v) as the first selection criterion. Taking into account this previous selection, we studied the diversity of yeasts highly tolerant to ethanol found in both types of ecosystem, oak forest and vineyards, showing clear differences between them, among species and sample types. *Q. robur* and *Q. faginea* were the main source of this type of yeast isolates and diversity. In connection with the sample type, leaf samples were the poorest source of yeast diversity. On the other hand, the samples from the vineyard provided a greater richness of species as well as a greater number of exclusive species. All the isolated species were ascomycetes except one species of basidiomycetes, probably due the enrichment step (presence of ethanol and temperature). Sylvester et al. (2015) reported that most basidiomycete are relatively sensitive to ethanol and that there is a strong relationship between the temperature used during the enrichment step and the phyla of the yeast isolates, which was supported by the preference of many basidiomycete yeasts for lower temperatures. These factors could explain the clear dominance of ascomycete species in the isolation process.

Not all the isolated species were expected to be suitable for beer production, since, among the detected species, some have been reported as disease-causing agents in humans such as *Candida norvegica*, *Candida parapsilosis*, *Candida subhashii* and *Candida tropicalis* (Adam et al., 2009; Miranda et al., 2009; Silva et al., 2012; Zuza-Alves et al., 2017).

Of the 35 identified species, 13 have been reported as species with interest for food fermentation processes, *Hanseniaspora osmophila*, *Kluyveromyces marxianus* (Beniwal et al., 2017; Bourdichon et al., 2012; Carlquist et al., 2014), *L. thermotolerans* (Domizio et al., 2016; Vaquero et al., 2021), *Metschnikowia fructicola*, *Metschnikowia sinensis*, *Ogataea* spp. (De Roos et al., 2018) *Pichia fermentans* (Bokulich et al., 2012; Piló et al., 2018; Spitaels et al., 2014), *Pichia kudriavzevii*, *S. paradoxus*, *S. cerevisiae*, *T. delbrueckii*, *W. anomalus*, *Z. florentina* (Callejo et al., 2017; Canonico et al., 2017; Domizio et al., 2011; Morata et al., 2016; Nikulin et al., 2020). The yeast species most frequently detected were *S. paradoxus*, *L. thermotolerans* and *T. delbrueckii.* In addition to being the most frequently isolated species, they are also among the ones that showed the greatest enzymatic activity in the implemented tests.

S. paradoxus, the closest relative to *S. cerevisiae*, was the first wild *Saccharomyces* species isolated from oaks and birch sap in Russia and Ukraine and has been broadly associated with bark and soil of *Quercus* sp. in Asia, Europe and North America (Alsammar and Delneri, 2020) and with acorns, as potential source of introduction of this species in New Zealand (Zhang et al., 2010). Furthermore, *S. paradoxus* has also been isolated from Croatian vineyards (Redzepovic et al., 2002), however, this species was not isolated from vineyard ecosystems in this work. In addition, Robinson et al. (2016) found an association of *S. paradoxus* isolation with summer temperature, showing highest isolation rates at intermediate temperatures. The geographic distribution predicted by this optimum temperature range is consistent with the distribution of locations where *S. paradoxus* has been isolated and could explain the low frequency of *S. cerevisiae* isolation taking into account the Mediterranean climate in Rioja and the sample type, grapes. The potential of *S. paradoxus* as a pure starter culture for beer fermentations has already been reported and the results were promising, with good brewing properties including intense fruity and floral aromas (Nikulin et al., 2020).

T. delbrueckii and, *L. thermotolerans* are currently produced at industrial level as dry yeast or liquid refrigerated starters, alone or combined with other yeast by international biotechnological companies (Vejarano and Gil-Calderón, 2021). *L. thermotolerans* strains produce lactic acid in large quantities during alcohol fermentation, reaching levels of up to 16 g/L, and improve the aroma of beer mainly through high ethyl butyrate and ethyl acetate production in both pure and mixed fermentations (Zdaniewicz et al., 2020). *T. delbrueckii* in mixed fermentations with *S. cerevisiae* is a suitable strategy to control flavour production in beer fermentation. Thus, *T. delbrueckii* can be used to obtain products with aroma compounds that are different from those obtained when pure *S. cerevisiae* starter strains are used (Canonico et al., 2017). *T. delbrueckii* shows the capacity to produce other metabolites of oenological interest, such as glycerol (Vejarano and Gil-Calderón,

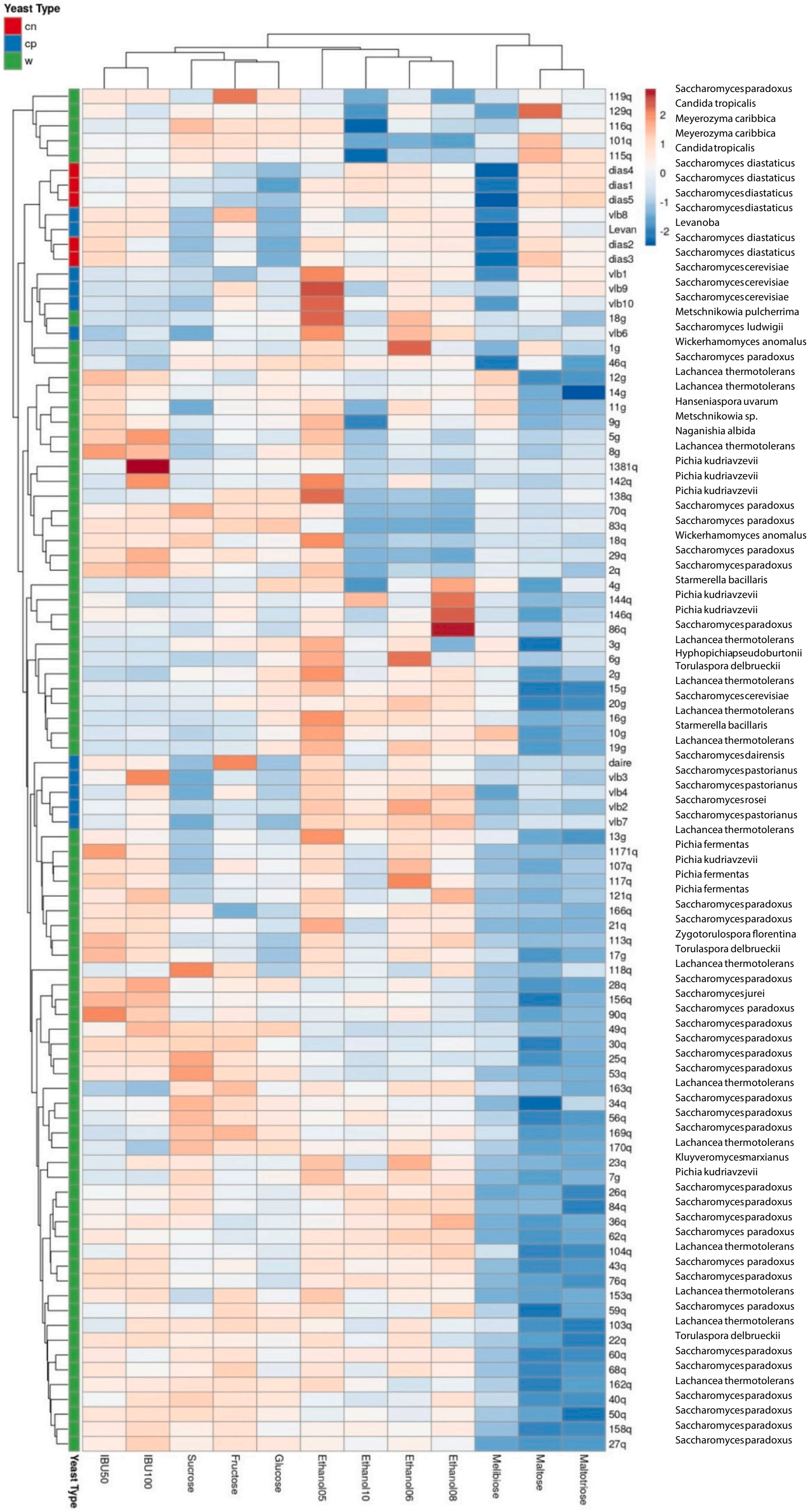
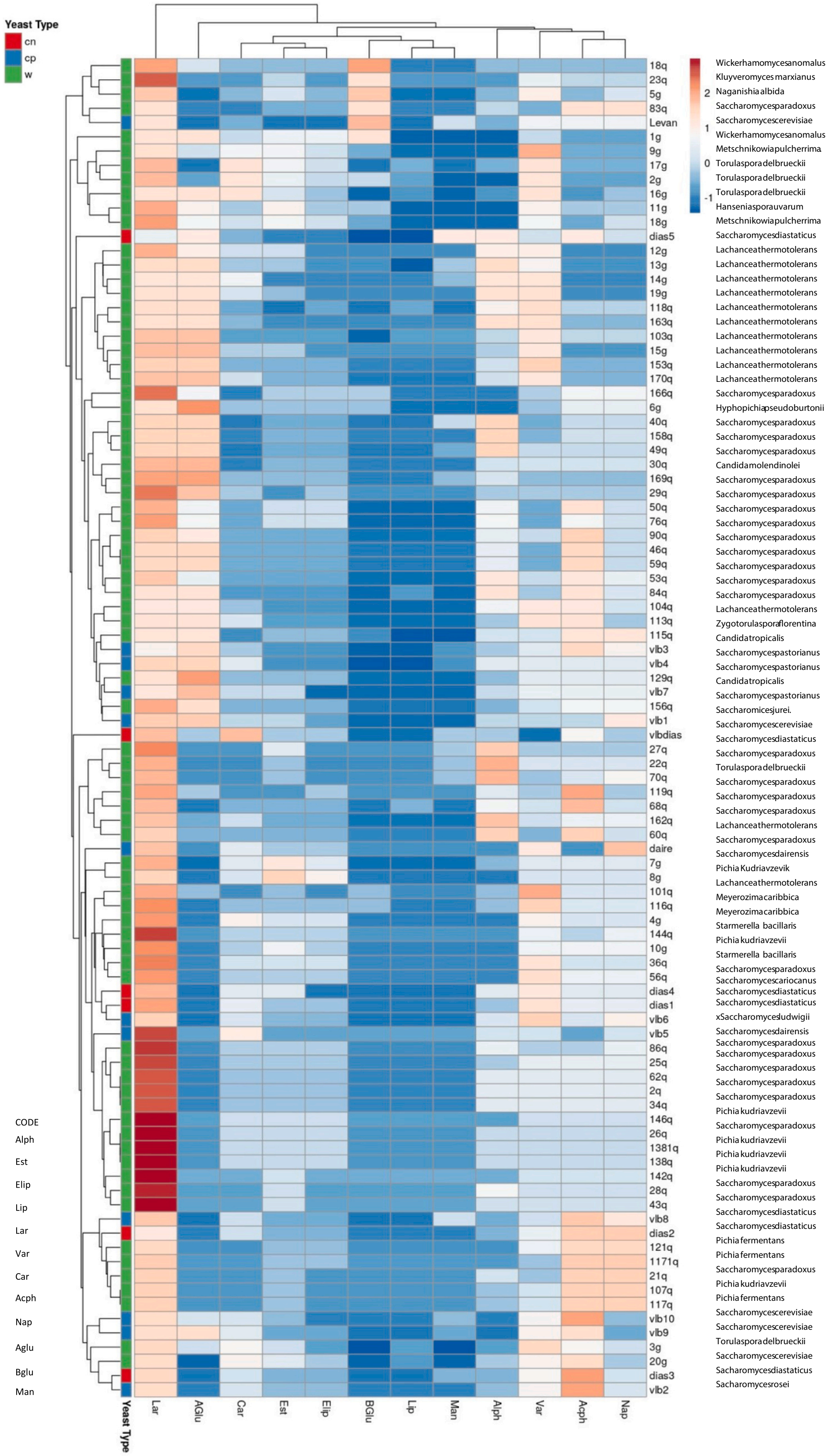


Fig. 3. Heat map to determine similarities between wild isolates and controls based on the growth of these isolates in different carbohydrates and in presence of different concentrations of ethanol and α-isoacids (miniaturised fermentation tests).
cn: negative controls, cp: positive controls, w: wild isolates.



ENZYME ASSAYED	CODE
Alkaline phosphatase	Alph
Esterase (C 4)	Est
Esterase Lipase (C 8)	Elip
Lipase (C 14)	Lip
Leucine arylamidase	Lar
Valine arylamidase	Var
Cystine arylamidase	Car
Acid phosphatase	Acph
Naphthol-AS-BI-phosphohydrolase	Nap
α-glucosidase	Aglu
ß-glucosidase	Bglu
α-mannosidase	Man

Fig. 4. Heat map to determine similarities between wild isolates and controls based on their API ZYM enzymatic profile. cn: negative controls, cp: positive controls, w: wild isolates.

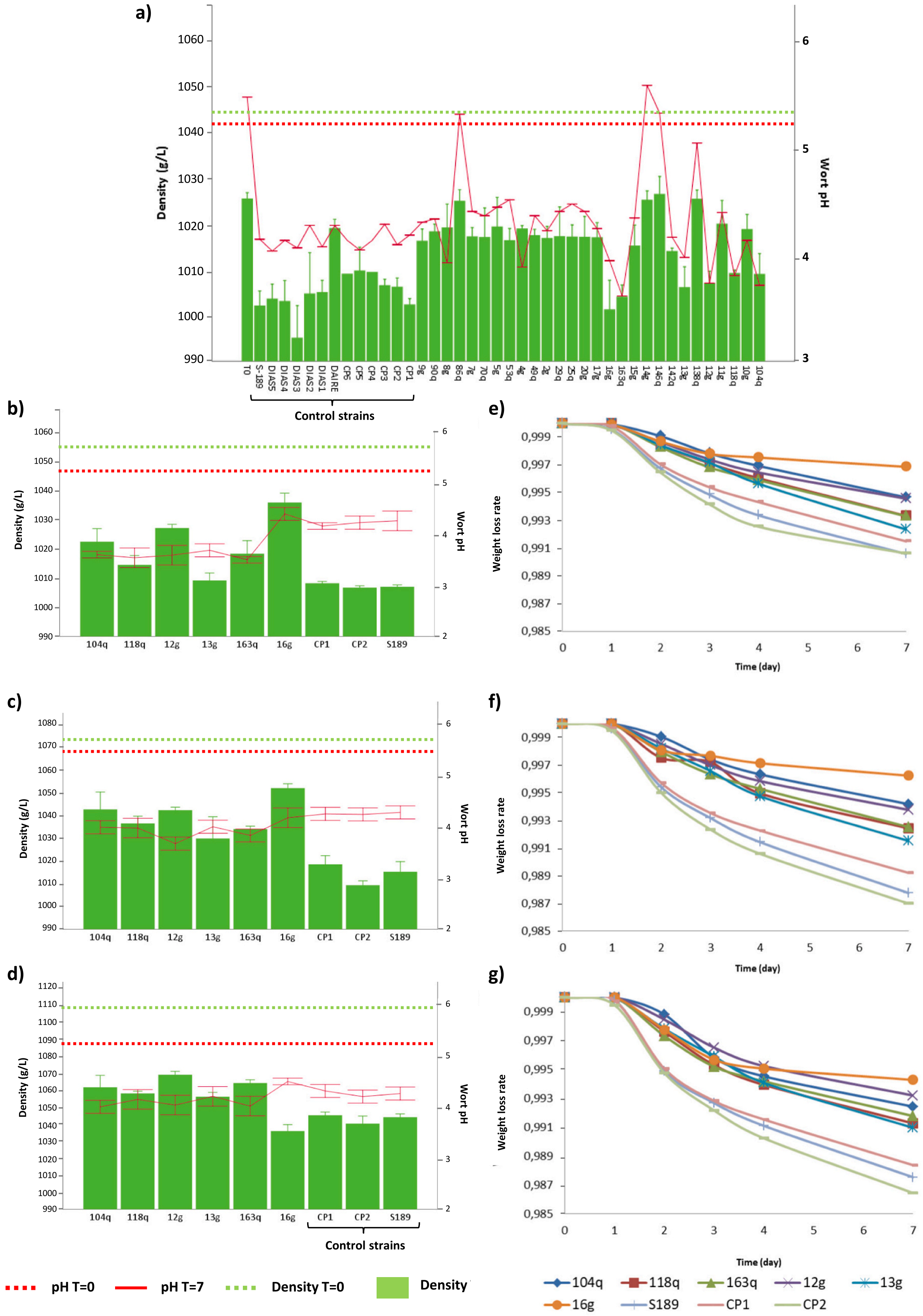


Fig. 5. Small-scale brewing fermentations. (a) A low-density wort was fermented with 28 wild isolates and 13 controls. (b–g) Three different style worts (European Lager (b–e), English IPA (c–f) and Belgian Dubbel (d–g)) were fermented with 6 wild isolates and 3 controls. The evolution of density and pH at the beginning and at the end (day 7) (a, b, c, d) and the weight loss throughout the process (e, f, g) is represented. Error bars indicate a 95 % confidence interval.

2021).

Once the collection of isolates was established, which was composed of 76 wild isolates (*Saccharomyces* and non-*Saccharomyces*), the brewing capabilities of these isolates were tested. In this screening, 12 strains were used as positive controls, belonging to different species of *Saccharomyces*, such as *S. cerevisiae* (ale yeast), *S. pastorianus* (lager yeast) and *S. dairensis*, *S rosei*, and *S. ludwigii* (yeast for low alcohol beers). On the other hand, 6 negative controls were also included, belonging to *S. cerevisiae* var. *diastaticus* (*S. diastaticus*), since this species is considered as a beer spoilage that could cause unwanted secondary fermentations due to extreme attenuating properties. However, recent studies have prompted a reassessment of this group as highly specialised, capable of hydrolysing and fermenting long-chain oligosaccharides such as dextrin and starch, with the potential to create new beers with new physicochemical properties (Krogerus and Gibson, 2020).

The first miniaturised screening determined the growth of these isolates based on the utilisation of carbohydrates that play an important role in the beer fermentation process, and on the inhibition caused by substances such as ethanol and α-isoacids. The latter are present in hops, and at certain concentrations could restrict the growth of yeasts. The fermentation and assimilation analysis showed that the isolates exhibited different inter and intra specific patterns and with different distance to the reference strain profiles. Therefore, the use of clustering tools, such as heatmaps and PCA analysis, was necessary to compare and evaluate the brewing characteristics of the different isolates in relation to the control strains, commercial yeasts used for beer elaboration, to investigate their potential as brewing starters. We included control strains for the production of all types of beers, which will allow us to select different isolates depending on the type of beer we want to elaborate. Interestingly, there were more strains similar to the negative controls than to the positive controls, especially strains isolated from oak trees. The grape isolates were more similar to yeasts used for the elaboration of lager and ales, which was expected since they come from a fermentative niche. Unexpectedly, most strains grew poorly in maltose, the main sugar in malt, even the commercial ones. Only the negative controls, strains belonging to *S. diastaticus*, and some oak isolates showed high growth in this sugar. Therefore, the high concentration of maltose used in this test, two to five times higher than the other sugars, may be selecting strains with a high attenuating capacity. These strains may have potential for the production of special beers, such as high gravity beers or light beers. In fact, the three sugars that were tested in higher concentrations (maltose, maltotriose and sucrose) were the ones that had the highest rates of growth inhibition, so it would be interesting to test all sugars at the same concentration to define the carbohydrate preferences of the different strains. In general, all strains exhibited good tolerance to α-isoacids and ethanol. In the case of ethanol, as expected, growth inhibition increased with higher concentrations of ethanol, although few strains were totally inhibited even by 10 % ethanol. Most of the isolates showed a good assimilation of common sugars (glucose, fructose, sucrose), which are main sugars found in natural environments. This property is the basis for choosing the appropriate strain to be used as starter in fermentations carried out with substrates containing a mixture of these carbohydrates, such as grape must in the wine industry (Wilkes et al., 2019), dough in the baking industry (Bell et al., 2001) or juice of sugar cane and sweet sorghum in bioethanol production (Jasman et al., 2012). In the case of brewing and distilling yeast, such as those for the Scotch whisky production (Waymark and Hill, 2021) assimilation of maltose and maltotriose should be also considered (Alves et al., 2008; Magalhães et al., 2016).

The second miniaturised screening analysed the presence of nineteen enzymatic activities in the yeast isolates, using the Api ZYM system, applied in studies of wine and beer yeasts (Escribano et al., 2017; Tofalo et al., 2011). These enzymes play an important role in the development of important wine and beer aroma compounds and improving their sensory properties (Fernández et al., 2000; Lee and Park, 2020). The group of aminopeptidases (leucine arylamidase, valine arylamidase and cysteine arylamidase), the group of esterases, esterase-lipase and lipases and the β-glucosidase are enzymes involved in the final aroma of wines (Escribano et al., 2017). Aminopeptidases catalyse the hydrolysis of N-terminal amino acids from peptides, improving the nutrient content of the wort and the synthesis of aroma compounds, since some amino acids, such as valine, leucine and phenylalanine, are precursors of active aroma compounds produced by yeast. All the wild isolates presented leucine and valine arylamidase activity and most of them also cysteine arylamidase. In fact, the former was the enzyme with the highest activity among all the enzymatic activities tested. These results agree with those of Escribano et al. (2017), who, using the same methodology also observed that the most and least frequent aminopeptidase activity in yeast was leucine arylamidase and cysteine arylamidase, respectively. On the other hand, esterase and lipase activities are involved in the formation of volatile aromatic ester compounds (Verstrepen et al., 2003), and in this study they were frequently detected but at significantly low intensity. β-glucosidase activity, which is related to the release of terpenes in wine (López et al., 2015), was detected in approximately half of the isolates, reaching maximum levels in two isolates of *W. anomalus* and one of *S. paradoxus*. These species have not previously been associated with high β-glucosidase activity, more related to *M. pulcherrima*, *Candida* spp. and *Cryptococcus* spp. (Escribano et al., 2017; Fernández et al., 2000; Rossouw and Bauer, 2016). The α-glucosidase (maltase) activity was observed practically in all the isolates of *L. thermotolerans* and half of the *S. paradoxus* in this study. The presence of this enzyme is interesting in beer strains since it hydrolyses maltose and maltotriose, the main malt sugars. In fact, most of the isolates of *L. thermotolerans* displayed the most interesting enzymatic activities (aminopeptidases, phosphatases, α-glucosidase), exhibiting a good potential in brewing. Strains of this species are considered optimal for beer production, despite needing more time than traditional ale yeast to finish fermentation but without negatively affecting the physical properties of beer (Domizio et al., 2016).

The presence of enzymatic activities, such as extracellular amylases, β-glucanases and β-glucosidase could be interesting for the development of new yeast strains for brewing (Stewart et al., 2013), because dextrins, β-glucan and soluble proteins are not metabolised by current brewer's yeast (Spier et al., 2016).

Finally, some of these isolates were tested in small-scale fermentation to assess their brewing potential, with the specific goal of generating a selection of strains for future brewing biotechnological applications. We identified three L. *thermotolerans* isolates (one isolated from grapes and two from oak) that showed great potential and adaptability to ferment different wort styles (European Lager, English IPA and Belgian Dubbel), although in the styles with higher density and bitterness, a mixed inoculation with *Saccharomyces* strains will be necessary. Nevertheless, further studies will be needed to evaluate the optimal fermentation conditions of each selected yeast (temperature, wort composition, etc.), and their impact on the flavour profile and other sensory properties of beers to determine the possible brewing applications of these isolates.

This study evidenced the great potential of this type of screening for creating an initial and rapid biochemical fingerprint of wild and commercial yeast to assess their functional diversity and to select isolates for further studies based on the target aim. Moreover, it is especially useful when a large number of isolates is handled and a previous selection is required in a short period of time. Furthermore, the final step of small-scale fermentation tests allowed a final and reliable selection of the best isolates.

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CRediT authorship contribution statement

Eugenia Iturritxa: Conceptualization, Investigation, Methodology, Data curation, Writing-Original Draft. Annie E. Hill: Writing-Review and Editing. María-Jesús Torija: Conceptualization, Supervision, Writing-Review and Editing.

All authors have read and agreed to the published version of the manuscript.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2023.110187.

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