Brewing and probiotic potential activity of wild yeasts *Hanseniaspora uvarum* PIT001, *Pichia kluveri* LAR001 and *Candida intermedia* ORQ001

Renan Eugenio Araujo Piraine1 · Gustavo Maas Retzlaf1 · Vitoria Sequeira Gonçalves1 · Rodrigo Casquero Cunha1 · Neida Lucia Conrad1 · Matthew L. Bochman2 · Fabio Pereira Leivas Leite1

Received: 10 May 2022 / Revised: 19 September 2022 / Accepted: 2 October 2022 / Published online: 15 October 2022 © The Author(s), under exclusive licence to Springer-Verlag GmbH Germany, part of Springer Nature 2022

**Abstract**
Non-conventional yeasts can be isolated from a wide range of environmental sources and are often found in the beverage industry in mixed fermentations, in which the microbial community is usually not fully known. However, it is important to know the compositions of these starter cultures because in addition to enabling reproducibility during fermentation, other properties can be discovered. Thus, the objective of this work was to identify and characterize non-conventional yeasts isolated from the environment, evaluating their probiotic potential and possible use in beer brewing. Isolates were obtained from flowers, fruits, leaves and mixed-fermentation beers, with the species being identified by PCR. Yeasts with promising activity were evaluated regarding their growth under different pHs, temperature and the presence of organic acids. To explore probiotic potential, in vitro tests were performed for antimicrobial activity and co-aggregation with food-spoiling microorganisms, auto-aggregation and survival in simulated gastrointestinal tract conditions. In this study, *Pichia kluveri* (LAR001), *Hanseniaspora uvarum* (PIT001) and *Candida intermedia* (ORQ001) were selected among 20 isolates for further study. *P. kluveri* was the only strain that tolerated pH 2.5. Lactic acid was not inhibitory, but acetic acid and incubation at 37 °C had partially inhibitory effects on yeast growth. All yeasts tolerated α-acids from hops and up to 1% NaCl. Our results also suggest that these isolates are able to adhere to intestinal cells and positively influence the host to combat pathogens, as they showed auto-aggregation rates > 99% and antagonistic activity to pathogenic bacteria. The yeasts tolerated gastric environment conditions, but were more sensitive to pancreatic conditions. We conclude that these non-conventional yeasts have probiotic potential and promising application in beer fermentation.

**Keywords** Beer · Non-conventional · Non-*Saccharomyces* · Antimicrobial activity · Yeasts · Fermentation

**Introduction**
Yeasts are ubiquitous in the environment, often being isolated from the microbiota of fruits, plant exudates, soil and insects [1]. Non-conventional yeasts, also referred to as non-*Saccharomyces* yeasts, represent an interesting alternative for the industrial development of new products [2–4]. For instance, yeasts like *Brettanomyces* spp., *Candida* spp. and *Pichia* spp. were historically considered as only contaminants in brewing environments, but they are currently considered important components in high-value added beers [5]. These fermenting microorganisms are often found in open fermentations (or fermentations exposed to the environment), a process that can be unpredictable and can generate large economic losses to breweries [2]. In the beer brewing industry, using pure monocultures in fermentations tends to be the first choice, but in other industries (e.g., wine and fermented dairy products), the addition of multiple strains in a controlled manner is a common practice. Mixed fermentations make it possible to create products with unique organoleptic bouquets and distinct nutritional characteristics,
obtained according to the concentration and identity of the strains used in the fermentation process [3, 6].

Among wild yeasts, Hanseniaspora spp. are described as predominant on some fruits surface (especially grapes) and are known to significantly contribute to the sensory profile of different wine styles [7]. Further, strains from other genera like Pichia and Candida are already used by researchers and breweries to produce beers with low or no alcohol content, representing an important application that can be protected by patents [8, 9]. Non-Saccharomyces yeasts have been increasingly investigated for their bioflavoring ability in fermented beverages, such as beer, wine and cider [3, 6, 9, 10]. This exploration has revealed that strains of Pichia kluyveri are able to enhance the levels of acetate esters, contributing fruity notes to beer and improving the quality of wines [3, 9, 11]. Strains of Hanseniaspora uvarum have generally been characterized for wine production, but when applied in co-fermentations with S. cerevisiae for beer fermentation, H. uvarum significantly impacts the glycerol and acetic acid levels, contributing to complexity and aroma intensity [12]. Candida intermedia strains have been identified in contaminated beers and other beverages (e.g., juices and dairy products) [13, 14], but there are no reports of fermented beverages using C. intermedia in a controlled manner. Therefore, its contribution to beer flavors is still unknown. Metabolic aspects regarding the use of different substrates, growth at different temperatures and pHs, halotolerance, osmotolerance and enzymatic activity must be well characterized before these yeast species can be determined as viable for brewing and/or other industrial applications [15, 16].

Some yeasts have the ability to produce antimicrobial compounds, capable of inhibiting the growth of pathogenic bacteria and other fungi [17, 18]. This characteristic and others are important for microbes to be classified as “probiotics”, which by definition are live microorganisms that, when administered in adequate amounts, confer benefits to the host’s health [19]. To be considered probiotics, these microorganisms need to have functional properties such as not being pathogenic, resisting harsh conditions found in the human gastrointestinal (GI) tract, cell adhesion capacity, or having immunostimulatory action, among others [20, 21]. Probiotic potential has been described for certain strains of P. kluyveri, H. uvarum and C. intermedia, involving their ability to produce antimicrobial proteins [17, 21, 22], growth repression of pathogens [23, 24], auto-aggregation and co-aggregation with pathogens [25, 26] and tolerance to gastrointestinal conditions [15, 25]. However, probiotic potential varies among strains, highlighting the importance of characterizing isolates through several in vitro and in vivo tests [27].

Saccharomyces cerevisiae is a yeast with known probiotic activity. The search for non-Saccharomyces yeasts with probiotic potential involves many different sources, such as plants, animals fermented foods and wild fermented beverages [15, 28, 29]. Depending on the isolate, different yeast species may have as much probiotic potential as species belonging to the Saccharomyces genus. Thus, the objective of this work was to isolate wild yeasts and then identify and characterize their fermentation capacity, sensory contribution to beer and their probiotic potential.

Materials and methods

Wild yeast isolation

A sterile swab soaked in YM medium (0.3% yeast extract, 0.3% malt extract, 0.5% bacteriological peptone and 1% glucose) was applied to the surface of a fruit—strawberry (Fragaria × ananassa), blackberry (Morus nigra), orange (Citrus × sinensis) or butiá (Butia capitata)—and subsequently used to inoculate a tube containing YM supplemented with 100 µg/µL ampicillin. Cultures were incubated for 48 h at 28 °C with constant agitation at 150 rpm. The same procedure was performed for flowers and the leaves of orchid (Aspasia lunata), pitaya (Hylocereus undatus) and a grape vine (Vitis vinifera), as well as Flanders Red- and Old Ale-style beers. All samples collected for yeast isolation were obtained in the city of Pelotas, Brazil (Latitude – 31.776, Longitude – 52.3594 31° 46’ 34” South, 52° 21’ 34” West), throughout the spring/summer period, with daily temperatures around 25–30 °C.

Yeasts were isolated by streaking samples from cultures on YM agar + ampicillin. Plates were incubated at 28 °C for 72 h for yeast growth. The yeast cell morphology was observed using a BLUE1600BA-L-BT (Biofocus, Brazil) optical microscope at 1000 × magnification. Isolates were stored at the Microbiology Laboratory Yeast Bank (Federal University of Pelotas, Brazil) by freezing (–80 °C) in glycerol 30%, with identification codes being given to each isolate.

Fermentation of synthetic beer must

To evaluate the fermentation ability of each yeast isolate in standard beer wort, a synthetic beer must was formulated with 400 mL of malt extract (Dry Brew, Liotecnica, Brazil) with a density of 11°P, pH 5.0, sterilized by autoclaving for 15 min at 121 °C. Iso-α-acid from hops (Hopsteiner, Germany) was added to a final concentration of 15 IBU (International Bitterness Units). A standardized a concentration of 10⁶ yeast cells (total) was used to ferment 400 mL of synthetic beer must by cultivating each strain in YM medium for 48 h at 28 °C and 150 rpm prior to fermentation tests. The yeast cell concentration was determined by counting...
in a Neubauer chamber. A volume corresponding to $10^9$ cells of each yeast culture was harvested by centrifugation at 1500 × g for 5 min in a DTR-16000 centrifuge (DAIKI, Korea), the cell pellet was resuspended in 15 mL of the synthetic beer must, and subsequently used to inoculate the 400 mL of medium for fermentation.

Fermentation was conducted for 14 days in an incubator at 22 °C with no agitation. The production and release of CO$_2$ was monitored through bubble formation in an air-lock that was used to cap each fermentation flask, while biofilm formation at liquid–gas interface of each flask was visually observed. After fermentation, the final density and pH of the beer were evaluated using a pH meter (KASVI, Brazil), a densimenter (Incoterm, Brazil) for density in g/cm$^3$ and a refractometer (AKSO, Brazil) to measure density in Brix degrees. These data used to calculate percentage of apparent attenuation (AA) by each isolate. AA was calculated using Beersmith™ v.3 software (Beersmith, United States), following the formula: AA% = 100 × (OG − FG)/(OG − 1.000), where OG is the original gravity of the beer must, and FG is the final gravity, both in g/cm$^3$. The area of each beer after the 14-day fermentation period was evaluated by simple sensory analysis consisting of multiple individuals, as conducted in the Osburn et al. [30] study. Saccharomyces cerevisiae YT001 (Yeastech, Brazil) was used in this experiment as a reference yeast, as well as a baseline for the sensory analyses.

**DNA extraction, PCR and sequencing**

Total genomic DNA from each isolate was extracted following the protocol described by Preiss et al. [31], and its concentration was quantified using a Nanovue™ (Biochrom, United States). Identification at the species level was performed by PCR using the ITS1 and ITS4 primers, which amplify the repetitive region of the 5.8S rRNA gene and the internal transcribed spacer (ITS) flanking regions. PCR was performed using 0.7 μL of genomic DNA (1 μg/μL), 22 μL of Master Mix (Ludwig Biotechnology, Brazil), 1 μL (0.5 μM) of ITS1 primer (5′TCCGTAGGTAACCTTGC GG) and 1 μL (0.5 μM) of ITS4 primer (5′TCCTCCGT TATTGATATGC), for a total volume of 24.7 μL in each reaction. The following incubation conditions for PCR were used: initial denaturation at 95 °C for 10 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 2 min and extension at 72 °C for 2 min, and then a final extension at 72 °C for 8 min. PCR product sizes were analyzed by electrophoresis of 10 μL of each reaction in 1% agarose gels (w/v) in TBE buffer (Tris 0.89 M, EDTA 0.02 M and boric acid 0.89 M) at 100 V, 500 mA for 2 h. Agarose gels were stained with 5 μL of ethidium bromide, and band sizes were determined by comparison with a 1 kb Plus DNA Ladder (ThermoFisher Scientific, United States).

The PCR product of each isolate was purified using a GFX PCR DNA and Gel Band Purification kit (GE Healthcare, United States), quantified with Nanovue™, and then submitted at an approximate concentration of 50 ng/μL for sequencing by ACTGene Análises Moleculares (Brazil) using an Applied Biosystems AB-3500 platform. Data regarding PCR product sequencing were used for alignment with the BlastN tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and querying the NCBI (National Center for Biotechnology Information) database for yeast identification.

**Characterization of yeast isolates**

**Evaluation of yeast growth under different stress conditions**

The yeasts *H. uvarum* (PIT001), *P. kluyveri* (LAR001) and *C. intermedia* (ORQ001) were selected based on observation of all data collected in the fermentation tests. In addition, the probiotic strain *S. boulardii* CNCM I-745 (Floratil®) (a S. cerevisiae isolate) that was already part of the microorganism bank of the Microbiology Laboratory in the Technological Development Center of the Federal University of Pelotas was also used during all tests.

Cultivation tests were performed in 10 mL of YPD medium (1% yeast extract, 2% bacteriological peptone and 2% glucose), following a protocol adapted from Zeng et al. [32]. The resistance to different conditions was tested: 1% lactic acid, 0.2% acetic acid and pHs 2.5, 6.0 and 8.0. Yeast cultures were incubated for 72 h at 28 °C in an orbital shaker at 150 rpm. In addition, yeast growth was evaluated in YPD pH 6.0 at 37 °C. Samples were collected at 0, 4, 24, 48 and 72 h, analyzing their biomasses by absorbance reads at 600 nm in a Biochrom Ultraspec-10 spectrophotometer (Amersham Biosciences, United States) and colony-forming units (CFU)/mL. Viable cells (CFU/mL) were counted by serial dilution using saline solution (0.9% NaCl) and plating on YPD agar medium, following a 48-h incubation period at 28 °C. The growth index (GI) was calculated based in Zeng et al. [32] and the Bevilacqua et al. [20] method, following the equation: GI = $\frac{AbSs}{AbSc} \times 100$, where AbSs is the absorbance of the samples under different pH, temperature, or organic acid conditions and AbSc is the absorbance of the control samples. GI < 25% was considered to indicate high inhibitory activity, GI from 25 to 75% was moderate, and GI > 75% growth indicated weak inhibition.

The selected yeasts were also cultivated in YPD supplemented with different concentrations of salt (NaCl), ranging from 0.5, 1, 5 and 10% (w/v), to assess their ability to tolerate ionic stress by halotolerance. Yeast growth was evaluated after 72 h at 28 °C by optical density, and if the OD$_{600nm}$ ≥ 1.0 (representing ≥ 1 x 10$^7$ cells), the yeast isolate was considered able to survive and grow in this condition.
Sensitivity to α-acids (spot dilution test)

The analysis of yeast tolerance to α-acids from hops was performed based on a protocol adapted from Samanfar et al. [33]. The tolerance of the isolated yeasts to 200 ppm of 30% isomerized hop extract (Hopsteiner, Germany) was evaluated on YM agar medium, onto which we applied 10 µL of tenfold serial dilutions of yeast cultures (initial concentration of 10³ CFU/mL). Plates were incubated for 48 h at 28 °C before scoring growth.

Determination of proteolytic activity

Protease activity was evaluated following the protocol established by Zeng et al. [32]. Briefly, 10 µL samples from yeast cultures at 10⁸ CFU/mL were applied to skim milk agar medium (1% skim milk, 0.1% glucose, 0.5% bacteriological peptone, 0.25% yeast extract and 1.5% agar). Protease-positive strains were defined by the observation of a halo forming around the drop of culture after incubation for 48 h at 28 °C.

Gelatinase test

Gelatinase activity was evaluated according to the Pereira et al. [34] protocol. Culture medium for gelatinase tests was prepared with 1% yeast extract, 1.5% bacteriological peptone and 12% gelatin. Strains that were previously cultured in YPD medium for 24 h at 28 °C were transferred to the medium containing gelatin with a platinum loop. Tubes were incubated at 28 °C for 7 days and then refrigerated (4–10 °C) for 30 min. Gelatinase-positivity (i.e., enzymatic activity) was defined as the conversion of the semi-solid medium into liquid. Staphylococcus aureus ATCC 25,923, previously cultivated in Brain Heart Infusion (BHI) medium, was used as a positive control in the gelatinase tests.

Auto-aggregation and co-aggregation

Auto-aggregation and co-aggregation tests were performed following the protocols of Collado et al. [35]. Briefly, yeasts were cultivated for 24 h at 28 °C in YM medium, centrifuged for 5 min at 8000 x g, and their OD₆₀₀nm was adjusted to 0.25 ± 0.02 using PBS. Equal volumes of pathogen and yeast were mixed (1:1) and incubated under the same conditions as performed in auto-aggregation test, and the OD₆₀₀nm was recorded likewise. Co-aggregation results are expressed as a percentage, originating from the equation \[ \left( \frac{(A_{\text{pat}} + A_{\text{isol}}) - (A_{\text{mix}})}{(A_{\text{pat}} + A_{\text{isol}})} \right) \times 100 \], in which “Aₚₐₜ + Aᵢₛᵲ” represents the OD₆₀₀nm value for pathogen + isolate at time 0 h, and “Aᵢₙₓ” is the OD₆₀₀nm of the mixed suspensions of microorganisms at different time periods.

Antimicrobial activity test

Bacteria related to foodborne diseases (FBDs; Pseudomonas aeruginosa ATCC 27,853, L. monocytogenes ATCC 7644, S. aureus ATCC 25,923 and E. coli ATCC 8739) and from genera related to beer spoilage (Pediococcus pentosaceus (wild isolate), Lactobacillus casei (wild isolate), Klebsiella sp. (wild isolate), Moraxella sp. (wild isolate) and Bacillus subtilis ATCC 6633) were used to test the capacity of the isolated yeasts to inhibit growth of bacterial contaminants. Antimicrobial activity was measured according to the double-layer protocol presented by Amorim et al. [28], with values of inhibition halos being expressed in millimeters (halo+drop).

In vitro gastrointestinal tract simulation

Gastrointestinal tract simulation tests were performed based on the protocol described by Bonatsou et al. [36], with some adaptations. Simulation of GI tract conditions was performed using two solutions: gastric digestion (GD) and pancreatic digestion (PD). The GD solution was prepared with NaCl (2.05 g/L), KH₂PO₄ (0.60 g/L), CaCl₂ (0.11 g/L) and KCl (0.37 g/L), with the pH adjusted to 2.0 with 1 M HCl, and autoclaved for 15 min at 121 °C. After sterilization, pepsin (0.0133 g/L) and lysozyme (0.01 g/L) were added. PD solution was prepared with bile salts (3.0 g/L), Na₂HPO₄ (26.9 g/L) and NaCl (8.5 g/L), with the pH adjusted to 8.0 with 1 M NaOH. After sterilization as described above, pancreatin enzyme (0.1 g/L) was added. Yeasts were exposed to the GD and PD simulations, with solutions applied separately and in sequence.

Initially, one colony was selected from fresh cultures streaked on YPD agar to be inoculated in 10 mL of YPD medium, with incubation at 28 °C until a 10⁸ CFU/mL concentration was reached. After collecting a sample, yeast cultures were centrifuged at 1500 x g for 10 min, washed with GD solution and then resuspended in 10 mL of the same solution. Incubation was performed at 37 °C for 2.5 h under agitation at 200 rpm to simulate peristaltic movements, and then an endpoint sample was collected. The cells...
were subsequently centrifuged again at 1500 × g for 10 min, washed with PD solution, and resuspended in 10 mL of the same solution. Yeast cultures remained at 37 °C for 3.5 h, under the same agitation condition as described in the previous test. Viable yeast cells were detected according to CFU counts before and after each treatment.

The concentrations of viable yeast cells were used to determine the viability index (VI) during these tests, based on the equation proposed by Zeng et al. [32]:

$$\text{VI} = \frac{\log N_t}{\log N_0} \times 100,$$

in which $N_t$ is the yeast concentration at a specific time, and $N_0$ is the initial cell concentration. The tests were also performed in an isolated manner, i.e., split in two independent tests, one with GD solution and another with PD solution, aiming to obtain specific VIs for the PD step. In both GD and PD solution the yeasts were inoculated at the same concentration of $10^8$ CFU/mL each.

**Statistical analysis**

Data were analyzed by analysis of variance (ANOVA) and Tukey’s test to determine significant differences ($p < 0.05$) between means. All statistical analyses were performed using GraphPad Prism 7 software.

**Results**

**Wild yeast isolation and identification**

Morphological characteristics of the yeast colonies and cells, as well as their respective origins, are shown in Table 1. The sequencing of PCR-amplified products was analyzed using the Blast N tool, which enabled the identification of 20 wild yeast isolates, comprising nine different species (> 99% similarity and identity). These sequences were deposited on GenBank® nucleotide database, with accession numbers OP392528;OP39253030 and OP469995;OP470012.

**Fermentation test**

We observed that *H. uvarum* isolates maintain as apparent attenuation between 5 and 18%, with *H. uvarum* PIT001 yielding the lowest final pH and the highest apparent attenuation (18%) among the *H. uvarum* isolates (Table 2). Isolates belonging to the *Candida* genus displayed important differences in aromatic profile, with *C. intermedia* producing wood, floral, spice and clove-like aromas, while *C. manasasensis* contributed citrus and tropical sensory notes. For both *C. terrestris* and *P. manshurica* yeasts, no apparent attenuation was observed nor the production of aromatic compounds that stood out. *P. kudriavzevit* (also known as *Issatchenkia orientalis*) displayed an apparent attenuation of 9%, with strong aromatic notes characterized as solvent, phenolic, grape and green apple. The *P. kluyveri* isolate yielded 5% apparent attenuation, with moderate acidification and an aromatic profile similar to commercial strains of *S. cerevisiae*, including banana-like aromatic esters.

*S. cerevisiae* isolate PIT002 was the only wild *Saccharomyces* yeast identified. This isolate displayed an apparent attenuation level > 50% (indicating the consumption of sugars other than just glucose), the presence of foam (or krausen) at the liquid–air interface due to intense fermentation, no biofilm formation, and the production of aromas with pleasant fruity notes and a variety of esters (mostly banana) normally detected at high levels in beers produced by specific *S. cerevisiae* strains [37, 38]. Low apparent attenuation (5%) was noted for *I. terricola*, but distinct sensory notes referred to as funky were detected, which were further described as barnyard and horse blanket-like aromas commonly produced by *Brettanomyces* spp. [39].

**Yeasts growth under different stress conditions**

**Biomass production and GI**

The isolates identified as *P. kluyveri* (LAR001), *H. uvarum* (PIT001) and *C. intermedia* (ORQ001) were selected for further characterization tests because they generated adequate final pHs for finished beers, low (thin layer of cells) or no production of biofilms and interesting post-fermentation aromas. Moreover, some strains of these species have previously been described regarding their ability to participate in co-fermentations of beers with *S. cerevisiae*, bioflavoring potential and antimicrobial properties. Therefore, these isolates and the control *S. boulardii* strain were cultured and evaluated under different stress conditions, in which biomass produced was analyzed by OD$_{600nm}$ over time. For GI determination, only the final OD$_{600nm}$ was evaluated.

When the isolated yeasts were incubated under optimal growth conditions (YPD medium at pH 6.0, 28 °C incubation temperature), we observed a similar behavior for all yeasts: their biomass increased until a maximum OD$_{600nm}$ was reached at 24 h after inoculation, with no significant difference for subsequent time points. After 72 h of culturing, *C. intermedia* showed the highest biomass production by the wild isolates under these conditions, followed by *P. kluyveri* and *H. uvarum* (Fig. 1a). A similar experiment conducted at 37 °C (Fig. 1b) revealed that *H. uvarum* is unable to tolerate this temperature, representing a limiting factor for its growth. The *C. intermedia* culture was also significantly impacted by this incubation temperature, maintaining an OD$_{600nm}$ < 2.0 at all time points. Although *P. kluyveri* displayed the best biomass production among the wild isolates, its growth was also negatively impacted at 37 °C, showing lower OD$_{600nm}$ than when incubated at 28 °C. Observing the GI values (Table 3), it was noted that incubation at 37 °C...
was an inhibitory condition for \( H. \textit{uvarum} \) and \( C. \textit{intermedia} \) growth and partially inhibitory for \( P. \textit{kluyveri} \).

\( C. \textit{intermedia} \) and \( H. \textit{uvarum} \) were similarly negatively impacted by YPD medium at pH 2.5 (Fig. 1c). In this condition, both \( P. \textit{kluyveri} \) and \( S. \textit{boulardii} \) displayed less growth compared to YPD at pH 6.0, but they were able to increase their biomass until peaking at an OD\textsubscript{600nm} of 5.6 after 48 h. During growth in YPD at pH 8.0, the cell concentration achieved its maximum at 24 h for all yeasts evaluated, with no significant differences (\( p > 0.05 \)) being noted after this time point (Fig. 1d). We found that the yeasts grew similarly to the control (GI > 75%) when incubated in YPD pH 8.0, indicating that the alkaline pH was a weakly or non-inhibitory condition for yeast growth. In contrast, low pH was a highly inhibitory growth condition for \( H. \textit{uvarum} \) and \( C. \textit{intermedia} \) (GI < 25%), causing no increase in their biomass over time when cultivated at pH 2.5. \( S. \textit{boulardii} \) growth was also impacted in this condition, causing partial growth inhibition (GI 25–75%). We observed that \( P. \textit{kluyveri} \) tolerated both the pH 2.5 and 8.0 media, with significant biomass inhibition (GI 25–75%). We observed that \( P. \textit{kluyveri} \) was also impacted in this condition, causing partial growth inhibition (GI 25–75%). We observed that \( P. \textit{kluyveri} \) was also impacted in this condition, causing partial growth inhibition (GI 25–75%). We observed that \( P. \textit{kluyveri} \) was also impacted in this condition, causing partial growth inhibition (GI 25–75%).

The \( P. \textit{kluyveri} \) and \( H. \textit{uvarum} \) responses to acetic acid-induced stress were similar (Fig. 1e), displaying less growth than when these yeasts were cultured in YPD with no acetic acid added (Fig. 1a). Further, these cultures only reached maximum density (OD\textsubscript{600nm} = 3.1 and 3.0, respectively) after 48 h of incubation, demonstrating that acetic acid is partially inhibitory to growth (GI 25–75%). No growth was detected for \( C. \textit{intermedia} \) when cultured in the presence of acetic acid, revealing its inability to tolerate acetic acid stress (high

### Table 1 Identification of wild yeast isolates

| Isolate | Isolation source | Colony morphology | Cell morphology | Species | AP* |
|---------|------------------|-------------------|-----------------|---------|-----|
| AMO001  | Blackberry (\textit{Morus nigra}) | Circular, flat, entire margin | Ellipsoidal, rarely ovoid | \textit{Hanseniaspora uvarum} | 750 bp |
| AMO003  | Blackberry (\textit{Morus nigra}) | Circular, flat, entire margin | Ellipsoidal, rarely ovoid | \textit{Hanseniaspora uvarum} | 750 bp |
| ORQ001  | Orchid (\textit{Aspasia lunata}) | Circular, flat, entire margin | Ovoid | \textit{Candida intermedia} | 420 bp |
| ORQ002  | Orchid (\textit{Aspasia lunata}) | Circular, flat, entire margin | Ovoid | \textit{Candida intermedia} | 420 bp |
| PAR001  | Vine (\textit{Vitis vinifera}) | Circular, flat, entire margin | Ellipsoidal, rarely ovoid | \textit{Hanseniaspora uvarum} | 750 bp |
| PAR002  | Vine (\textit{Vitis vinifera}) | Circular, flat, entire margin | Ellipsoidal, rarely ovoid | \textit{Hanseniaspora uvarum} | 750 bp |
| PIF001CR| Pitaya (\textit{Hylocereus undatus}) | Circular, raised, undulated margin | Ovoid, rarely ovoid | \textit{Candida manassasensis} | 650 bp |
| PIF2.001| Pitaya (\textit{Hylocereus undatus}) | Circular, raised, undulated margin | Ovoid, rarely ovoid | \textit{Candida manassasensis} | 650 bp |
| PITS002 | Cherry (\textit{Eugenia uniflora}) | Circular, raised, entire margin | Ovoid | \textit{Saccharomyces cerevisiae} | 880 bp |
| PIT001  | Cherry (\textit{Eugenia uniflora}) | Circular, flat, entire margin | Ellipsoidal, rarely ovoid | \textit{Hanseniaspora uvarum} | 750 bp |
| PIT004  | Cherry (\textit{Eugenia uniflora}) | Circular, flat, entire margin | Ellipsoidal, rarely ovoid | \textit{Hanseniaspora uvarum} | 750 bp |
| MOR001  | Strawberry (\textit{Fragaria × ananassa}) | Circular, flat, entire margin | Ellipsoidal, rarely ovoid | \textit{Hanseniaspora uvarum} | 750 bp |
| MOR003  | Strawberry (\textit{Fragaria × ananassa}) | Circular, flat, undulated margin | Elongated | \textit{Issatchenka terricola} | 450 bp |
| COQ001  | Jelly palm fruit (\textit{Butia capitata}) | Circular, brilliant, raised, entire margin | Ovoid | \textit{Cryptococcus terrestris} | 510 bp |
| LAR001  | Orange (\textit{Citrus × sinensis}) | Irregular, flat, filiform margin | Ovoid, rarely ellipsoidal | \textit{Pichia kluyveri} | 450 bp |
| REF003  | Flanders Red beer (mixed fermentation) | Circular, flat, entire margin | Ovoid | \textit{Pichia manshurica} | 500 bp |
| REF005  | Flanders Red beer (mixed fermentation) | Circular, flat, entire margin | Ovoid | \textit{Pichia manshurica} | 500 bp |
| JRO001  | Old Ale beer (mixed fermentation) | Circular, raised, filiform margin | Elongated or ovoid, sometimes forming chains | \textit{Pichia kludriavzezi} | 350 bp |
| CAR001  | Old Ale beer (mixed fermentation) | Circular, raised, filiform margin | Elongated or ovoid, sometimes forming chains | \textit{Pichia kludriavzezi} | 350 bp |
| AMB001  | Old Ale beer (mixed fermentation) | Circular, raised, filiform margin | Elongated or ovoid, sometimes forming chains | \textit{Pichia kludriavzezi} | 350 bp |
| CNCM I-745| Yeast bank of Microbiology Laboratory | Circular, raised, entire margin | Ovoid | \textit{Saccharomyces boulardii} | 850 bp |
| YT001   | Yeast bank of Yeastech Laboratory | Circular, raised, entire margin | Ovoid or spherical | \textit{Saccharomyces cerevisiae} | 880 bp |

*AP—Amplified product on PCR using ITS1 and ITS4 primers
inhibition). In similar experiments with lactic acid added to YPD, *P. kluyveri* and *H. uvarum* cultures reached maximum OD_600nm at 24 h, while *C. intermedia* required a prolonged period of adaptation in this condition, between 0 and 24 h, before beginning to increase biomass until the end of the experiment (Fig. 1f). Lactic acid at a 1% concentration was not inhibitory to any yeast as demonstrated by their GI values. Viable cell counts measured during these tests are presented in Supplementary Information S1.

### Halotolerance

Regarding salinity (ionic) tolerance, all yeasts were able to grow in media containing up to 1% NaCl, but only *C. intermedia* was able to tolerate 5% NaCl. It was also observed in *C. intermedia* cultures a substantial inhibitory effect of the highest NaCl concentration tested (10%), however an OD_600nm ≥ 1.0 was detected over the course of the experiment (Table 4).

### Auto-aggregation and co-aggregation

We found that auto-aggregation was temperature dependent, with all isolates displaying a lower percentage of auto-aggregation (<90%) at 18 °C than that observed at 28 and 37 °C (p < 0.05). The yeasts showed high auto-aggregation capacity within 20 h (> 98%) at incubation temperatures of 28 and 37 °C, with no significant differences among the three isolates (Table 5).

Co-aggregation analyses with *E. coli* (Table 6) and *L. monocytogenes* (Table 7) revealed that temperature also influenced aggregation, which was < 60% for all yeasts at 18 °C. However, co-aggregation increased at 28 and 37 °C, remaining between 65 and 70% after 20 h at 37 °C for all isolates. We also observed that co-aggregation was increased...
**Fig. 1** Evaluation of biomass produced by *P. kluyveri* (LAR001), *H. uvarum* (PIT001), *C. intermedia* (ORQ001) and *S. boulardii* cultures in YPD under different conditions. Graphs show the absorbance of the cultures at different time points for the four yeasts incubated in YPD medium at pH 2.5 and 8.0, containing 1% lactic acid, containing 0.2% acetic acid, and at a temperature of 37 °C. Yeast cultivation in YPD medium at pH 6.0 and incubated at 28 °C was considered as optimal for growth, representing a control culture. The total experiment time, time points for sample collection, and agitation were maintained the same among all tests. Graphs were created with GraphPad Prism 7 software.

**Table 3** Growth index (GI) of yeasts cultured under different growing conditions

| pH 2.5 | pH 8.0 | 1% Lactic acid | 0.2% Acetic acid | 37 °C | Control |
|--------|--------|----------------|-----------------|-------|---------|
|        | O.D.600 nm | G.I | O.D.600 nm | G.I | O.D.600 nm | G.I | O.D.600 nm | G.I | O.D.600 nm | G.I | O.D.600 nm | G.I |
| *P. kluyveri* | 5.15 | > 75 | 6.50 | > 75 | 5.60 | > 75 | 3.05 | 25–75 | 4.40 | 25–75 | 6.50 |
| *H. uvarum* | 0.04 | < 25 | 4.90 | > 75 | 4.65 | > 75 | 3.15 | 25–75 | 0.14 | < 25 | 4.90 |
| *C. intermedia* | 0.03 | < 25 | 6.65 | > 75 | 9.00 | > 75 | 0.10 | < 25 | 1.79 | < 25 | 8.35 |
| *S. boulardii* | 4.35 | 25–75 | 6.65 | > 75 | 6.00 | > 75 | 5.20 | 25–75 | 5.70 | > 75 | 7.25 |

GI < 25% = high inhibitory activity
GI on 25–75% range = moderate inhibitory activity
GI > 75% = weak inhibitory activity
O.D.600 nm = final optical density of the yeast cultures
Fig. 2 Evaluation of the sensitivity of P. kluyveri, H. uvarum and C. intermedia to α-acids from hop extract. Cultures were serially diluted tenfold from 10^8 CFU/mL samples, and then diluted samples were spotted in decreasing order on agar plates.

Table 4 Tolerance of yeasts to different concentrations of NaCl in YPD medium

| Yeast     | NaCl 0.5%       | NaCl 1%       | NaCl 5%       | NaCl 10%      |
|-----------|-----------------|--------------|--------------|--------------|
| P. kluyveri | OD_{600 nm} 6.40 | OD_{600 nm} 5.90 | OD_{600 nm} 0.03 | OD_{600 nm} 0.01 |
| H. uvarum  | OD_{600 nm} 5.00 | OD_{600 nm} 4.70 | OD_{600 nm} 0.02 | OD_{600 nm} 0.02 |
| C. intermedia | OD_{600 nm} 8.80 | OD_{600 nm} 8.60 | OD_{600 nm} 6.80 | OD_{600 nm} 1.03 |
| S. boulardii | OD_{600 nm} 7.20 | OD_{600 nm} 6.50 | OD_{600 nm} 0.51 | OD_{600 nm} 0.08 |

(+) Cell growth with O.D_{600 nm} ≥ 1.0

(−) Cell growth was not sustained in the condition tested

Table 5 Auto-aggregation of P. kluyveri, H. uvarum and C. intermedia at different time points and temperatures

| Yeast | 18 °C | 28 °C | 37 °C |
|-------|-------|-------|-------|
|       | 2 h   | 20 h  | 2 h   | 20 h  | 2 h   | 20 h  |
| P. kluyveri | 16.04 ± 0.08 SC | 88.22 ± 0.46 bB | 43.80 ± 0.54 aA | 98.25 ± 1.70 aA | 26.10 ± 4.90 bB | 99.88 ± 0.12 aA |
| H. uvarum  | 9.02 ± 0.12 dB | 84.25 ± 0.64 cB | 20.00 ± 0.24 bB | 98.57 ± 1.38 aA | 25.62 ± 6.82 bA | 99.92 ± 0.08 aA |
| C. intermedia | 13.51 ± 1.91 CC | 83.75 ± 0.50 cB | 23.93 ± 0.09 cA | 99.95 ± 0.04 aA | 18.12 ± 1.65 bA | 99.87 ± 0.06 aA |
| S. boulardii | 22.02 ± 1.63 aB | 95.87 ± 0.25 aB | 38.05 ± 1.63 aA | 99.87 ± 0.07 aA | 42.25 ± 4.92 aB | 99.83 ± 0.16 aA |

Different lowercase letters within the same column indicate significant differences according to Tukey’s test (p < 0.05), comparing isolates to each other at the same temperature and incubation period. Different uppercase letters in the same row indicate significant differences (p < 0.05) in auto-aggregation percentages at different incubation temperatures, for each isolate at the same incubation period

Table 6 Co-aggregation of P. kluyveri, H. uvarum and C. intermedia with E. coli ATCC8739 at different time points and temperatures

| Yeast | 18 °C | 28 °C | 37 °C |
|-------|-------|-------|-------|
|       | 2 h   | 20 h  | 2 h   | 20 h  | 2 h   | 20 h  |
| P. kluyveri | 16.06 ± 3.16 aA | 58.03 ± 1.63 aB | 11.98 ± 0.02 bB | 64.02 ± 0.05 bB | 12.07 ± 0.15 bB | 66.02 ± 1.63 bA |
| H. uvarum  | 14.07 ± 1.64 aA | 50.15 ± 1.66 cC | 10.00 ± 1.63 cB | 60.30 ± 0.47 cB | 14.00 ± 1.59 bA | 68.11 ± 0.08 bA |
| C. intermedia | 8.12 ± 3.27 bA | 58.10 ± 1.82 aB | 0.18 ± 0.03 dB | 68.08 ± 0.17 aA | 4.15 ± 0.30 cC | 66.13 ± 1.65 bA |
| S. boulardii | 18.11 ± 1.61 ab | 57.98 ± 1.50 dC | 20.08 ± 0.17 aAB | 63.81 ± 0.16 bB | 22.10 ± 1.53 aA | 72.12 ± 0.11 aA |

Different lowercase letters within the same column indicate significant differences according to Tukey’s test (p < 0.05), comparing isolates to each other at the same temperature and incubation period. Different uppercase letters in the same row indicate significant differences (p < 0.05) in co-aggregation percentages at different incubation temperatures, for each isolate at the same incubation period.
by 5–12% when yeasts were mixed with \textit{L. monocytogenes} compared to \textit{E. coli} \((p < 0.05)\).

**Enzymatic activity and antagonistic effect against pathogens**

The enzyme activity analyses revealed that none of the yeasts were positive for gelatinase. Similarly, all of the isolates were negative for proteolytic activity. However, all of the yeasts were able to inhibit bacterial growth to different degrees. This was especially apparent for \textit{P. kluyveri} inhibition of \textit{L. monocytogenes} and \textit{H. uvarum} inhibition of \textit{S. aureus}. High inhibitory activity by the yeasts was also noted for other food contaminants (\textit{e.g.}, \textit{P. aeruginosa}), yielding similar results to those observed for the probiotic \textit{S. boulardii} (Fig. 3). \textit{P. kluyveri} LAR001, \textit{H. uvarum} PIT001 and \textit{C. intermedia} ORQ001 were also responsible for growth inhibition of the lactic acid bacteria \textit{P. pentosaceus} and \textit{L. casei}, members of genera that are frequently detected in spoiled beers [41].

**Yeasts tolerance to in vitro gastrointestinal tract simulated conditions**

The isolated yeasts were next evaluated for survival after exposure to GD and PD solutions, which simulated travel through the gastrointestinal tract. Figure 4a presents the viable cell concentrations (CFU/mL) before and after incubation in GD solution. There was no significant difference \((p > 0.05)\) in viability for any yeast after GD passage.
demonstrating that although a harsh condition, it was not enough to significantly impact cell viability.

Similarly, we measured viable cell counts before and after exposure to PD solution, in which *H. uvarum* and *C. intermedia* displayed a decrease from $10^8$ CFU/mL to $10^6$ CFU/mL ($1 \log_{10}$ decrease), while *P. kluyveri* experienced a 2 $\log_{10}$ decrease (final concentration of $10^6$ CFU/mL). The data collected were also analyzed by VI (Fig. 4b), in which all isolates presented VI $\geq 93\%$ for GD solution. In contrast, after PD solution, these VI values decreased to 84% for *H. uvarum*, 88% for *C. intermedia* and 75% for *P. kluyveri*.

**Discussion**

Wild yeasts are easily found near sugar-rich sources in the environment [1]. The challenge is to isolate them from existing microbial communities and to explore their potential industrial use. Malt extract and beer worts contain a high concentration of maltose, and to a lesser extent glucose, maltotriose, fructose and sucrose [42]. Some yeasts are not able to ferment maltose, so their attenuation profile is reduced to the fermentation of simple sugars. However, some yeasts that poorly attenuate beer wort significantly contribute to beer flavor [43, 44], are used in the production of other fermented beverages (e.g., wine, spirits and mead), or can be used in the formulation of beers with low or no alcohol content. We observed through preliminary sensory analysis that our novel isolates had the ability to produce flavors characterized as floral, fruity and phenolic, among others. In this aspect, *P. kluyveri* can be highlighted for generating a remarkable esterified banana aroma upon fermentation, similar to the organoleptic profiles described by Saerens and Swiegers [45] and Methner et al. [44]. Thus, even though non-*Saccharomyces* yeasts often display poor attenuation of beer wort, these yeasts show potential for use in sequential or co-fermentations with *Saccharomyces* yeasts. For “normal” beers, it is suggested that they be used in (a) sequential fermentations, where the wort in inoculated with the non-*Saccharomyces* strain first after cooled wort is transferred to the fermenter so that it can use the available oxygen, nutrients and simple sugars to convert them into aromatic compounds, and then a highly attenuative *S. cerevisiae* strain can be inoculated to consume the complex sugars to finish the fermentation process [3]. Alternatively, (b) co-fermentations (or controlled mixed fermentations) can be used in which there is a consortium of different yeast strains (non-*Saccharomyces* + *Saccharomyces*) combined in a single inoculum, fermenting the wort at the same time. Michel et al. [43], Lu et al. [46], Holt et al. [3], and Matraxia et al. [12] report that using these approaches, it is possible to enhance the flavor complexity and intensity of beers, wines and other fermented beverages. Also, non-*Saccharomyces* are trending for the production of non-alcoholic beers, in which they are used to inoculate low gravity worts [47].

Here, three non-*Saccharomyces* yeasts were identified and selected from among all of our novel isolates to perform characterization tests: *P. kluyveri* (LAR001), based on its probiotic potential and the production of low-alcohol beers [45, 48]; *H. uvarum* (PIT001), for its aromatic profile, contributions to beverage fermentation [7] and GRAS status (Generally Regarded as Safe) [49]; and *C. intermedia* (ORQ001), for its distinct flavors and potential antimicrobial activity [17]. Synthetic beer must fermented by these yeasts showed final pH between 4.0 and 5.5, a normal pH range for traditional ales and lagers [50], which is important to inhibit the growth of several contaminants through the enhancement of inhibitory effects of hops and affecting enzyme activity [51]. Biofilm formation represents a concern for breweries due to the difficulty of cleaning and removing cell aggregates after fermentation, so yeasts such as *H. uvarum* (PIT001) that do not display this characteristic are favored in beer production [40, 52]. However, the existence of extracellular matrices participating in the composition of...
biofilms is related to protection against spoiling microorganisms and from environmental disturbances [53], which positively impacts beer brewing. Moreover, when prospecting for probiotic properties in microorganisms, biofilm formation may indicate an extended residence time and promote the exchange of nutrients in the gut [54]. Thus, _P. kluyveri_ LAR001 and _C. intermedia_ ORQ001 were also chosen for further characterization even though they created a thin biofilm at the liquid–gas interface during fermentation.

Characterizing wild isolates permits one to explore their physiological properties, tolerance to stressful conditions, secondary metabolite production and biotechnological performance, as well as to predict their suitability for use as starter cultures for various fermentation processes [2, 5, 43]. Proteolytic activity is usually analyzed because it is related to specific flavor production in fermented products, like meat [55]. Concerning beer, proteolytic activity may prevent the formation of hazes upon chilling and during storage [56]. Not all microorganisms have this activity, as observed for our yeast isolates and in Zeng et al. [32].

Being able to withstand different pH ranges is also of great importance, for instance, for sour beer production [57] and to characterize yeasts regarding their tolerance to gastrointestinal tract conditions. Growth media formulated at pH 6.0 favors yeast growth because it is a normally tolerable characteristic when exploring new starter cultures, because some fermented foods such as meat, olives and “salted” beers require yeasts that resist high-salt conditions [20]. Different concentrations of salt can be used in industrial fermentations to favor yeast growth, ethanol production and reduce contamination risks [40, 64]. We observed that all isolates were able to grow in conditions up to 1% NaCl, while only _C. intermedia_ grew in YPD supplemented with 5% and 10% NaCl. This is a common feature observed in some species of _Candida_, which have been pointed regarding their halotolerance, as observed by Bevilacqua et al. [20] and Stratford et al. [65].

When applied in beer fermentations, it is interesting that yeasts are tolerant to hop α-acids (one of the main hop compounds with antimicrobial properties) because it can inhibit their growth [66]. In hop tolerance tests, we observed that the yeasts displayed similar growth to the control (YM medium without isomerized α-acids), suggesting resistance of these strains to high concentrations (200 ppm) of the compound. Michel et al. [43] and Methner et al. [67] demonstrate that hop compounds can have a negative effect on yeasts growth (even restricting their application in conventional beers like IPAs), but we observed sufficient iso-α-acid tolerance by all analyzed yeasts, suggesting their applicability in a wide range of extremely hopped beers.

Microorganism auto-aggregation is a necessary property for adhesion to intestinal epithelial cells, in which _P. kluyveri_ and _C. intermedia_ sustained their growth at 37 °C. The survival and tolerance of yeasts to 37 °C are also important when considering their probiotic potential, as yeasts are exposed to this temperature during transit in the gastrointestinal tract [61].

Lactic acid bacteria and acetic acid bacteria are present in the gastrointestinal tract microbiome and are able to produce significant amounts of organic acids, which can affect the viability of yeasts cells [32]. Lactic and acetic acids are also by-products released by yeasts during alcoholic fermentation [62]. Narendranath et al. [63] note that in minimal media supplemented with these organic acids, lactic acid concentrations of 0.8–1% sharply reduce yeast growth, and lower concentrations of acetic acid (0.05–0.1%) have the same effect. Based on Narendranath et al. [63] and Zeng et al. [32], we tested moderate levels of these organic acids to evaluate the acid tolerance of our isolated yeasts. We observed that lactic acid did not significantly impact the final biomass of any yeast tested, but acetic acid impacted both the biomass and viability of all strains. Considering yeast susceptibility to acetic acid is important because the food industry has explored the cytotoxic effect of this acid as a food preservative and for CIP (clean-in-place) procedures, in which it is included in cleaning products for the disinfection of brewery equipment [62].

Tolerance to ion stress (halotolerance) is an important characteristic when exploring new starter cultures, because some fermented foods such as meat, olives and “salted” beers require yeasts that resist high-salt conditions [20]. Yeast tolerance to stress caused by increases in growth temperature is well described for several _S. cerevisiae_ strains [60], but similar data for non-_Saccharomyces_ yeasts is limited to a few studies. In our work, incubation at 37 °C was limiting for _H. uvarum_ growth and partially inhibited _C. intermedia_. Steensels and Verstrepen [2] report that in cocoa fermentations and mixed-fermentations beers, when the temperature remains around 30 °C, _Hansensiaspora_ spp. are among the first microorganisms to multiply. However, when fermenting at temperatures > 35 °C, _Pichia_ spp., _Candida_ spp. and _Saccharomyces_ spp. are the main yeasts acting during fermentation. These data correlate with our findings, in which yeast isolates were able to grow in conditions up to 1% NaCl, while only _C. intermedia_ grew in YPD supplemented with 5% and 10% NaCl. This is a common feature observed in some species of _Candida_, which have been pointed regarding their halotolerance, as observed by Bevilacqua et al. [20] and Stratford et al. [65].

When applied in beer fermentations, it is interesting that yeasts are tolerant to hop α-acids (one of the main hop compounds with antimicrobial properties) because it can inhibit their growth [66]. In hop tolerance tests, we observed that the yeasts displayed similar growth to the control (YM medium without isomerized α-acids), suggesting resistance of these strains to high concentrations (200 ppm) of the compound. Michel et al. [43] and Methner et al. [67] demonstrate that hop compounds can have a negative effect on yeasts growth (even restricting their application in conventional beers like IPAs), but we observed sufficient iso-α-acid tolerance by all analyzed yeasts, suggesting their applicability in a wide range of extremely hopped beers.
rates > 80% are considered good auto-aggregators [68]. In our study, all isolates showed values > 80% after 20 h of incubation, even being > 99% at the warmer temperatures tested. Co-aggregation is an alternative mechanism to inhibit pathogenic bacterial growth in the human intestine [32]. P. kluveri, H. uvarum and C. intermedia demonstrated co-aggregation values > 50%, but we observed that temperature was a key factor during the process. Indeed, at 28 and 37 °C, the yeasts displayed greater co-aggregation values for L. monocytogenes than E. coli (p < 0.05), regardless of the yeast isolate. Antagonistic activity is one of the main properties desired in a probiotic because it represents the ability to hinder or even prevent the development of pathogenic microorganisms that penetrate through an organism mucosal sites [28]. The ability to inhibit bacterial pathogen growth by our isolated yeasts was similar—or even superior—to that observed for S. boulardii, demonstrating the probiotic potential of these yeasts. This characteristic is also important for the control of contaminants during wort production, beer fermentation, in finished beers and on brewery equipment because strains of Escherichia, Klebsiella, Pseudomonas, Staphylococcus and mainly Pediococcus and Lactobacillus are often detected in spoiled beers or participating in the mixed-fermentations of sour beers [41, 69, 70]. Gram-negative bacteria from the Enterobacteriaceae group are indirect spoilers because even though they are not usually found in finished beers, these microorganisms can cause a negative sensorial impact by off-flavor production (and promoting slow fermentation) if present throughout the process [41, 71].

The production of gelatinase by probiotics is generally analyzed in relation to a strain’s safety to use, because pathogenic microorganisms usually produce this enzyme as part of their pathogenesis [68]. As reported by Syal and Vohra [68] and Fakruddin et al. [21] in wild yeast isolates, gelatinase enzyme activity was not found in our isolates, acting as a preliminary indication of their safety. Among the three isolated yeasts evaluated here, only H. uvarum is a current member of the QPS list (Qualified Presumption of Safety), but there are no mentions for the other yeasts on this list [72]. Thus, safety tests must be conducted prior to their use in commercial products, focusing on specific characteristics such as resistance to antimicrobial agents and biogenic amine production [40].

Several microorganisms demonstrate their probiotic potential through pathogen inhibition, but they still need to overcome barriers during passage through the gastrointestinal tract, which involves gastric juice, digestive enzymes, organic acids, bile salts and considerable variations in temperature and pH, such as the acidic pH of gastric juice and alkaline pH existing in the intestine [19, 61]. Bonatsou et al. [36] and Cassanego et al. [15] demonstrate that GD solution is not generally inhibitory to yeasts, as also observed in our study. Nevertheless, our isolates were sensitive to pancreatic conditions, which decreased cell viability by 1–2 orders of magnitude. In the study by Cassanego et al. [15], isolates classified as S. cerevisiae and H. uvarum were not able to survive after exposure to pancreatic conditions, but our yeast isolates were able to resist the PD solution. Cell viability analysis during in vitro gastrointestinal tract simulation proves to be important because it is believed that the effects related to probiotics are dose-dependent, with a suggested effective dose between 10⁷ and 10⁹ CFU/mg per day [73]. Thus, our results suggest that the concentration of viable cells after gastrointestinal tract passage is within that expected for probiotic effects in humans.

Yeasts with probiotic activity are being explored in the development of functional beers, i.e., low-alcohol and non-alcoholic (NA) beers produced with herbs, amino acids, vitamins, minerals, vegetables, and/or fruits, focusing on the health benefits and nutritional value for consumers [74]. NA beers are generally produced by thermal and membrane-based methods, depending on alcohol removal after fermentation by traditional Saccharomyces yeasts, or through a biological method consisting of a controlled mashing and fermentation process that limits ethanol formation by the yeast [8, 74]. These production techniques can negatively impact beers, modifying the aromas, body and acidity of the final product [8]. Alternatively, our isolates of P. kluveri, H. uvarum and C. intermedia could be employed to produce beers with low levels of ethanol that maintain aromas and desired characteristics because they are able to ferment only simple sugars and, consequently, generate less alcohol during fermentation. In this sense, beer may also work as a delivery system for probiotic microorganisms, as demonstrated by Calumba et al. [75]. However, aiming for the development of functional beers, their probiotic potential must be confirmed, requiring more in vitro and in vivo studies.

In summary, the wild yeast isolates Pichia kluveri (LAR001), Hanseniaspora uvarum (PIT001) and Candida intermedia (ORQ001) demonstrated probiotic potential, both in relation to inhibition of pathogenic microorganisms and their tolerance to harsh conditions simulating the human gastrointestinal tract. Their biotechnological properties regarding application for beer production were also evaluated, demonstrating their contribution to the organoleptic profile and fermentation ability in a synthetic beer must, as well as indicating them as promising candidates for application in other fermentation processes (e.g., that require halotolerance). Future perspectives of this work are in vivo tests to confirm their probiotic action, larger-scale beer fermentations to stabilize them as starter cultures, and to evaluate these beers as delivery systems for probiotic microorganisms.
Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00217-022-04139-z.

Acknowledgements The present work was carried out with the support from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES)—Brazil—financial code 001, and support from Conselho Nacional de Desenvolvimento Científico (CNPq). We thank all students involved directly or indirectly with this study.

Author contributions All authors contributed to the study conception and design. Material preparation was performed by REAP and GMR. Data collection and analysis were performed by REAP. The first draft of the manuscript was written by REAP and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Funding The present work was carried out with the support from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES)—Brazil—financial code 001, and support from Conselho Nacional de Desenvolvimento Científico (CNPq).

Availability of data and material All data generated or analyzed during this study are included in this published article.

Code availability Not applicable.

Declarations

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

Compliance with ethics requirements This article does not include any human subjects or animal experiments.

Consent to participate Not applicable.

Consent for publication Not applicable.

References

1. Tikka C, Otsuru HP, Aituri N et al (2013) Isolation and characterization of ethanol tolerant yeast strains. Bioinformation 9:421–425. https://doi.org/10.6026/97320630009421
2. Steensels J, Verstrepen KJ (2014) Taming wild yeast: potential of conventional and nonconventional yeasts in industrial fermentations. Annu Rev Microbiol 68:61–80. https://doi.org/10.1146/annurev-micro-091213-113025
3. Holt S, Mukherjee V, Lieveens B et al (2018) Bioflavoring by nonconventional yeasts from wine. Fermentation 8:280–302. https://doi.org/10.3390/fermentation8060280
4. Martin V, Valera M, Medina K et al (2018) Oenological impact of the Hanseniaspora/Kloeckera yeast genus on wines—a review. Fermentation 4:76. https://doi.org/10.3390/fermentation4030076
5. Bellut K, Michel M, Zarnkow M et al (2018) Application of non-saccharomyces yeasts isolated from kombucha in the production of alcohol-free beer. Fermentation 4:66. https://doi.org/10.3390/fermentation4030066
6. Methner Y, Hutzler M, Zarnkow M, Prowald A, Endres F, Jacob F (2022) Investigation of non-Saccharomyces yeast strains for their suitability for the production of non-alcoholic beers with novel flavor profiles. J Am Soc Brew. https://doi.org/10.1008/03610470.2021.2012747
7. Gutiérrez A, Boekhout T, Gojkovicz Z, Katz M (2018) Evaluation of non-Saccharomyces yeasts in the fermentation of wine, beer and cider for the development of new beverages. J Inst Brew 124:389–402. https://doi.org/10.1002/jib.512
8. Vicente J, Calderón F, Santos A et al (2021) High potential of Pichia kluyveri and other Pichia species in wine technology. Int J Mol Sci 22:1–15. https://doi.org/10.3390/ijms22031196
9. Mattaxia M, Alfonso A, Prestianni R et al (2021) Non-conventional yeasts from fermented honey by-products: Focus on Hanseniaspora uvarum strains for craft beer production. Food Microbiol 99:103806. https://doi.org/10.1016/j.fm.2021.103806
10. Pinti FO, Lopes T, Vieira AM, Fabricio MF, Ayub MAZ, Mendes SDC, Pagani DM, Valente P (2022) Isolation, selection and characterization of wild yeasts with potential for brewing. J Am Soc Brew. https://doi.org/10.1008/03610470.2022.2031777
11. Cassanego D, Richards N, Valente P et al (2017) Identification by PCR and evaluation of probiotic potential in yeast strains found in kefir samples in the city of Santa Maria, RS, Brazil. Food Sci Technol 38:59–65. https://doi.org/10.1590/1678-457x.2017.103617
12. Pinti FO, Lopes T, Vieira AM, Oliveira RO, Gomes FG, Fabricio MF, Ayub MAZ, Mendes SDC, Pagani DM, Valente P (2022) Isolation, selection and characterization of wild yeasts with potential for brewing. J Am Soc Brew. https://doi.org/10.1008/03610470.2022.2031777
13. Younis G, Awad A, Dawod RE, Yousef NE (2017) Antimicrobial activity of yeasts against some pathogenic bacteria. Vet World 10:979–983. https://doi.org/10.14202/vetworld.2017.979-983
14. Sampaoleisi S, Briand LE, Antoni G, Peláez AL. (2022) The synthesis of soluble and volatile bioactive compounds by selected brewer’s yeasts: antagonistic effect against enteropathogenic bacteria and food spoiler – toxigenic Aspergillus sp. 13:100193. https://doi.org/10.1016/j.fochx.2021.100193
15. FAO/WHO (2001) Health and nutritional properties of probiotics in foods including power milk with live lactic acid bacteria. American Córdoba Park Hotel, Cordoba, Argentina. Accessed at: http://www.who.int/foodsafety/publications/fs_management/en/probiotics.pdf?ua¼1
16. Bevilacqua A, Perricone M, Cannarsi M et al (2009) Technological and spoiling characteristics of the yeast microflora isolated from Bella di Cerignola table olives. Int J Food Sci Technol 44:2198–2207. https://doi.org/10.1111/j.1365-2621.2009.02060.x
17. Fakruddin M, Hassain MN, Ahmed MM (2017) Antimicrobial and antioxidative activities of Saccharomyces cerevisiae. IS'TO62013, a potential probiotic. BMC Complement Altern Med 17:64. https://doi.org/10.1186/s12906-017-1591-9
18. Labbani FZK, Turchetti B, Bennamoun L et al (2015) A novel killer protein from Pichia kluyveri isolated from an Algerian soil:
purification and characterization of its in vitro activity against food and beverage spoilage yeasts. Antonie van Leeuwenhoek Int J Gen Mol Microbiol 107:961–970. https://doi.org/10.1007/s10482-015-0388-4

23. Goerges S, Aigner U, Silakowski B, Scherer S (2006) Inhibition of Listeria monocytogenes by food-borne yeasts. Appl Environ Microbiol 72:313–318. https://doi.org/10.1128/AEM.72.1.313-318.2006

24. Tiago FCP, Martins FS, Rosa CA et al (2009) Physiological characterization of non-Saccharomyces yeasts from agro-industrial and environmental origins with possible probiotic function. World J Microbiol Biotechnol 25:657–666. https://doi.org/10.1007/s11274-008-9934-9

25. Ogunremi OR, Sanni AI, Agrawal R (2015) Probiotic potentials of yeasts isolated from some cereal-based Nigerian traditional fermented food products. J Appl Microbiol 119:797–808. https://doi.org/10.1111/jam.12875

26. Yildiran H, Basyiğit Kılıç G, Karahan Çakmakçi AG (2019) Characterization and comparison of yeasts from different sources for some probiotic properties and exopolysaccharide production. Food Sci Technol 39:646–653. https://doi.org/10.1590/fst.29818

27. Staniszewski A, Kordowska-Wiater M (2021) Probiotic and potentially probiotic yeasts—characteristics and food application. Foods 10:1306–1319. https://doi.org/10.3390/foods10061306

28. Amorim JC, Piccoli RH, Duarte WF (2018) Probiotic potential of yeasts isolated from pineapple and their use in the elaboration of potentially functional fermented beverages. Food Res Int 107:518–527. https://doi.org/10.1016/j.foodres.2018.02.054

29. Zivkovic M, Cadez N, Uroic K et al (2014) Evaluation of probiotic potential of yeasts isolated from traditional cheeses manufactured in Serbia and Croatia. J Interdisc Ethnopharmacol 4:12. https://doi.org/10.5455/jie.20141128051842

30. Osburn K, Ahmad NN, Bochman ML (2016) Bio-prospecting, selection, and analysis of wild yeasts for ethanol fermentation. Zymurgy 39:81–89. https://doi.org/10.13140/RG.2.2.16952.14080

31. Preiss R, Tyrawa C, Krogerus K et al (2018) Traditional Norwegian Kveik are a genetically distinct group of domesticated Saccharomyces cerevisiae brewing yeasts. Front Microbiol. https://doi.org/10.3389/fmicb.2018.02137

32. Zeng X, Fan J, He L et al (2019) Technological properties and probiotic potential of yeasts isolated from traditional low-salt fermented Chinese fish Suan yu. J Food Biochem 43:1–14. https://doi.org/10.1111/jbfc.12865

33. Samanfar B, Shostak K, Moteshareie H et al (2017) The sensitivity of the yeast, Saccharomyces cerevisiae, to acetic acid is influenced by DOM34 and RPL36A. PeerJ. https://doi.org/10.7717/peerj.4037

34. Pereira V, Lopes C, Castro A et al (2009) Characterization for enterotoxin production, virulence factors, and antibiotic susceptibility of Staphylococcus aureus isolates from various foods in Portugal. Food Microbiol 26:278–282. https://doi.org/10.1016/j.fm.2008.12.008

35. Collado MC, Meriluoto J, Salminen S (2008) Adhesion and aggregation properties of probiotic and pathogen strains. Eur Food Res Technol 226:1065–1073. https://doi.org/10.1007/s00217-007-0632-x

36. Bonatsou S, Benítez A, Rodríguez-Gómez F et al (2015) Selection of yeasts with multifunctional features for application as starters in natural black table olive processing. Food Microbiol 46:66–73. https://doi.org/10.1016/j.fm.2014.07.011

37. Schneiderbanger H, Kooi J, Poltinger S et al (2016) Gene expression in wheat beer yeast strains and the synthesis of acetate esters. J Inst Brew 122:403–411. https://doi.org/10.1002/jib.337

38. Holt S, Miks MH, De Carvalho BT et al (2019) The molecular biology of fruity and floral aromas in beer and other alcoholic beverages. FEMS Microbiol Rev 43:193–222. https://doi.org/10.1093/femsrev/fuy041

39. Menoncin M, Bonatto D (2019) Molecular and biochemical aspects of Brettanomyces in brewing. J Inst Brew 125(4):402–411. https://doi.org/10.1002/jib.580

40. Piraine RE, Nickens DG, Sun DJ et al (2022) Isolation of wild yeasts from Olympic National Park and Moniellia megachilensis ONP131 physiological characterization for beer fermentation. Food Microbiol 104:103974. https://doi.org/10.1016/j.fm.2021.103974

41. Bokulich NA, Bamforth CW (2013) The microbiology of malting and brewing. Microbiol Mol Biol Rev 77:157–172. https://doi.org/10.1128/mmbrr.00060-12

42. Hansen B, Wasdovitch B (2005) Malt ingredients in baked goods. Cereal Foods World 50:18–22

43. Michel M, Kopecká J, Meier-Dörnberg T et al (2016) Screening for new brewing yeasts in the non-Saccharomyces sector with Torulaspora delbrueckii as model. Yeast 33:129–144. https://doi.org/10.1002/yea.3146

44. Methner Y, Hutzler M, Zarnkow M et al (2022) Investigation of non-saccharomyces yeast strains for their suitability for the production of non-alcoholic beers with novel flavor profiles. J Am Soc Brew Chem. https://doi.org/10.1080/03610470.2021.2012747

45. Saerens SMG, Swiegers JH (2017) Production of low-alcohol or alcohol-free beer with Pichia kluyveri yeast strains. Patent, US009580675B2.

46. Lu Y, Voon MKW, Chua JY et al (2017) The effects of co- and sequential inoculation of Torulaspora delbrueckii and Pichia kluyveri on chemical compositions of durian wine. Appl Microbiol Biotechnol 101:7853–7863. https://doi.org/10.1007/s00253-017-8527-7

47. Capece A, Romaniello R, Siesto G, Romano P (2018) Conventional and non-conventional yeasts in beer production. Fermentation 4:38. https://doi.org/10.3390/fermentation4020038

48. Fai AEC, da Silva JB, de Andrade CJ et al (2014) Production of probiotic galactooligosaccharides from lactose by Pseudozyma tsukubaensis and Pichia kluyveri. Biocatal Agric Biotechnol 3:343–350. https://doi.org/10.1016/j.bcab.2014.04.005

49. López S, Mateo JJ, Maicas SM (2016) Characterisation of Hanseinispora Isolates with Potential Aroma-enhancing Properties in Muscat Wines. South African J Enol Vitic 35:292–303. https://doi.org/10.21548/35-2-1018

50. Bamforth C (2001) pH in brewing: an overview. Mbaa Tq 38:1–8

51. Vrieze Kokop F, Krahl M, Hacker B, Menz G (2012) 125th Anniversary review: bacteria in brewing: The good, the bad and the ugly. J Inst Brew 118:335–345. https://doi.org/10.1002/jib.49

52. Riedl R, Fütterer J, Guderbauer P et al (2019) Combined yeast biofilm screening-characterization and validation of yeast related biofilms in a brewing environment with combined cultivation and specific real-time PCR screening of selected indicator species. J Am Soc Brew Chem 77:99–112. https://doi.org/10.1080/03610470.2019.1579036

53. Branda SS, Vik A, Friedman L, Kolter R (2005) Biofilms: The matrix revisited. Trends Microbiol 13:20–26. https://doi.org/10.1016/j.tim.2004.11.006

54. Deng Z, Luo XM, Liu J, Wang H (2020) Quorum sensing, biofilm, and intestinal mucosal barrier: involvement the role of probiotic. Front Cell Infect Microbiol 10:1–10. https://doi.org/10.3389/fcimb.2020.538077

55. Zeng X, Xia W, Jiang Q, Yang F (2013) Effect of autochthonous starter cultures on microbiological and physico-chemical characteristics of Suan yu, a traditional Chinese low salt fermented fish. Food Control 33:344–351. https://doi.org/10.1016/j.foodcont.2013.03.001
56. Priest F, Campbell I (1996) Brewing Microbiology. Springer US, Boston, MA. https://doi.org/10.1007/978-1-4757-4679-2
57. Rogers CM, Veatch D, Covey A et al (2016) Terminal acidic shock inhibits sour beer bottle conditioning by Saccharomyces cerevisiae. Food Microbiol 57:151–158. https://doi.org/10.1016/j.fm.2016.02.012
58. Murakami CJ, Wall V, Basisty N, Kaeberlein M (2011) Composition and acidification of the culture medium influences chronological aging similarly in vineyard and laboratory yeast. PLoS ONE. https://doi.org/10.1371/journal.pone.0024530
59. Reis VR, Bassi APG, da Silva JCG, Ceccato-Antonini SR (2013) Characteristics of Saccharomyces cerevisiae yeasts exhibiting rough colonies and pseudohyphal morphology with respect to alcoholic fermentation. Brazilian J Microbiol 44:1121–1131. https://doi.org/10.1590/S1517-83822014005000020
60. Munna MS, Humayun S, Noor R (2015) Influence of heat shock and osmotic stresses on the growth and viability of Saccharomyces cerevisiae SUBSC01 Microbiology. BMC Res Notes 8:1–8. https://doi.org/10.1186/s13104-015-1355-x
61. Czerucka D, Piche T, Rampal P (2007) Review article: Yeast as probiotics - Saccharomyces boulardii. Aliment Pharmacol Ther 26:767–778. https://doi.org/10.1111/j.1365-2036.2007.03442.x
62. Ludovico JMP, Rodrigues F, et al (2012) Stress and Cell Death in Yeast Induced by Acetic Acid. In: Cell Metabolism - Cell Homeostasis and Stress Response. InTech https://doi.org/10.5772/27726
63. Narendranath NV, Thomas KC, Ingledew WM (2001) Effects of acetic acid and lactic acid on the growth of Saccharomyces cerevisiae in a minimal medium. J Ind Microbiol Biotechnol 26:171–177. https://doi.org/10.1038/sj.jim.7000090
64. Corte L, Rellini P, Lattanzi M et al (2006) Diversity of salt response among yeasts. Ann Microbiol 56:363–368. https://doi.org/10.1007/BF03175033
65. Stratford M, Steels H, Novodovorska M et al (2019) Extreme osmo-tolerance and halotolerance in food-relevant yeasts and the role of glycerol-dependent cell individuality. Front Microbiol 10:1–14. https://doi.org/10.3389/fmicb.2018.03238
66. Osburn K, Amaral J, Metcalf SR et al (2018) Primary souring: A novel bacteria-free method for sour beer production. Food Microbiol 70:76–84. https://doi.org/10.1016/j.fm.2017.09.007
67. Methner Y, Hutzler M, Matouklová D et al (2019) Screening for the brewing ability of different non-Saccharomyces yeasts. Fermentation. https://doi.org/10.3390/fermentation5040101
68. Syal P, Vohra A (2013) Probiotic potential of yeasts isolated from traditional indian fermented foods. Int J Microbiol Res 5:390–398. https://doi.org/10.9735/0975-5276.5.2.390-398
69. Ashtavinayak P, Elizabeth HA (2016) Review: gram negative bacteria in brewing. Adv Microbiol 06:195–209. https://doi.org/10.4236/am.2016.63020
70. Piraine REA, Leite FPL, Bochman ML (2021) Mixed-culture metagenomics of the microbes making sour beer. Fermentation 7:174. https://doi.org/10.3390/fermentation7030174
71. Rodhouse L, Carbonero F (2019) Overview of craft brewing specificities and potentially associated microbiota. Crit Rev Food Sci Nutr 59:462–473. https://doi.org/10.1080/10408398.2017.1378616
72. Panel EB, Koutsoumanis K, Allende A, et al (2021) Updated list of QPS-recommended biological agents for safety risk assessments carried out by EFSA. https://doi.org/10.5281/ZENODO.4917383
73. Minelli EB, Benini A (2008) Relationship between number of bacteria and their probiotic effects. Microb Ecol Health Dis 20:180–183. https://doi.org/10.1080/08910600802408095
74. Habschied K, Živković A, Krstanović V, Mastanjević K (2020) Functional beer—a review on possibilities. Beverages 6:1–15. https://doi.org/10.3390/beverages6030051
75. Calumba KF, Vondel R, Bonilla F et al (2021) Ale beer containing free and immobilized Lactobacillus brevis, a potential delivery system for probiotics. Food Produc Process and Nutr 3:8–24. https://doi.org/10.1186/s43014-021-00051-32

Publisher’s Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.