Brewing and Probiotic Potential Activity of Wild Yeasts *Hanseniaspora Uvarum* PIT001, *Pichia Kluyveri* LAR001 and *Candida Intermedia* ORQ001

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Research Article

Keywords: beer, Candida intermedia, Hanseniaspora uvarum, non-conventional, Pichia kluyveri, probiotic potential, yeasts

Posted Date: August 11th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-772136/v1

Abstract

Non-conventional yeasts can be isolated from a wide range of environmental sources, often found in beverage industry in mixed fermentations, in which the microorganisms' inoculum usually is not fully known. It is important to know starter cultures, since in addition to favoring reproducibility, other properties can be discovered. Thus, the objective of this work was to identify and characterize yeasts isolated from environment, evaluating their probiotic potential and possible use in brewery. Isolates were obtained from flowers, fruits, leaves and mixed-fermentation beers, being identified by PCR. Yeasts with promising activity were evaluated regarding their growth under different pHs, temperature and presence of organic acids. To explore probiotic potential, *in vitro* tests were performed of antimicrobial activity and co-aggregation with food pathogens, auto-aggregation, and survival in simulated gastrointestinal tract conditions. In our study, *Pichia kluyveri* (LAR001), *Hanseniaspora uvarum* (PIT001) and *Candida intermedia* (ORQ001) were selected among 20 isolates. *P. kluyveri* was the only one that tolerated pH 2.5. Lactic acid was not inhibitory, while acetic acid and incubation at 37 °C had a partially inhibitory effect on yeasts growth. All yeasts tolerated α-acids from hops and NaCl up to 1%. It is suggested that isolates are able to adhere to intestinal cells and influence positively the organism in combating pathogens, as they showed auto-aggregation rates above 99% and antagonistic activity to pathogenic bacteria. The yeasts tolerated gastric environment conditions, however were more sensitive to pancreatic conditions. We conclude that isolated non-conventional yeasts showed probiotic potential and promising application in beer fermentation.

Introduction

Yeasts are ubiquitous in the environment, being often isolated from the microbiota of fruits, plant exudates, soil and insects (Tikka et al., 2013). Non-conventional yeasts, also named non-*Saccharomyces*, represent an interesting alternative for industry in the development of new products (Steensels and Verstrepen, 2014; Holt et al. 2018). Yeasts like *Brettanomyces* spp., *Candida* spp. and *Pichia* spp. were considered only contaminants in brewing environments, however they are currently considered important components in high-value added beers (Michel et al., 2016). These fermenting microorganisms are often found in open fermentations (or exposed to environment), a process which can be unpredictable and can generate large economic losses to breweries (Steensels and Verstrepen, 2014). In the brewing industry, applying pure cultures in fermentations tends to be the first choice, however it is observed in other industries (wine, fermented dairy products, among others) that addition of multiple strains in a controlled manner is a common practice. Mixed fermentations make possible to create products with unique aromatic *bouquets* and distinct nutritional characteristics, obtained according to the concentration of strains used in fermentation process (Holt et al., 2018).

Among wild yeasts, *Hanseniaspora* spp. have been described as predominant on some fruits surface (especially grapes) and are known for significantly contribute in the sensory profile of different wine styles (Martin et al., 2018). Also, strains from other genera like *Pichia* spp. and *Candida* spp. have already been used by researchers and breweries to produce beers with low or no alcohol content, representing an important application which can be protected by patents (Bellut et al., 2018). Metabolic aspects regarding the use of different substrates, growth at different temperatures and pHs, halotolerance, osmotolerance and enzymatic activity must be well characterized, then industrial application of these isolates can be determined as viable (Cassanego et al., 2017).

Some yeasts have the ability to produce antimicrobial compounds, capable of inhibiting the growth of pathogenic bacteria and other fungi (Younis et al., 2017). This characteristic and others are important to confer the classification as "probiotics", which by definition are live microorganisms that, when administered in adequate amounts, confer benefits to host's health (FAO, WHO, 2001). To be considered probiotics, these microorganisms need to have some functional properties such as: not being pathogenic, resisting harsh conditions found on human gastrointestinal (GI) tract, cell adhesion capacity, having immunostimulatory action, among others (Bevilacqua et al., 2009; Fakruddin et al., 2017).

Saccharomyces cerevisiae and *S. boulardii* are two yeasts that stand out for their probiotic activity. The search for non-*Saccharomyces* yeasts with probiotic potential arises from different sources, such as plant and animal fermented foods as

well as wild fermented beverages (Amorim et al., 2018; Cassanego et al., 2017; Zivkovic et al., 2014). Depending on the isolate, different yeast species may have as much probiotic potential as those of *Saccharomyces* genus. Thus, the objective of this work was to isolate wild yeasts, identifying and characterizing their fermentation capacity, sensory contribution for beer worts and their probiotic potential.

Materials And Methods

Wild yeasts isolation

Using a sterile swab soaked in YM medium (0.3% yeast extract, 0.3% malt extract, 0.5% bacteriological peptone and 1% glucose) there was a friction on surface of the fruits strawberry (*Fragaria ^{x} ananassa*), blackberry (*Morus nigra*), cherry (*Eugenia uniflora*), orange (*Citrus ^{x} sinensis*) and butiá (*Butia capitata*), being subsequently inoculated in a tube containing YM added with ampicillin (100 µg/µL). Cultures were incubated for 48 h at 28 °C with constant agitation of 150 rpm. For flowers and leaves of orchid (*Aspasia lunata*), pitaya (*Hylocereus undatus*), vine (*Vitis vinífera*), and *Red Flanders* and *Old Ale* beers, the same procedure was performed. All samples collected for yeasts isolation were obtained in the city of Pelotas (Latitude -31.776, Longitude -52.3594 31° 46’ 34” South, 52° 21’ 34” West), throughout spring/summer period, with daily temperatures around 25 to 30 °C.

Yeasts were isolated by streaking samples from cultures on YM agar + ampicillin. Plates were kept at 28 °C for 72 h for yeasts growth. The yeast morphology was observed using optical microscope BLUE1600BA-L-BT (Biofocus, Brazil) at 1000x magnification. Isolates were stored by freezing (- 80°C) using glycerol 30%, with identification codes being given to each isolate.

Fermentation of standard beer wort

In order to evaluate fermentation ability in standard beer worts, 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 was used. Iso-α-acid from hops (HOPSTEINER, Germany) was added to final 15 IBU (International Bitterness Units). All isolates were cultivated in YM medium over 48 h, 28 °C and 150 rpm condition prior fermentation test. Yeast cells concentration was obtained by counting in a Neubauer chamber, then inoculum for fermentation was standardized at a concentration of 10^9 cells, which were obtained by centrifugation at 1.500 × g for 5 min in DTR-16000 centrifuge (DAIKI, Korea). Cells were suspended with 15 mL of malt extract 11 °P, subsequently inoculated in the total volume for fermentation.

Fermentation was conducted during 14 days, in an incubator at 22 °C, with no agitation. Production and release of CO_2 was monitored through bubble formation in an air-lock piece, while biofilm formation, on medium surface, was visually observed. After this period, final density and pH were evaluated using a pHmeter (KASVI, Brazil), a densimeter (INCOTERM, Brazil) for density in g/cm³ measure and a refractometer (AKSO, Brazil) for measure in Brix degrees, data used to calculate percentage of attenuation by each isolate. Aroma resulting from malt extract fermentation was evaluated by simple sensory analysis, as conducted in Osburn *et al.* (2016) study. *Saccharomyces cerevisiae* YT001 (YEASTECH, Brasil) was used in this experiment as a reference yeast.

DNA extraction, PCR and sequencing

Total DNA from each isolate was extracted following the protocol described by Preiss et al. (2018)and its concentration quantified in Nanovue™ (Biochrom). Identification at species level was performed using ITS1 and ITS4 primers, which are responsible for amplifying the repetitive region of the 5.8S rRNA gene and ITS flanking regions (Internal Transcribed Spacer). PCR reaction was performed using 0,7 uL of previously extracted DNAs (1 ug/uL), 22 uL of Master Mix (Ludwig Biotecnology, Brazil), 1 uL (0.5 uM) of ITS1 primer (5'TCCGTAGGTGAACCTTGCGG) and 1 uL (0,5 uM) of ITS4 primer (5'TCCTCCGCTTATTGATATGC). Following incubation conditions for PCR were used: initial denaturation at 95 °C for 10 min, 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 final

extension at 72 °C for 8 min. PCR products sizes were analyzed by electrophoresis of 10 uL in 1% agarose gel (w/v) in TBE buffer (Tris 0.89 M, EDTA 0.02 M, Boric Acid 0.89 M), under 100 V, 500 mA over 2 h. Agarose gels were stained with 5 uL of ethidium bromide and bands sizes were predicted by comparison with 1 kb Plus DNA Ladder (ThermoFischer Scientific, United States).

The PCR product of each isolate was purified using GFX PCR DNA and Gel Band Purification (GE Healthcare, United States), quantified with Nanovue^{TM} and then submitted at an approximate concentration of 50 ng/l uL for sequencing by ACTGene Análises Moleculares company (Brazil) through Applied Biosystems AB-3500 platform.

Characterization of yeasts isolates

Evaluation of yeast growth in different stress conditions

Wild yeasts *Hanseniaspora uvarum* (PIT001), *Pichia kluyveri* (LAR001) and *Candida intermedia* (ORQ001) were selected in addition to *S. boulardii*, a yeast which was already part of microorganism bank of Microbiology Laboratory in the Technological Development Center of the Federal University of Pelotas.

Cultivation tests were performed in 10 mL of YPD medium (1% yeast extract, 2% bacteriological peptone, 2% glucose), following a protocol adapted from Zeng et al. (2019). Analysis of resistance to different conditions were tested: lactic acid 1%, acetic acid 0.2% and pHs 2.5, 6.0 and 8.0. Yeast cultures were maintained for 72 h, with samples being collected at 0 h, 4 h, 24 h, 48 h and 72 h, analyzing their biomasses by absorbance reads at 600nm in Biochrom Ultrospec-10 spectrophotometer (Amersham Biosciences, United States) and CFU/mL (Colony Forming Units). Incubation temperature was maintained constant at 28 °C, in an orbital shaker at 150 rpm. In addition, yeast growth was evaluated in YPD pH 6.0 at 37 °C, following same procedures for samples collection and incubation of previous tests. Growth index (GI) was calculated based in Zeng et al. (2019) and Bevilacqua et al. (2009) method, following the equation: GI= AbSs/AbSc × 100, where AbSs is the absorbance of the samples in different pH, temperature and acid type presence, and AbSc is the absorbance of the control samples. Values < 25% were considered inhibitory for microorganisms' growth, between 25% - 75% partially inhibitory and > 75% growth was similar to respective yeast culture in optimal conditions (YPD pH 6.0, incubated at 28 °C).

Selected yeasts were also cultivated in YPD with different concentrations of Sodium Chloride (NaCl) salt, ranging from 0.5%, 1%, 5% and 10% (p/v), to assess the ability to tolerate ionic stress by halotolerance. Yeast growth was evaluated after 48 h at 28 °C by optical density, and if $OD_{600nm} \geq 1.0$, it was considered that yeast isolate was able to survive and grow in this condition.

Sensitivity to α-acids (spot test)

The analysis of yeast tolerance to α-acids from hops was performed based on adapted protocol from (Samanfar et al., 2017). Aiming the evaluation of yeast sensitivity to α-acids, it was added to YM agar medium 200 ppm of isomerized hop extract 30% (HOPSTEINER, Germany), and into plates containing this medium were applied 10 µL of 10X dilutions of yeast cultures in initial concentration of 10^8 CFU/mL. Plates were incubated for 48 h at 28 °C.

Determination of proteolytic activity

Protease activity was evaluated following protocol established by Zeng et al. (2019). Briefly, skim milk agar medium was used (1% skim milk, 0.1% glucose, 0.5% bacteriological peptone, 0.25% yeast extract, 1.5% agar), in which 10 µL from yeast cultures at 10^8 CFU/mL were applied. Positive result should be observed with formation of a halo around the drop, after an incubation during 48 h at 30 °C.

Gelatinase test

Gelatinase activity was evaluated according to Pereira et al. (2009) protocol. Culture medium was prepared with 1% yeast extract, 1.5% bacteriological peptone and 12% gelatin. Isolates were cultured for 24 h at 28 °C, then transferred to the medium containing gelatin with a platinum loop. Tubes were incubated at 30 °C for 7 days, and after that were maintained under refrigeration (4 °C – 10 °C) for 30 minutes. Positivity (i.e. enzymatic activity) in the test was based on the conversion of semi-solid medium into liquid. As a positive control, *Staphylococcus aureus* ATCC 25923 was used.

Auto-aggregation and co-aggregation

Auto-aggregation and co-aggregation tests were performed following Collado et al. (2008) protocols. Briefly, yeasts were cultivated for 24 h at 30 °C in YM medium, centrifuged for 5 min at 2.000 × g and absorbance OD_{600nm} adjusted to 0.25 ± 0.02 using Phosphate-Buffered Saline (PBS). Auto-aggregation test was conducted under 18 °C, 30 °c and 37 °C. Absorbance at 600nmwas read at 2h and 20 h and these values were used in the equation [1-A_{600nm} of final suspension/A_{600nm} of initial suspension) x 100] for results as percentage.

In the co-aggregation test, pathogens *Escherichia coli* ATCC8739 (Gram-negative bacterium) and *Listeria monocytogenes* ATCC 7644 (Gram-positive bacterium) were cultivated for 24 h at 37 °C in Brain Heart Infusion (BHI) medium, centrifuged during 5 min at 8.000 × g, then their absorbances at 600nm were adjusted to 0.25 ± 0.02 using PBS solution. Equal volumes of pathogen and yeast were mixed (1:1) and incubated under the same conditions as performed in auto-aggregation test, as well as absorbance reads times. Results for co-aggregation were expressed as percentage, originated from the equation $[(A_{pat} + A_{isol}) - (A_{mix})/(A_{pat} + A_{isol})] \times 100$, in which "$A_{pat} + A_{isol}$" represents the absorbance value for pathogen + isolate in time 0 h and "A_{mix}" is the absorbance of microorganisms' mixed suspensions in different periods of time.

Antimicrobial activity test

Bacteria related to foodborne diseases (FBDs) *Pseudomonas aeruginosa* ATCC27853, *L. monocytogenes* ATCC 7644, *S. aureus* ATCC25923, *E. coli* ATCC8739 and a wild isolate of *Klebsiella* sp. were used to test the capacity of isolated yeasts to inhibit growth of bacterial pathogens. Antimicrobial activity test was carried out according to the double-layer technique protocol presented by Amorim et al. (2018), with values of inhibition halos being expressed in millimeters (halo + drop). Following identifications were designated for inhibition capacity: + + + (inhibition zone diameter ≥ 24 mm), + + (16 – 23 mm), + (8 – 16 mm) and – (≤ 7mm); so, the larger the halo, greater the inhibitory power by yeast.

Gastrointestinal tract *in vitro* simulation

Gastrointestinal tract simulation tests were performed based on protocol described by Bonatsou et al. (2015), with some adaptations. Simulation of GI tract conditions was performed using two solutions: Gastric Digestion (GD) and Pancreatic Digestion (PD). Gastric digestion solution was prepared with NaCl (2.05 g/L), KH_2PO_4 (0.60 g/L), $CaCl_2$ (0.11 g/L), KCl (0.37 g/L), pH 2.0 adjusted with HCl 1 M and autoclaved for 15 min at 121 °C; after sterilization, pepsin (0.0133 g/L) and lysozyme (0.01 g/L) were added. Pancreatic digestion solution was prepared with bile salts (3.0 g/L), Na_2HPO_4 (26,9 g/L), NaCl (8,5 g/L), pH 8.0 adjusted with HCl 1 M; after sterilization, pancreatin enzyme (0.1 g/L) was added. Yeasts were submitted to simulations of GD and PD, with solutions applied separately and in sequence.

Initially, 1 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 reaching 10^8 CFU/mL concentration. After collecting a sample, yeast cultures were centrifuged at 1.500 × g for 10 min, washed with GD solution, and then suspended in 10 mL of the same solution. Incubation was carried out at 37 °C for 2.5 h, under agitation at 200 rpm in order to simulate peristaltic movements, and at test ending a sample was collected. Then cultures were centrifuged again at 1.500 × g for 10 min, washed with PD solution, and suspended 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 previous test. Viable yeast cells were detected according to yeast counts before and after each treatment.

Concentrations of viable yeast cells were used to determine viability index (VI) during the test, based on the equation proposed by Zeng et al. (2019): VI=$log\ N_t / log\ N_0$ x 100, in which N_t is related to yeast concentration at specific time and N_0 refers to the initial cell concentration. The test was also performed in an isolated manner, in which it was split in two independent tests, one with GD solution and another one with PD solution, aiming to obtain specific VI for PD step. Again, yeasts started these tests with a concentration of 10^8 CFU/mL.

Statistical analysis

Data were analyzed by analysis of variance (ANOVA) and Student's *t* test to determine significant difference ($p<0.05$) between means. All statistical analyzes were performed using GraphPad Prism 7 software.

Results

Wild yeast isolation and identification

Morphological characteristics of colonies and cells, as well as the respective source of isolation are shown in table 1. PCR-amplified products were used in Blast N tool, which enabled the identification of 20 wild yeast isolates, with 9 different species (>99% similarity and identity).

Fermentation of standard beer wort

We observed that *H. uvarum* isolates maintain as apparent attenuation between 5% - 18%, with emphasis on *H. uvarum* PIT001, responsible for the lower final pH and the highest apparent attenuation (18%) among *H. uvarum* isolates (table 2). Isolates classified as composing *Candida* genus showed important differences in aromatic profile, with *C. intermedia* producing aromas similar to wood, floral, spice and clove-like, while *C. manassasensis* contributed with citrus and tropical sensory notes. For both *C. terrestris* and *P. manshurica* yeasts, no apparent attenuation was observed, as well as a production of aromatic notes that stood out. *P. kludriavzezii* (also known as *Issatchenkia orientalis*) showed an apparent attenuation of 9%, with strong aromatic notes referring to solvent, phenolic, grape and green apple. *P. kluyveri* isolate presented 5% of apparent attenuation, with moderate acidification and an aromatic profile similar to commercial strains of *S. cerevisiae*, being possible to note esters referring to banana.

The *S. cerevisiae* isolate PITS002 was the only one identified as wild *Saccharomyces* yeast, with a fermentative profile very similar to commercial strain YTOO1, however the main difference was observed related to aroma produced, which wild strain originated fruity notes and a variety of esters, mostly banana. It was noted for *I. terricola* a low apparent attenuation (5%), however remarkable sensory notes referring to *funky* were detected, descripted as barnyard and horse blanket-like.

Yeasts culture under different stress conditions

Growth index (GI)

The cultures of *P. kluyveri* (LAR001), *H. uvarum* (PIT001), *C. intermedia* (ORQ001) and the control *S. boulardii* were evaluated under different stress conditions, in which biomasses produced were analyzed by absorbance at OD_{600nm}. For GI determination, only final absorbance was evaluated. Observing growth index (Table 3), it was possible to observe that yeasts presented growth similar to the control (GI > 75%) when incubated in YPD pH 8.0. Low pH was an inhibitory condition to *H. uvarum* and *C. intermedia* development (GI < 25%), causing to these yeasts not to increase their biomass over time when cultivated at pH 2.5; it was detected that *S. boulardii* culture was also impacted in this condition, it being partially inhibitory for its growth (GI 25% - 75%). We observed that *P. kluyveri* adapted itself and tolerated both media with pH 2.5 and 8.0, since its biomass had significant increase already at the beginning of the cultivation, reaching OD600nm greater than 5.

Lactic acid at 1% concentration was not inhibitory to any yeast, what was demonstrated on growth index values. Yeast cultivation in YPD medium containing 0.2% acetic acid revealed that *P. kluyveri* and *H. uvarum* were not able to overcome

biomass above OD_{600nm} 3.2 throughout the test, demonstrating this was a condition with partial growth inhibition (GI 25% - 75%). *C. intermedia* was the only yeast that failed to multiply its biomass, which was an inhibitory condition for this yeast. As observed in GI for 37 °C condition, this temperature was inhibitory for *H. uvarum* and *C. intermedia* growth, and partially inhibitory for *P. kluyveri*.

Cell viability evaluation

When yeasts were incubated in the condition considered optimal for their growth (YPD pH 6.0, incubation temperature 28 °C), it was observed a same behavior for all yeasts: they started the test (4 h) with 10^5 CFU/mL, increased their growth to 10^8 CFU/mL in 24 h and 48 h time points and then ending (72 h) with 10^7 CFU/mL (Fig. 1).

C. intermedia and *H. uvarum* presented respectively 10^4 CFU/mL and 10^5 CFU/mL at the beginning (4h) of YPD pH 2.5 test, however due to inability to withstand this hostile environment, no live cells were found in the next time points. Both *P. kluyveri* and *S. boulardii* already had 10^5 CFU/mL at the beginning of their cultivation, with a viability peak being reached at 48 h (10^7 CFU/mL). However, after 72 h *P. kluyveri* presented a decrease of 1 $\log_{10}$ in CFU/mL concentration, ending the test with 10^6 CFU/mL. During YPD pH 8.0 test, only *P. kluyveri* started (4h) with 10^6 CFU/mL, while all other yeasts presented 10^5 CFU/mL. *P. kluyveri*, *C. intermedia* and *H. uvarum* showed the highest live cell concentration at 24 h time point (10^8 CFU/mL), maintaining same concentration at 48 h for *C. intermedia* and *H. uvarum* or decreasing to 10^7 CFU/mL in *P. kluyveri* culture. The only yeast that maintains a concentration of 10^8 CFU/mL after 72 h was *C. intermedia*, proving to be the most tolerant and well-adapted to basic pH.

Responses of *P. kluyveri* and *H. uvarum* to acetic acid-induced stress were similar, where both yeasts reached 10^4 CFU/mL at 24 h of culture and then remained at 10^6 CFU/mL in 48 h and 72 h time points. *C. intermedia* started the test with 10^4 CFU/mL (4 h) and no longer showed capacity to increase live cell concentration, revealing its difficulty in resisting acetic acid stress. Lactic acid presence in culture medium revealed that although *P. kluyveri* and *H. uvarum* reached a concentration of 10^7 CFU/mL in 48 h time point, after 72 h their cultures had a decrease of 1 $\log_{10}$ in cell concentration, totaling 10^6 CFU/mL for both. The yeast *C. intermedia* was the only one that maintained a prolonged period of adaptation in this condition, between 0 h – 24 h (10^4 CFU/mL), and then keeping growing until the end of the experiment, presenting 10^8 CFU/mL at 72 h time point.

Experiment conducted at 37 °C revealed that *C. intermedia* was able to grow up to 10^6 CFU/mL in 24 h, *P. kluyveri* and *S. boulardii* up to 10^7 CFU/mL and only *H. uvarum* demonstrated the inability to tolerate this harsh condition, representing a limiting factor for its growth. Concentration of 10^7 CFU/mL after 72 h was observed only for *S. boulardii*, while *P. kluyveri* and *C. intermedia* remained at 10^6 CFU/mL at the experiment ending.

Tolerance to α-acids from hops

In the analysis of yeasts tolerance to hop α-acids we observed that 200 ppm of this compound was tolerated by the three isolates, as well as *S. boulardii*, a yeast genotipically close to *S. cerevisiae* and with similar biological behavior. Figure 2 shows different dilutions of yeasts culture applied in solid culture medium containing isomerized hop extract, from which we could determine that *P. kluyveri, H. uvarum* and *C. intermedia* were resistant to high concentrations of this compound.

Halotolerance

Regarding salinity (ionic) tolerance, all yeasts were able to grow up to a concentration of NaCl 1%, however only *P. kluyveri* and *C. intermedia* are able to tolerate the ionic stress created by NaCl at a concentration of 5%. Also, at the highest concentration tested (NaCl 10%) only *C. intermedia* was able to maintain its growth, obtaining $O.D._{600nm}$ > 1.0 (as shown in table 4).

Auto-aggregation and co-aggregation

It was possible to observe auto-aggregation characteristic was affected when at 18 °C, in which all yeasts presented a lower percentage of auto-aggregation (< 90%) than those observed at 28 °C and 37 °C ($p<0.05$). Yeasts showed high auto-aggregation capacity within 20 h (> 98%) under temperatures of incubation between 28 to 37 °C, with no statistical differences among the three isolates and incubation temperatures (table 5).

Co-aggregation analysis with *E. coli* (table 6) and *L. monocytogenes* (table 7) revealed temperature also influenced on percentage of aggregation, it being less than 60% for all yeasts at 18 °C. Values for co-aggregation increase when evaluated at temperatures of 28 °C and 37 °C, remaining between 65 – 70% after 20 h at 37 °C for all isolates. It could be also observed in these two temperatures that co-aggregation percentages are higher when yeasts are mixed with *L. monocytogenes,* increasing the rates between 5 – 12% when compared to *E. coli* ($p<0.05$).

Enzymatic activity and antagonistic effect against pathogens

The test performed to analyze enzyme activity showed that none of the yeasts had positivity for gelatinase, thus all of them were considered negative for its activity (table 8). During yeast characterization, proteolytic activity was also evaluated, and the negative reaction was found again for all isolates.

We observed all yeasts were able to inhibit bacterial growth in different degrees (table 8). We could highlight the inhibition performed by *P. kluyveri* for *L. monocytogenes* and by *H. uvarum* for *S. aureus*, in which a high capacity of inhibition was detected. For other food contaminants, for example *P. aeruginosa*, it was also found high inhibitory activity being performed by isolates, even having similar results to those observed for probiotic *S. boulardii* (Fig. 3).

Yeasts tolerance to *in vitro* GI tract simulated conditions

Isolated yeasts were evaluated regarding their survival after submission to GD and PD solutions, what simulated a complete trajectory in GI tract. Figure 4a presents live cells concentration (CFU/mL) before and after yeasts incubation in GD solution. There was no statistical difference ($p<0.05$) for any yeast after GD passage, demonstrating that although a harsh condition, it was not enough to significantly decrease cell viability count.

We observed viable cell count before and after pancreatic digestion solution, in which *H. uvarum* and *C. intermedia* showed a decrease from 10^8 CFU/mL to 10^7 CFU/mL (1 $\log_{10}$ decrease), while for *P. kluyveri* it was observed a final concentration of 10^6 CFU/mL (2 $\log_{10}$ decrease). Data collected could also be analyzed by viability index (Fig. 4b), in which all isolates presented VI ≥ 93% for GD solution. Meanwhile, after PD solution, these rates decreased to around 88% for *H. uvarum* and *C. intermedia*, and 75% for *P. kluyveri.*

Discussion

Wild yeasts are easily found in sugar-rich sources (Tikka et al., 2013), the challenge is to isolate them from existing microbiota and to explore their potential for use in industry. Malt extract is composed of maltose, and to a lesser extent glucose, maltotriose, fructose and sucrose (Hansen and Wasdovitch, 2005). Some yeasts are not able to ferment maltose, so their attenuation profile is reduced to fermentation of simple sugars. However, yeasts with a low percentage of attenuation in beer wort contribute to their flavors (Michel et al., 2016), being also used in the production of other fermented beverages (e.g. wine, spirits, mead) or even they can be used in the formulation of beers with low or no alcohol content. We observed through preliminary sensory analysis that isolates had the ability to produce flavors like floral, fruity, phenolic, among others. In this aspect, *P. kluyveri* can be highlighted, which in its fermentation presented a remarkable esterified banana aroma, similar to flavors described by Saerens and Swiegers (2017).

Three yeasts were selected from all isolates to perform characterization tests: *P. kluyveri* (LAR001), based on its probiotic potential and production of low-alcohol beers (Fai et al., 2014; Saerens and Swiegers, 2017), *H. uvarum* (PIT001), for its aromatic profile, contributions to the fermentation of beverages (Martin et al., 2018) and GRAS status (Generally Regarded as Safe) (López et al., 2016) and *C. intermedia*, for their distinct flavors and potential antimicrobial activity (Younis et al., 2017).

Proteolytic activity is usually analyzed because it is related to specific flavors production in fermented products, like meat (Zeng et al., 2013), however not all microorganisms have this activity as observed in our study and in Zeng et al. (2019). Properties like ability to withstand different pH ranges are of great importance, for sour beer production (Rogers et al., 2016) and mainly to characterize yeasts regarding their tolerance to GI tract conditions. Media formulated with pH 6.0 favors yeast growth because it is a normally tolerable acidity (Murakami et al. 2011), configuring it as a standard pH in many culture media for these microorganisms. In YPD pH 8.0 test, all yeast cultures had the same cell concentration as observed at pH 6.0 after 72 h, what indicates the adaptability to basic pH without significant impacts in their cell viability. It was observed not all yeasts were able to maintain cell multiplication in different pH ranges, as noted for *C. intermedia* and *H. uvarum*, which did not tolerated pH 2.5. Analyzing cell viability at pH 2.5, it was observed for *P. kluyveri* 1 $\log_{10}$ less in cell concentration value than that observed in other pHs, what could be related to physiological disturbances caused by extremely low pH, such as loss of minerals to the extracellular environment, decreased in trehalose levels and cell aging (Murakami et al., 2011; Reis et al., 2013).

Yeasts tolerance to stress caused by increase in incubation temperature is well described for several *S. cerevisiae* strains (Munna et al., 2015), however data for non-*Saccharomyces* yeasts are still reduced to a few studies. In our work, incubation temperature of 37 °C was shown to be a limiting growth condition for *H. uvarum*, and partially inhibiting *C. intermedia*. Steensels and Verstrepen (2014) reported for cocoa fermentations and mixed fermentations beers, when temperature remains around 30 °C, *Hanseniaspora* spp. is among the first microorganisms to multiply, however following fermentation process with temperatures above 35 °C, *Pichia* spp., *Candida* spp. and *Saccharomyces* spp. are main yeasts acting in fermentation. These data corroborate with our findings, in which *P. kluyveri* and *C. intermedia* sustained their growth in 37 °C incubation test. Survival and tolerance of yeasts to 37 °C incubation are also important to know their probiotic potential, as yeasts will be submitted to this temperature during transit in GI tract (Czerucka et al., 2007).

Lactic acid bacteria and acetic acid bacteria compose the microbiota present in GI tract and are able to produce significant amounts of organic acids, which can affect viability of yeasts cells (Zeng et al., 2019). We observed that lactic acid presence did not significantly impact final biomasses of any yeasts tested; meanwhile, acetic acid impacted both biomass and viable cell concentration of all yeasts.

Tolerance to ionic stress (halotolerance) is an important characteristic when exploring new starter cultures, since some fermented foods such as meat, olives and “salted” beers demand yeasts in their process that resist this condition (Bevilacqua et al., 2009). Thus, it was observed that all isolates were able to grow in concentrations up to 1% of NaCl, while only *P. kluyveri* and *C. intermedia* had same ability in YPD with NaCl 5% added. As described in Bevilacqua et al. (2009) and Stratford et al. (2019) in which were highlighted some species of *Candida* spp. regarding its tolerance to NaCl, we observed in our study *C. intermedia* was the only one able to grow in YPD with NaCl 10%. When applied in beer fermentations, it is interesting that yeasts are tolerant to hop α-acids, since it can inhibit their growth (Osburn et al., 2018). In the hop tolerance test, it was observed yeasts having similar growth to the control (YM medium without iso- α-acids), what suggested resistance by yeasts at high concentrations (200 ppm) of the compound.

Microorganism auto-aggregation is a necessary property for adhesion to intestinal epithelial cells, in which rates above 80% are considered good auto-aggregators (Syal and Vohra, 2013). In our study all isolates showed values higher than 80% after 20 h of assay, even being above 99% at the warmer temperatures. Co-aggregation is an alternative mechanism to inhibit pathogenic bacteria growth in human intestine (Zeng et al., 2019). *P. kluyveri, H. uvarum* and *C. intermedia* demonstrated co-aggregation values above 50% in co-aggregation tests, however it was observed that temperature was a key factor during the process. It was also possible to identify in 28 °C and 37 °C incubation temperatures yeasts showed greater co-aggregation

values for *L. monocytogenes* than *E. coli* ($p<0.05$), regardless the isolate. Antagonistic activity is one of the main properties desired in a probiotic, because represents the ability to hinder or even prevent the development of pathogens that penetrate through organism mucosal sites (Amorim et al., 2018). The ability to inhibit bacterial pathogens growth by isolated yeasts was similar to observed for *S. boulardii*, and for some bacteria even superior, what demonstrates the probiotic potential of these yeasts.

The production of gelatinase by probiotics is generally analyzed for being related to safety in its use, because pathogenic microorganisms usually produce this enzyme as part of their pathogenesis (Syal and Vohra, 2013). As observed by Syal and Vohra (2013) and Fakruddin et al. (2017) in wild yeast isolates, gelatinase enzyme activity was not found in our isolates, it being a preliminary indication of its safety.

Several microorganisms demonstrate their probiotic potential through pathogens inhibition, however they still need to overcome barriers during passage through GI tract, which involves gastric juice, digestive enzymes, organic acids, bile salts and considerable variations in temperature and pH, such as acidic pH of gastric juice and alkaline pH existing in the intestine (FAO/WHO, 2001; Czerucka et al. 2007). Bonatsou et al. (2015) and Cassanego et al. (2017) demonstrated GD solution is not usually aggressive to yeasts, as also observed in our study; nevertheless, isolates showed to be sensitive to pancreatic conditions, with a decrease of living cells between 1 $\log_{10}$ - 2 $\log_{10}$. In the studies of Cassanego et al. (2017), isolates classified as *S. cerevisiae* and *H. uvarum* were not able to survive after exposure to this condition, however we presented in our work that yeast isolates were able to resist to PD solution. Cell viability analysis during *in vitro* GI tract simulation proves to be important because it is believed that effects related to probiotics are dose-dependent, it being suggested as effective a dosage between 10^7-10^9 CFU/mg per day (Minelli and Benini, 2008). Thus, results suggest the concentration of viable cells after GI tract passage is within that expected for probiotic effect in organism.

Wild yeasts isolate *Pichia kluyveri* (LAR001), *Hanseniaspora uvarum* (PIT001) and *Candida intermedia* (ORQ001) demonstrated probiotic potential, both in relation to inhibition of pathogenic microorganisms and tolerance to harsh conditions of human GI tract. Technological properties regarding application for beer production were also evaluated, demonstrating their contribution on sensory profile and fermentation ability in beer wort, as well as them as promising candidates for application in other fermentation processes. Future perspectives of this work are *in vivo* tests to confirm their probiotic action and larger scale beer fermentations to stablish them as probiotic starter cultures.

Declarations

Funding

This work was partial supported by CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brazil) and CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico – Brazil) with scholarships.

Conflicts of interest

The authors declare no competing interests

Availability of data and material

All data generated or analysed during this study are included in this published article.

Code availability

Not applicable

Author's contributions

All authors contributed to the study conception and design. Material preparation was performed by Renan Eugênio Araujo Piraine and Gustavo Maas Retzlaf. Data collection and analysis were performed by Renan Eugênio Araujo Piraine. The first draft of the manuscript was written by Renan Eugênio Araujo Piraine and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Ethics approval

Not applicable

Consent to participate

Not applicable

Consent for publication

Not applicable

Acknowledgements

This work was partial supported by CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brazil) and CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico – Brazil) with scholarships. We thank all students involved directly or indirectly with this study.

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Tables

Table 1: Identification of wild yeast isolates. Description of macromorphological aspects of colonies and cells observed through microscopy. It was possible to identify isolates at species level through PCR using ITS1 and ITS4 primers, with subsequent sequencing of PCR-amplified product (AP).

Isolate	Isolation source	Colony morphology	Cell morphology	Species	AP*
AMO001	Blackberry (*Morus nigra*)	Circular, flat, entire margin	Ellipsoidal, rarely ovoid	*Hanseniospora uvarum*	750 pb
AMO003	Blackberry (*Morus nigra*)	Circular, flat, entire margin	Ellipsoidal, rarely ovoid	*Hanseniospora uvarum*	750 pb
ORQ001	Orchid (*Aspasia luneta*)	Circular, flat, entire margin	Ovoid	*Candida intermedia*	420 pb
ORQ002	Orchid (*Aspasia luneta*)	Circular, flat, entire margin	Ovoid	*Candida intermedia*	420 pb
PAR001	Vine (*Vitis vinífera*)	Circular, flat, entire margin	Ellipsoidal, rarely ovoid	*Hanseniospora uvarum*	750 pb
PAR002	Vine (*Vitis vinífera*)	Circular, flat, entire margin	Ellipsoidal, rarely ovoid	*Hanseniospora uvarum*	750 pb
PIF001CR	Pitaya (*Hylocereus undatus*)	Circular, raised, ondulated margin	Ovoid, rarely ovoid	*Candida manassasensis*	650 pb
PIF2.001	Pitaya (*Hylocereus undatus*)	Circular, raised, ondulated margin	Ovoid, rarely ovoid	*Candida manassasensis*	650 pb
PITS002	Cherry (*Eugenia uniflora*)	Circular, raised, entire margin	Ovoid	*Saccharomyces cerevisiae*	880 pb
PIT003	Cherry (*Eugenia uniflora*)	Circular, flat, entire margin	Ellipsoidal, rarely ovoid	*Hanseniospora uvarum*	750 pb
PIT004	Cherry (*Eugenia uniflora*)	Circular, flat, entire margin	Ellipsoidal, rarely ovoid	*Hanseniospora uvarum*	750 pb
MOR001	Strawberry (*Fragaria x ananassa*)	Circular, flat, entire margin	Ellipsoidal, rarely ovoid	*Hanseniospora uvarum*	750 pb
MOR003	Strawberry (*Fragaria x ananassa*)	Circular, flat, ondulated margin	Elongated	*Issatchenkia terricola*	450 pb
COQ001	Jelly palm fruit (*Butia capitata*)	Circular, brillant, raised, entire margin	Ovoid	*Cryptococcus terrestris*	510 pb
LAR001	Orange (*Citrus x sinensis*)	Irregular, flat, filiform margin	Ovoid, rarely ellipsoidal	*Pichia kluyveri*	450 pb
REF003	Red Flanders beer (mixed fermentation)	Circular, flat, entire margin	Ovoid	*Pichia manshurica*	500 pb
REF005	Red Flanders beer (mixed fermentation)	Circular, flat, entire margin	Ovoid	*Pichia manshurica*	500 pb
JRO001	Old Ale beer (mixed fermentation)	Circular, raised, filiform margin	Elongated or ovoid, sometimes forming chains	*Pichia kludriavzezii*	350 pb
CAR001	Old Ale beer (mixed fermentation)	Circular, raised, filiform margin	Elongated or ovoid, sometimes forming chains	*Pichia kludriavzezii*	350 pb
AMB001	Old Ale beer (mixed fermentation)	Circular, raised, filiform margin	Elongated or ovoid, sometimes forming chains	*Pichia kludriavzezii*	350 pb
SBO	Yeast bank of Microbiology Laboratory	Circular, raised, entire margin	Ovoid	*Saccharomyces boulardii*	850 pb

Table 2: Results of standard beer wort fermentation. Fermentation profile and sensory contribution were obtained after 14 days of fermentation at 22 °C. All yeast isolates were inoculated at the same cell concentration (10^9 cells/total).

Isolate	Species	Apparent attenuation	Final pH	Biofilm	Sensory notes (aroma)
AMO001	*Hanseniospora uvarum*	9%	4.46	-	Grapes, wine, floral, sour
AMO003	*Hanseniospora uvarum*	9%	4.41	-	Grapes, wine, floral, sour
ORQ001	*Candida intermedia*	5%	4.40	+/-	Wood, floral, spice, clove
ORQ002	*Candida intermedia*	9%	4.49	+/-	Wood, floral, spice, clove
PAR001	*Hanseniospora uvarum*	9%	4.40	-	Grapes, wine, floral, sour
PAR002	*Hanseniospora uvarum*	5%	4.41	-	Grapes, wine, floral, sour
PIF001CR	*Candida manassasensis*	5%	4.58	+	Citrusy and tropical notes
PIF2.001	*Candida manassasensis*	14%	4.28	+	Citrusy and tropical notes
PITS002	*Saccharomyces cerevisiae*	50%	4.13	-	Fruity, ester, banana
PIT001	*Hanseniospora uvarum*	18%	4.00	-	Grapes, wine, floral, sour
PIT004	*Hanseniospora uvarum*	14%	4.39	-	Grapes, wine, floral, sour
MOR001	*Hanseniospora uvarum*	9%	4.40	-	Grapes, wine, floral, sour
MOR003	*Issatchenkia terricola*	5%	4.38	+	*Funky*, phenolic, barnyard, horse blanket
COQ001	*Cryptococcus terrestris*	0%	4.35	-	Neutral
LAR001	*Pichia kluyveri*	5%	4.35	+/-	Slightly esterified, banana
REF003	*Pichia manshurica*	0%	4.59	-	Neutral
REF005	*Pichia manshurica*	0%	4.51	-	Neutral
JRO001	*Pichia kludriavzezii*	9%	4.43	+	Solvent, phenolic, grapes, green apple
CAR001	*Pichia kludriavzezii*	9%	4.44	+	Solvent, phenolic, grapes, green apple
AMB001	*Pichia kludriavzezii*	9%	4.47	+	Solvent, phenolic, grapes, green apple
SBO	*Saccharomyces boulardii*	32%	3.8	-	Slightly esterified, alcoholic, spice, clover

Table 3: Growth index of yeasts culture under different growing conditions. GI values < 25% represent conditions considered inhibitory for microorganism's growth, between 25% - 75% partially inhibitory and > 75% growth was similar to the control, i.e. there was no growth inhibition by this condition. G.I. = Growth Index. OD600nm = final absorbance of cultures after 72 h of cultivation.

	pH 2.5		pH 8.0		Lactic acid 1%		Acetic acid 0.2%		37 °C		Control
	O.D.$_{600nm}$	G.I.	O.D.$_{600nm}$	G.I.	O.D.$_{600nm}$	G.I.	O.D.$_{600nm}$	G.I.	O.D.$_{600nm}$	G.I.	O.D.$_{600nm}$
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

Table 4: Tolerance of yeasts to different concentrations of NaCl in YPD media. Concentrations of NaCl ranging between 0.5% to 10% were added to YPD media, aiming to characterize halotolerance in isolated yeasts. A plus sign (+) represents cell growth above O.D.$_{600nm}$ 1.0, while minus sign (-) indicates there was no growth in the culture tested.

	NaCl			
	0,5%	1%	5%	10%
P. kluyveri	+	+	+	-
H. uvarum	+	+	-	-
C. intermedia	+	+	-	+
S. boulardii	+	+	-	-

Table 5: Auto-aggregation of *P. kluyveri*, *H. uvarum* and *C. intermedia* at different time points and temperatures. Results refers to two independent tests, performed in duplicate. Statistical difference was analyzed using Tukey test ($p<0.05$).

	Auto-aggregation (%)					
	18 °C		28 °C		37 °C	
	2h	20h	2h	20h	2h	20h
P. kluyveri	16.04 ±0.08	88.22 ±0.46	43.80 ±0.54	98.25 ±1.70	26.10 ±4.90	99.88 ±0.13
H. uvarum	9.02 ±0.12	84.25 ±0.64	20.00 ±0.24	98.57 ±1.89	25.62 ±6.82	99.92 ±0.09
C. intermedia	13.51 ±1.91	83.75 ±0.50	23.93 ±0.09	99.95 ± 0.10	18.12 ±1.65	99.87 ±0.25
S. boulardii	22.02 ±1.63	95.87 ±0.25	38.05 ±1.63	99.87 ±0.25	42.25 ±4.92	99. 83 ±0.23

Table 6: Co-aggregation of *P. kluyveri*, *H. uvarum*, *C. intermedia* with *E. coli* ATCC8739 at different time points and temperatures. Results refers to two independent tests, performed in duplicate. Statistical difference was analyzed using

Tukey test (p<0.05).

	Co-aggregation *E. coli* ATCC 8739 (%)					
	18 °C		28 °C		37 °C	
	2h	20h	2h	20h	2h	20h
P. kluyveri	16.06 ±3.16	58.03 ±1.63	11.98 ±0.02	64.02 ±0.05	12.07 ±0.15	66.02 ±1.63
H. uvarum	14.07 ±1.64	50.15 ±1.66	10.00 ±1.63	60.30 ±0.47	14.00 ±1.59	68.11 ±0.08
C. intermedia	8.12 ±3.27	58.10 ±1.82	0.18 ± 0.23	68.08 ±0.17	4.15 ±0.30	66.13 ±1.65
S. boulardii	18.11 ±1.61	57.98 ±1.50	20.08 ±0.17	63.81 ±0.16	22.10 ±1.53	72.12 ±0.11

Table 7: Co-aggregation of *P. kluyveri*, *H. uvarum, C. intermedia* with *L. monocytogenes* ATCC7644 in different time points and temperatures. Results refers to two independent tests, performed in duplicate. Statistical difference was analyzed using Tukey test (p<0.05).

	Co-aggregation *L. monocytogenes* ATCC 7644 (%)					
	18 °C		28 °C		37 °C	
	2h	20h	2h	20h	2h	20h
P. kluyveri	2.14 ±1.64	54.05 ±1.55	20. 01 ±0.02	70.03 ±1.53	26.10 ±1.61	73.25 ±2.21
H. uvarum	1.62 ±1.09	50.12 ±1.84	20.04 ±0.04	68.00 ±0.01	22.00 ±1.63	76.00 ±0.01
C. intermedia	0.27 ±0.20	52.01 ±0.02	0.36 ±0.304	70.12 ±1.65	12.12 ±0.25	78.02 ±1.63
S. boulardii	4.06 ±0.11	54.03 ±1.63	26.06 ±1.57	74.13 ±1.60	32.23 ±0.25	78.60 ±1.95

Table 8: Characterization of enzymatic and antimicrobial activity by isolated yeasts. Results refers to two independent tests, performed in duplicate. Statistical difference was analyzed using Tukey test (p<0.05). In the analysis of antimicrobial activity: + + + (very high inhibition): inhibition zone diameter ≥ 24 mm, + + (high): 16 – 23 mm, + (medium): 8 – 16 mm e – (no inhibition/to low): ≤ 7mm. N = no enzymatic activity was observed.

	Enzymatic activity		Antimicrobial activity				
	Protease	Gelatinase	*L. monocytogenes*	*S. aureus*	*P. aeruginosa*	*E. coli*	*Klebsiella* sp.
P. kluyveri	N	N	+ + +	+ +	+ +	+	+
H. uvarum	N	N	+ +	+ + +	+ +	+	+
C. intermedia	N	N	+	+ +	+ +	+	+
S. boulardii	N	N	+ +	+ +	+ +	+	+

Figures

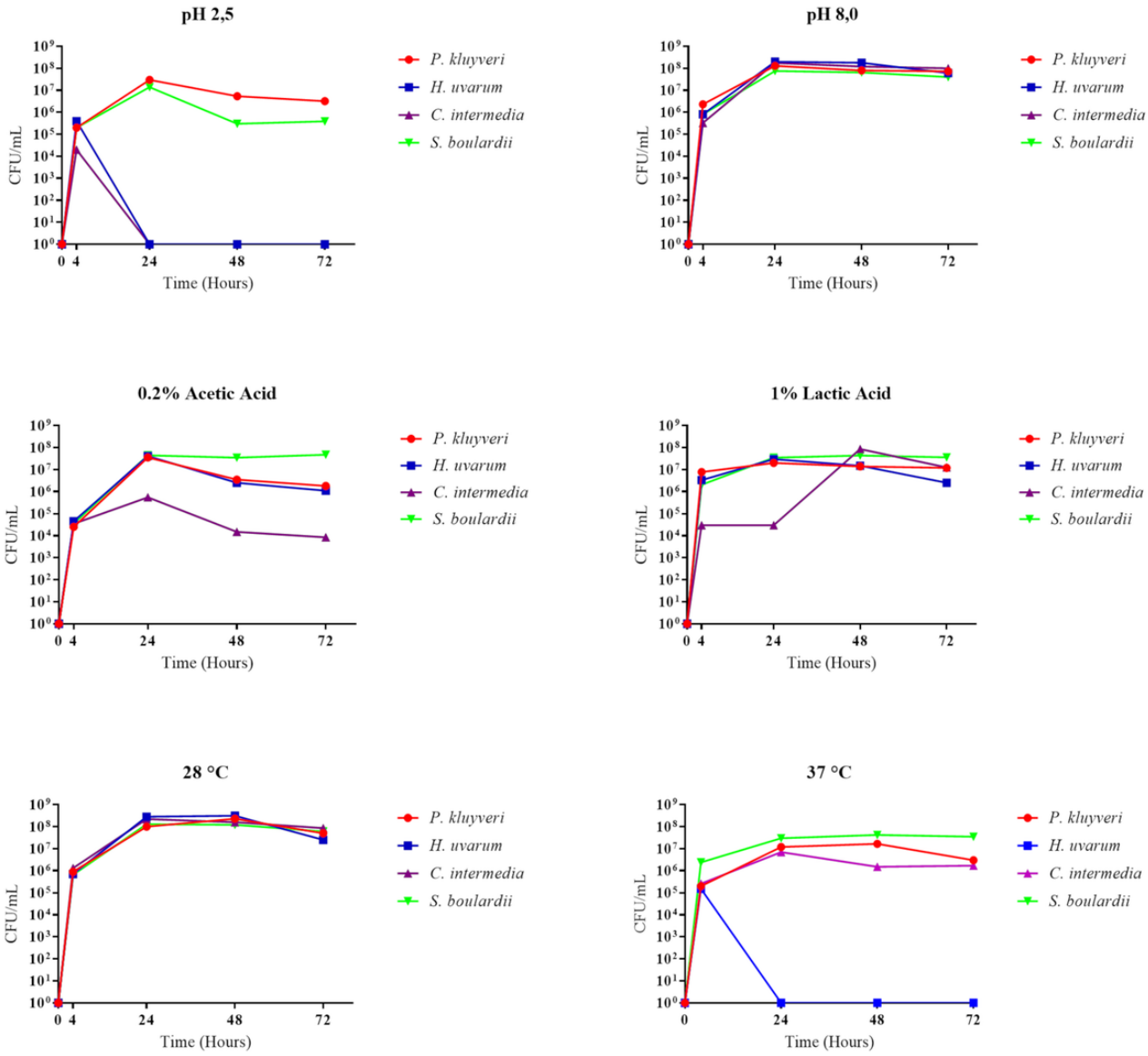

Figure 1

Evaluation of cell viability of P. kluyveri (LAR001), H. uvarum (PIT001), C. intermedia (ORQ001) and S. boulardii cultivated in YPD under different conditions. Graphs show CFU/mL concentration for the four yeasts incubated with YPD media at pH 2.5 and 8.0, lactic acid 1%, acetic acid 0.2% and at a temperature of 37 °C. Yeast cultivation in YPD medium pH 6.0, incubated at 28 °C was considered as optimal condition for yeast growth, representing a control culture. Total experiment time, time points for samples collection and agitation were maintained the same in all tests. Graphs were created in GraphPad Prism 7 software

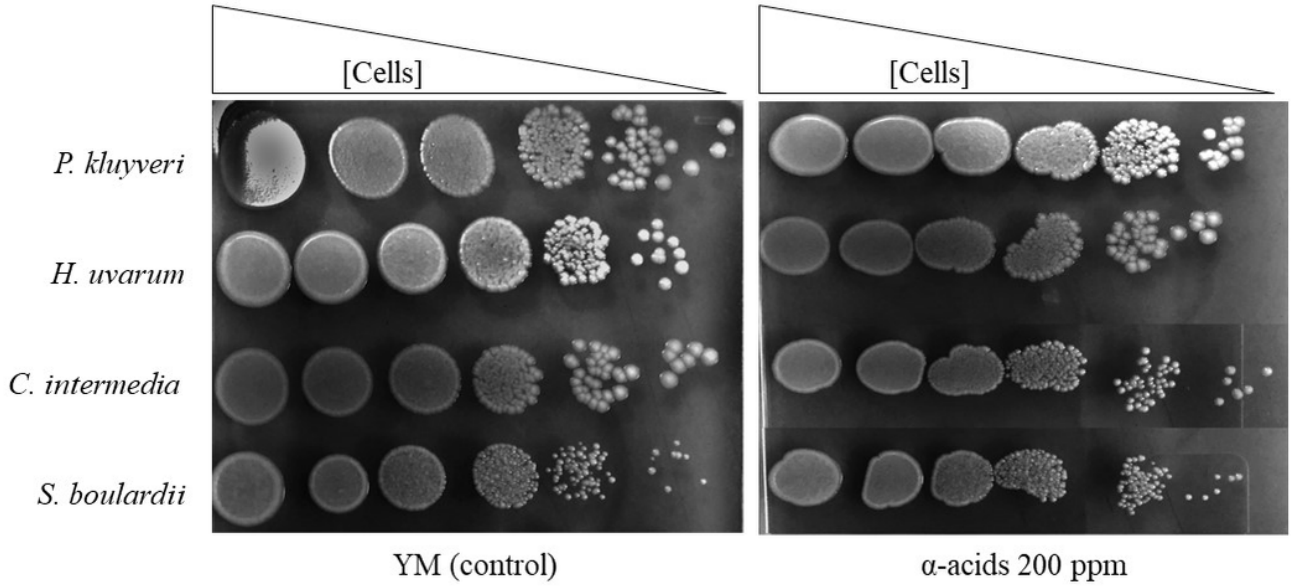

Figure 2

Evaluating the sensitivity of P. kluyveri, H. uvarum and C. intermedia to α-acids from hop extract. There were no differences visualized between cells grown on YM (control) and YM medium containing 200 ppm of α-acids, evidencing tolerance by them. Culture dilutions were made in 10x, from 108 UFC/mL samples

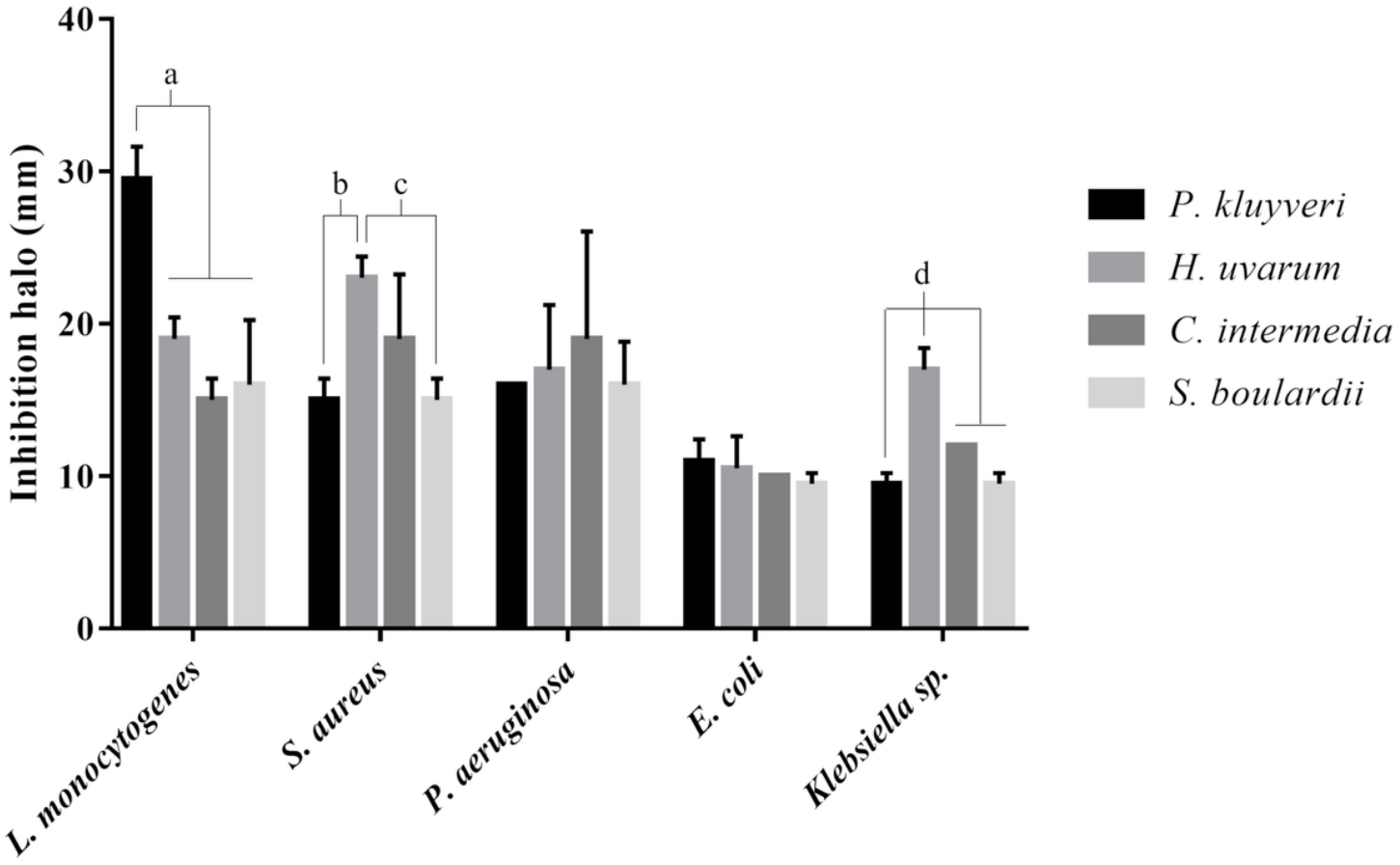

Figure 3

Antimicrobial activity by P. kluyveri, H. uvarum, C. intermedia and S. boulardii against pathogen bacteria related to FBD. Bacterial growth inhibition was detected through visualization of halo formation, which represents antimicrobial activity by isolated yeasts. Statistical difference was established by analysis of variance ANOVA ($p<0.05$), in which groups with differences are indicated in the graph by letters: a: P. kluyveri was different from other groups; b: P. kluyveri differs from H. uvarum; c: H. uvarum was different from S. boulardii; d: H. uvarum differs from the other yeasts

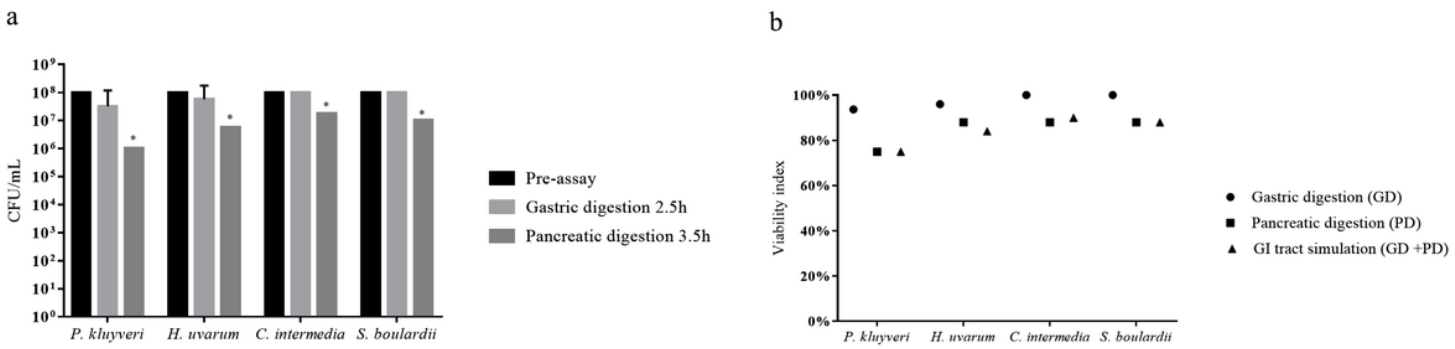

Figure 4

Cell viability analysis before and after incubation in GI tract simulated conditions. A) Yeast tolerance was evaluated under adverse conditions found in GI tract, with incubation of yeast cells on gastric digestion (GD) and later in pancreatic digestion (PD) solution. No statistical difference was observed between cell viability before and after GD solution, for all yeasts

cultures. After incubation under PD conditions, a decrease in viable cell concentration was observed for H. uvarum, C. intermedia and P. kluyveri. Statistical difference in data was evaluated by analysis of variance ANOVA ($p<0.05$). * indicates significant difference in cell viability during the test. B) Viability Index (VI) of yeasts under GD, PD and GD + PD conditions: all yeasts showed VI ≥ 93% for GD solution, while a decrease to 88% after PD solution (tested in isolated method) could be observed for H. uvarum and C. intermedia. Since it was observed a decrease of 2 log10 in P. kluyveri cell count, a VI = 75% was determined for PD and GD + PD test

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- graphicalabstract.png
- checklistwjmb.pdf