

Research article

Technological characterisation and probiotic traits of yeasts isolated from *Sha'a*, a Cameroonian maize-based traditional fermented beverage



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GRAPHICAL ABSTRACT



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ABSTRACT

The current trend in starter selection is to combine both technological and probiotic properties to standardise and make functional artisanal fermented beverages such as *Sha'a* whose properties are very variable due to the lack of a known starter. The objective of this work was to study technological and probiotic properties of yeasts isolated from *Sha'a* sold in Bamenda, Bafoussam, Bonabérié, Dschang, Foumbot, Mbouda and Njombé (Cameroon). The isolated yeasts were studied for their ability to produce CO₂ from glucose, to grow in the presence of 8% ethanol, 20% glucose and pH 3, to assimilate maltose and to produce ethanol. Then, the survival of the pre-selected isolates was assessed in simulated gastric (pH 2 and 3) and intestinal juices, followed by self-aggregation, co-aggregation, hydrophobicity, haemolysin, gelatinase, biogenic amine production, antibiotic and antifungal susceptibility, bile salt hydrolase and antiradical activity. The selected isolates were identified by sequencing the 5.8S/28S rRNA gene. From the 98 isolates obtained, 66 produced CO₂ from glucose and 16 were then selected for their ability to grow in the presence of 8% ethanol, 20% glucose, pH 3 and maltose. The overall survival of isolates ranged from 4.12 ± 1.63 to $104.25 \pm 0.19\%$ (LT16) and from 0.56 ± 0.20 to $96.74 \pm 1.60\%$ (LT66) at pH 3 and pH 2 respectively. All of them have remarkable surface hydrophobicity properties. Based on principal component analysis, 5 isolates were selected as the best. However, only 3 of them, LT16 (the most promising), LT25 identified as *Saccharomyces cerevisiae* and LT80 as *Nakaseomyces delphensis*, do not produce a virulence factor. The latter can deconjugate bile salts with a maximum percentage of $60.54 \pm 0.12\%$ (LT16) and the highest inhibition of DPPH[•] radicals was $55.94 \pm 1.14\%$ (LT25). In summary, the yeast flora of *Sha'a* contains yeasts capable of fermenting and producing ethanol while producing bioactive compounds that would benefit the consumer.

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1. Introduction

The production of fermented beverages is one of the oldest practices in which sugars are converted into ethanol through the action of yeasts (Dalia et al., 2017). Traditionally carried out, they include a multitude of microorganisms, some of which would not be beneficial for the quality of the final product (Solange et al., 2014). This characteristic is specific to traditional fermented beverages widely produced around the world, Africa and Cameroon particularly. In this country, we find mainly palm wine, *Bili Bili*, banana beer and *Sha'a* (Kaktcham et al., 2012). The latter is a brown alcoholic or non-alcoholic drink made from corn, produced and marketed mainly by housewives (Kaktcham et al., 2012). Like its counterparts (*Pito* in Ghana, *Tchapalo* in Ivory Coast, *Dolo* in Burkina Faso and *Tella* in Ethiopia etc.), the production and marketing of *Sha'a* face many difficulties, the fermentation stage being the final critical point (Minamor et al., 2017; Coulibaly et al., 2016; Solange et al., 2014). As a result, *Sha'a* has a non-regular taste, dubious hygienic quality and a very short shelf life of about 3 days, leading to a product of low market value and significant losses for traders (Zran et al., 2017; Nemo and Bacha, 2021). To solve these problems common to crafting fermented beverages, many researchers have proposed the development of starter cultures by selecting efficient yeasts to produce beverages in a reproducible manner.

According to Bevilacqua et al. (2012), the selection of microorganisms is a complex process and can be adapted depending on the nature of the matrix to be fermented. Technically, this selection consists of three steps: *in vitro* selection, laboratory-scale validation and then industrial scale. Commonly, isolates should be derived from the application matrix and should have the ability to metabolise the various major sugars found there (Lee et al., 2011). Coulibaly (2016) developed a starter consisting of *Saccharomyces* and non-*Saccharomyces* strains based on technological criteria such as the ability of isolates to grow under different stress conditions (pH, temperature), to metabolise glucose, to produce ethanol and their viability after freeze-drying. Given the rapidity of the selection, Michel et al. (2020) adopted a selection based on fermentation performance by evaluating CO₂ production in order to optimise propagation on an industrial scale. In addition to these qualities, fermentation speed, attenuation and ethanol production are insufficient, since they also affect the fate of the ingested yeast, whose beneficial functional effect could help prevent some chronic pathologies, in this case, metabolic stress and hypercholesterolemia (Abid et al., 2022; Suyash Kathade et al., 2020). These benefits have led to an increased demand for beverages made functional through the action of some microorganisms and/or metabolites (Palla et al., 2020).

The current trend is the use of functional starters, which in addition to their technological properties, are full of other functions such as toxin reduction, production of essential vitamins and/or amino acids and probiotic effect (Tamang and Lama, 2022; Palla et al., 2020; Bevilacqua et al., 2012). The term probiotic includes a range of microorganisms largely represented by bacteria and a minority of yeasts. Although several yeast genera have been recently recognised as safe by EFSA (EFSA, 2017). Probiotics are viable microorganisms that confer a beneficial effect on their host when consumed in adequate quantity (FAO/WHO, 2002). To reach their site of action, these microorganisms must overcome the different barriers of the digestive tract.

Several authors have focused their attention on the lactic flora with probiotic properties, leaving out the yeast flora (Kim et al., 2022; Simatende et al., 2019; Romero-Luna et al., 2017; Magala et al., 2016). Knowing that yeast is the key element of alcoholic fermentation, it would be wise to conduct investigations to develop a multifunctional starter capable of effectively driving fermentation and able to prevent chronic diseases on the rise, such as cardiovascular diseases (Navi and Segal, 2009), which according to the WHO (2019), killed more than 18 million people in 2019 and could decimate 23 million by 2030. However, the yeast flora of starch drinks, although largely explored in the rest of the African continent, has not yet been characterized in order to formulate a

starter. Therefore, a techno-functional characterisation of the isolates providing *Sha'a* would allow firstly to control the fermentation and secondly to promote a certain beneficial effect for the consumer. Whereas, yeasts are naturally resistant to low pH, survive in the presence of bile salts by hydrolysing them (Fadda et al., 2017), can be consumed at the same time as antibiotics, reduce the intake of antibiotics and consequently the development of antibiotic resistance (Agarbati et al., 2020). They also have the advantage of persisting for only a few days in the intestine (Weichselbaum, 2009), which facilitates their regulation. These characteristics make yeasts an appropriate tool to improve the functional properties of *Sha'a*. The objective of this work was to study the technological and probiotic properties of yeasts isolated from *Sha'a* sold in six localities of three Cameroonian regions.

2. Materials and methods

2.1. Sample collection and yeasts isolation

Fifteen samples of *Sha'a* aged at least 48 h (backslopes) were aseptically collected from different localities of Cameroon especially Bamenda (North-West Region), Njombé, Bonabéri (Littoral Region), Bafoussam, Dschang, Foubot and Mbouda (West Region) of Cameroon from January to February 2020. After collection, the samples were immediately sent to the laboratory for yeast isolation. Briefly, a decimal dilution series was performed from the samples in a 0.9% (w/v) NaCl solution in 100 µl of the dilution considered was spread on apple agar, PDA (Oxoid) supplemented with chloramphenicol (0.005%) and then incubated aerobically for 48 h at 30 °C. Based on their morphological criteria, distinct colonies were picked and streaked until identical colonies were obtained (Kurtzman et al., 2011).

2.2. Selection of yeasts isolates based on technological traits

2.2.1. CO₂ production test

CO₂ production was assessed using a modelled gasometer as described by Weinberg (2018). After 24 h of reactivation, cells were obtained by centrifugation and washed twice in physiological solution. The resulting pellet was resuspended in the physiological solution until McFarland standard 1 was obtained (approximately 3×10^8 CFU/ml). Then, the suspension was used to inoculate Yeast extract Peptone Dextrose (YPD, Merck, Germany) broth medium at 1% (v/v), followed by incubation at 30 °C for 24 h. The amount of CO₂ produced was proportional to the amount of water received in the graduated cylinder. Isolates not producing CO₂ were excluded from the study.

2.2.2. Stress exclusion test

Resistance to different stresses was performed using a protocol adapted from Carrasco et al. (2001) and Lee et al. (2011). From pre-cultures of isolates, suspensions of optical density 0.2 were obtained following two successive washes in 0.9% NaCl solution. The isolates were then tested for their ability to grow in YPD broth in the presence of 8% (v/v) ethanol, 20% (w/v) glucose and pH 3. The cultures were incubated at 30 °C for 24 h and then the optical densities were read at 600 nm against the blank. Any increase in optical density was considered as growth and the ratio (rOD) of the final optical density to the initial optical density has permitted the selection of isolates.

2.2.3. Ability to grow in the presence of maltose

Maltose uptake was determined according to the method of Lee et al. (2011). Cell suspensions were obtained as before. YPM broth (1% yeast extract, 2% peptone, 2% maltose) was inoculated at 1%, subsequently incubated at 30 °C for 24 h and optical densities were read using a spectrophotometer (Thermo Scientific BioMate 3S UV-Visible spectrophotometer, Thermo Scientific, USA) at 600 nm against the blank value (Lee et al., 2011).

2.2.4. Quantification of ethanol produced by the selected isolates

The fermentation medium consisted of YEP (1% Yeast extract, 2% Peptone) supplemented with 10% glucose and then inoculated with 1% and the fermentation lasted 24 h at 30 °C. The amount of ethanol produced was determined by the spectrophotometric method described by Rattanaporn et al. (2019). Briefly, 1 ml of tri-n-butyl phosphate (TPB, Lobal Chemie, India) and 1 ml of sample were mixed, stirred vigorously for 1 min and then centrifuged (3420g, 5 min). Subsequently, 500 µl of the upper phase (clear and transparent) and potassium dichromate solution (10% w/v K₂Cr₂O₇, 5M H₂SO₄) were mixed in a new microtube, shaken vigorously for 1 min, then allow to stand for 10 min at room temperature. Finally, 100 µl of the lower (green) phase was diluted in 900 µl of deionised water and the optical densities were read at 595 nm using a spectrophotometer (Thermo Scientific BioMate 3S UV-Visible spectrophotometer, Thermo Scientific, USA). The concentration of ethanol in the sample was estimated from the calibration curve showing the relationship between absorbance at 595 nm and ethanol concentrations.

2.3. Functional characterisation of the probiotic potential of pre-selected isolates

2.3.1. Assessment of the ability of isolates to survive in simulated gastrointestinal juices

Isolates were evaluated for their ability to cross the barriers of the gastrointestinal tract according to the method of Pisano et al. (2014). From a 24 h preculture, a suspension of approximately 10⁷ colony forming unit (CFU/ml) was obtained following two successive washes in physiological water (NaCl, 0.9%). From the latter, 1 ml was introduced into 9 ml of simulated gastric juice (6.2 g.l⁻¹ NaCl, 2.2 g.l⁻¹ KCl, 0.22 g.l⁻¹ CaCl₂, 1.2 g.l⁻¹ NaHCO₃, 0.3% pepsin, pH 2 and 3) then incubated at 37 °C for 120 min, the time recommended by Minekus et al. (2014). After this time, the number of viable cells was assessed on YPD agar. The remaining volume was centrifuged (3500 g, 15 min) and the supernatant was discarded. Then, 8.75 ml of synthetic intestinal juice (6.4 g.l⁻¹ NaHCO₃, 0.239 g.l⁻¹ KCl, 1.28 g.l⁻¹ NaCl, 0.1% pancreatin, pH 7.4) supplemented with 2 ml of 10% (w/v) bile salts (Sigma, New Zealand) was added to simulate passage through the first portion of the intestine. Incubation was carried out for 180 min at 37 °C after which the survival percentages were calculated.

2.3.2. Assessment of the cell surfaces characteristics of isolates

2.3.2.1. Auto-agrégation. It was performed following the modified method of del Re et al. (2000). Cells of about 24 h old were obtained by centrifugation (3500 g, 15 min) and then washed twice in phosphate-buffered saline (PBS). These cells were resuspended in PBS to reach McFarland's scale 4. The suspensions were vigorously homogenised and allowed to stand at room temperature and the optical densities were read after 2 h and 4 h. An aliquot (0.1 ml) of the top suspension from each tube was taken and mixed with 500 µl of PBS for measurement of absorbance at 600 nm (A_t). Self-aggregation (Eq. (1)) was expressed as the percentage decrease in absorbance after 2 and 4 h compared to the initial absorbance (A₀).

$$\%Au = \frac{A_0 - A_t}{A_0} \times 100 \quad (1)$$

where: % Au = percentage of auto-aggregation after 2 or 4 h; A₀ = initial absorbance of the yeast suspension; A_t = absorbance after 2 or 4 h of incubation.

2.3.2.2. Co-aggregation. Following the method of Yadav et al. (2017), the coaggregation of isolates was assessed. Yeast isolates were grown in YPD broth and *E. coli* ATCC 11775 in brain heart infusion broth (BHI) and

incubated at 30 °C and 37 °C respectively for 24 h. After incubation, the cultures were centrifuged at 3500 g for 15 min and then washed in PBS. The resulting cells were resuspended in the same buffer, homogenised and the absorbance was adjusted to 0.5 ± 0.05 read at 600 nm. Then, 2 ml of yeast and *E. coli* suspensions were mixed, and the mixture was incubated at room temperature without shaking for 4 h and the absorbance was also read at 600 nm. The percentage of co-aggregation was calculated by applying the following formula (Eq. (2)):

$$\%Co = \frac{(DO_y + DO_{Eco}) - DO_{mix}}{(DO_y + DO_{Eco})} \times 100 \quad (2)$$

where: %Co = Percentage of co-aggregation; OD_y = Optical density of the yeast suspension OD_{Eco} = Optical density of the *E. coli* suspension; OD_{mix}: Optical density of the mixed suspension of the two strains.

2.3.2.3. Hydrophobicity. From a 24 h-old culture, cells were collected by centrifugation (3500 g, 15 min), washed twice in PBS and subsequently resuspended therein until McFarland's scale 4 was obtained (approximately 12 × 10⁸ CFU/ml). The absorbance of each of these cell suspensions (A₀) was measured at 600 nm. Afterwards, 1 ml of solvent (Hexane or Chloroform) was added to 3 ml of cell suspension. After 10 min of pre-incubation at room temperature, the different mixtures were vigorously homogenised with a vortex (Thermolyne, USA) for 2 min. The tubes were allowed to stand at room temperature for 20 min. The aqueous phase was recovered and the absorbance was measured at 600 nm (A_t) (Ekmekci et al., 2009). The percentage adhesion of the yeast isolates to the solvent was calculated as follows (Eq. (3)):

$$\%Hy = \frac{A_0 - A_t}{A_0} \times 100 \quad (3)$$

where: %Hy = Percentage of hydrophobicity in the presence of hexane or chloroform; A₀ = Initial absorbance of the yeast suspension; A_t = Absorbance after 20 min incubation.

2.3.2.4. Safety evaluation of isolates.

- Hemolytic activity

The non-virulent nature of the isolates toward blood cells was assessed on YPD agar supplemented with 5% sheep blood. On this medium, 24 h-old cultures were deposited as spots and incubated at 37 °C for 48 h. The development of clear (β-haemolysis), and greenish (α-haemolysis) areas were looked for around the colonies (Fadda et al., 2017).

- Gelatinase production

Gelatinase production was carried out on Nutrient Agar containing 7% (w/v) gelatin (Oxoid, UK) (Rohde et al., 1978). Two microlitres (2 µl) of culture from each isolate was deposited as a spot on the surface of the Nutrient Agar and incubated at 37 °C for 72 h. Following this incubation, the surface of the Nutrient Agar was flooded with a saturated solution of ammonium sulphate and the clear areas around the yeast spots were searched for.

- Biogenic amine production

The tested isolates were grown on YPD broth for 24 h were collected by centrifugation and then spotted onto a test medium consisting of YEP supplemented with each of the corresponding biogenic amine precursors (lysine, phenylalanine, arginine and histidine) at 0.5% (w/v), bromocresol purple (0.006%, w/v) and the pH was adjusted to 5.3 (Mete et al., 2016). The negative control was constituted with YPD + bromocresol purple and without amino acids. The whole set was incubated for 5 days.

The formation of purple colour around the spots in the media containing the amino acids while yellow was present in the control was considered as biogenic amine production.

- Susceptibility to antibiotics and antifungals

It was performed by the diffusion method according to the CLSI protocol (2009). Fresh yeast cultures were used to prepare the MC Farland 0.5 densities (1.5×10^8 CFU/ml). This was grown in Mueller-Hinton agar plates supplemented with 2% glucose and 0.5 mg/l Methylene Blue. Discs were then placed on the surface of the medium and incubated for 24 h. Blue areas around the discs were measured and the isolates were classified as susceptible (S), intermediate (I), or resistant (R).

2.3.3. Bile salt deconjugation

2.3.3.1. Screening of bile salt hydrolase producing isolates. Culture medium consisted of YPD supplemented with 0.5% bile salts (consisting of 50% taurodeoxycholate and glycodeoxycholate) and $0.37 \text{ g.l}^{-1} \text{ CaCl}_2$. After a culture of 24 h, 2 μl of each isolate was deposited as a spot on the surface of the medium, followed by incubation at 37 °C for 48 h. The presence of clear and opaque halos around the spots was considered as glycodeoxycholate and taurodeoxycholate hydrolyses (Fadda et al., 2017).

2.3.3.2. Bile salt hydrolase activity. The bile salt hydrolase production assay was evaluated on supernatants and intracellular contents (ICC) by determining the quantity of amino acids released following the hydrolysis of bile salts (Glycodeoxycholate and Taurodeoxycholate, 50%) using the Ninhydrin assay (Jiang et al., 2010). Thus, BSH activity was expressed as U/mg, representing the amount of enzyme that releases 1 μmol of amino acids (Glycine and/or Taurine) from the substrate per minute.

2.3.4. Reduction of 2,2-diphenyl-1-picryl hydrazyl

The antioxidant activity of the different extracts was assessed in culture supernatants, ICCs and whole cells by the 2,2-diphenyl-1-picryl hydrazyl radical method as described by Chen et al. (2010). The selected isolates were grown in YPD broth for 24 h at 30 °C. Then the culture supernatants were obtained by centrifugation (3500 g, 15min) and the ICCs by cell bursting in citrate buffer pH 5 at 50 °C. An aliquot (800 μl) of supernatant or ICC and 1000 μl of DPPH (0.2 mM) were mixed, left in the dark at 25 °C for 30 min. Optical densities were measured at 517 nm using a spectrophotometer (Thermo Scientific Bio-Mate 3S UV-Visible spectrophotometer, Thermo Scientific, USA). The test was performed in triplicate and the blank was prepared in deionised water. Antiradical activity of the extracts (A) was calculated according to the formula (Eq. (4)):

$$\%A = \frac{(1 - \text{Abs sample})}{\text{Abs blank}} \times 100 \quad (4)$$

where: A = Antiradical activity.

2.3.5. Molecular identification of isolates

The amplification of the gene coding for rRNA ITS1-5.8S-ITS4 and NL1-28S-NL4 was performed by Polymerase Chain Reaction (PCR) on pure yeast colonies using the following primer pair: ITS1 (5'-TCCGTAGGTGAACCTCGCG-3') and ITS4 (5'-TCCTCCGCTTATGATATGC-3') for LT16 and NL-1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and NL-4 (5'-GGTCCGTGTTTCAAGACGG-3') for LT26 and LT80 (White et al., 1990). PCR reactions were performed in a final volume of 50 μl consisting of 21 μl of distilled and deionised water (ddH₂O), 2 μl of each primer, one yeast colony of the pure isolate and 25 μl of Dream-Taq™ PCR (Thermo Fischer Scientific, Lithuania) (Weisburg et al., 1991). Amplification was performed in a thermocycler (SimpliAmp,

Thermo Fischer Scientific) programmed as follows: initial denaturation at 95 °C for 5 min; 30 cycles each comprising a denaturation step at 94 °C for 2 min, hybridisation at 60 °C for 1 min and elongation at 72 °C for 2 min and a final extension at 72 °C for 7 min (van der Aa Kühle et al., 2001). The PCR products were separated on a 1.5% agarose gel containing 0.4 $\mu\text{g/ml}$ ethidium bromide in Tris Borate EDTA (0.5 \times), visualised under UV and a 100 bp DNA weight marker (New England BioLabs, Inc., USA) was used to estimate the amplicon sizes. The amplicons were thereafter sequenced thanks to the commercial services of MacroGen Europe. The obtained sequences were submitted to QC and subsequently trimmed before being compared for similarities with existing strains in the Genbank database using the BLAST programme.

2.4. Statistical analysis

All the quantitative data were expressed as mean \pm standard deviation using Microsoft Excel version 13. Statistical analysis was performed with Minitab 19 using one-way ANOVA followed by Fisher test to determine significant differences between isolates and LSD test within different treatments for overall, survival, auto-aggregation, hydrophobicity, BSH and DPPH° scavenging percentage at the 0.05 probability level. Additionally, Principal Component Analysis was performed using XLSTAT 2007 software to determine the best isolates able to colonise the digestive tract.

3. Results and discussion

3.1. Isolation and selection of isolates

Assessing CO₂ production is a quick and acceptable approach to study yeast fermentation performance since the amount of CO₂ is positively correlated to yeast potential (Olowonibi, 2017; Michel et al., 2020). A total of 98 yeast isolates were obtained from *Sha'a* back slopes samples and only 66 isolates were able to produce CO₂ from glucose as sole carbon source. These isolates are thus said to be alcoholic and are among the microbiota involved in the alcoholic fermentation of *Sha'a*. Similar results were obtained by Channipa et al. (2018) who showed that fermentative yeasts accumulate large amounts of CO₂ when they metabolise glucose. Fermentative yeasts are able, under anaerobic conditions, to metabolise glucose to produce CO₂ and ethanol. Especially in the species *S. cerevisiae*, alcoholic fermentation occurs even in the presence of oxygen and high sugar concentrations (Hagman et al., 2013; Pfeiffer and Morley, 2014). Although CO₂ is produced when the yeast performs respiration, the amount is relatively very small and requires the presence of O₂ (Walker and Stewart, 2016). However, the strains are required to initiate fermentation of wort with high sugar and acid concentrations and must tolerate high levels of ethanol.

3.2. Stress tolerance

The selection of fermentation yeasts must consider their ability to tolerate the initial matrix conditions and physicochemical changes that occur during fermentation (Lee et al., 2011) while possessing the necessary enzymatic machinery to metabolise the major fermentable sugars. In the present study, only sixteen isolates were pre-selected for their ability to grow under different stress conditions (alcoholic, osmotic and acid stress) as mentioned in Table 1. Among the 98 initial yeasts isolates, 41 were able to grow in the presence of 8% ethanol, 30 in the presence of 20% glucose and 16 at pH 3 successively. In fact, at the beginning of the fermentation, the high sugar content, particularly glucose and sucrose, causes osmotic stress, leading to passive diffusion of water from the cells to the surrounding environment; this leads to cell death. When osmotic stress occurs, a modification of the cell wall (Slaninová et al., 2000) or the production of glycerol and trehalose (Hohmann, 2002) can counteract the escape of water molecules from the yeast cells. Furthermore, during fermentation, the consumption of the

Table 1. Ability of isolates to grow under different stress conditions, to produce ethanol and assimilate maltose.

Isolates	Ethanol (g.l ⁻¹)	Ethanol 8% (rOD>2)	Glucose 20% (rOD>4)	pH3 (rOD>2)	Maltose assimilation (rOD)	Origins
LT1	34.50 ± 0.00 ^g	5.40 ± 0.02	10.67 ± 0.04	5.92 ± 0.40	9.69 ± 0.03	Bamenda
LT3	40.91 ± 0.82 ^d	3.83 ± 0.02	10.71 ± 0.09	2.13 ± 0.11	9.92 ± 0.01	
LT4	45.95 ± 1.38 ^{abc}	4.14 ± 0.01	9.24 ± 0.05	2.17 ± 0.13	8.80 ± 0.06	
LT11	38.65 ± 0.03 ^{de}	3.33 ± 0.03	11.65 ± 0.16	2.17 ± 0.08	11.62 ± 0.03	
LT16	23.95 ± 0.75 ^g	2.08 ± 0.04	12.95 ± 0.01	6.81 ± 0.03	9.73 ± 0.04	
LT22	38.64 ± 0.33 ^{de}	2.60 ± 0.02	9.47 ± 0.08	4.93 ± 1.05	8.37 ± 0.04	Bonabérie
LT25	34.79 ± 0.56 ^f	3.31 ± 0.01	8.75 ± 0.11	4.88 ± 0.90	6.09 ± 0.02	
LT80	35.27 ± 0.28 ^f	2.98 ± 0.02	4.23 ± 0.08	5.33 ± 1.10	8.33 ± 0.01	
LT30	48.32 ± 3.29 ^a	5.50 ± 0.03	8.36 ± 0.06	7.93 ± 0.00	7.38 ± 0.06	
LT53	39.95 ± 0.42 ^{de}	2.52 ± 0.07	6.10 ± 0.00	3.73 ± 0.06	4.09 ± 0.39	Dschang
LT54	25.27 ± 0.37 ^g	2.15 ± 0.06	6.43 ± 0.07	3.07 ± 0.45	5.78 ± 0.07	
LT62	37.89 ± 0.89 ^e	2.56 ± 0.01	9.93 ± 0.02	1.64 ± 0.03	9.42 ± 0.00	Bafoussam
LT66	43.93 ± 1.24 ^c	2.99 ± 0.06	10.87 ± 0.07	2.88 ± 0.11	9.93 ± 0.00	
LT70	45.57 ± 0.61 ^{bc}	3.39 ± 0.07	10.58 ± 0.05	4.09 ± 0.58	9.92 ± 0.05	
LT72	46.74 ± 0.90 ^{ab}	2.29 ± 0.04	7.04 ± 0.07	2.74 ± 0.35	10.05 ± 0.01	
LT76	43.68 ± 0.74 ^c	3.74 ± 0.06	6.89 ± 0.09	2.10 ± 0.09	8.92 ± 0.00	

^{a-g} Within the same column, values with different lower case letters differ significantly ($p < 0.05$); i: initial, f: final.

substrate and the excretion of metabolites cause a modification of the physicochemical properties of the matrix, resulting in the accumulation of metabolites such as ethanol and the lowering of the pH, leading to fermentation arrest (Bai et al., 2004). Yeasts also have mechanisms for adapting to these stresses. Once the ethanol concentration becomes critical, yeasts can metabolise it to reduce its concentration in the medium, as demonstrated by Coulibaly (2016) in his work, where *S. cerevisiae* and *Candida tropicalis* strains were able to reduce the ethanol content from 7.5% to 4.5%. As well as in the presence of ethanol, yeast growth is influenced by low pH levels, as at pH levels below 3, Thomas et al. (2002) demonstrated significant inhibition of non-*Saccharomyces* strains.

3.3. Maltose assimilation

Sprouted cereals, especially maize, are largely made up of maltose and thus the starters need to be able to assimilate the latter. As depicted by Table 1, all the 16 isolates pre-selected in the previous step interestingly grew in the presence of maltose as the only carbon source. These results are in agreement with those of Olowonibi (2017) who demonstrated that 12 isolates from fresh palm wine were all able to assimilate maltose as the sole carbon source. Using maltose assimilation as a selection criterion for yeasts belonging to the species *S. cerevisiae*, Marongiu et al. (2015) demonstrated that only 10 out of 13 strains were able to ferment maltose. This indicates that this property is not present in all yeasts (Methner et al., 2019). The ability to assimilate maltose can be lost when yeasts are regularly grown in a glucose-rich medium, inducing repression of genes involved in maltose transport and hydrolysis so that when inoculated into a maltose-rich medium; thus unable to grow (Kuthan et al., 2003). Yeasts with this ability are highly present in cereal-based fermented beverages (Canonico et al., 2014); but some may lose their ability to metabolise maltose completely during evolution or selection (Ohdate et al., 2018).

3.4. Ethanol production

The production of alcoholic beverages involves the selection of strains able to produce ethanol (Tarimo and Kaale, 2022). In this study, all the pre-selected yeast isolates were able to produce ethanol at varying concentrations in the presence of glucose as the sole carbon source. This production is isolate dependent and reaches concentrations ranging from 23.95 ± 0.75 g.l⁻¹ to 48.32 ± 3.29 g.l⁻¹ after 24 h of fermentation (Table 1). Indeed, fermentative yeasts carry out alcoholic fermentation, a

biotechnological process occurring under anaerobic conditions, during which sugars are converted into ethyl alcohol and CO₂ (Huang et al., 2015; Walker and Stewart, 2016). The same approach was used by Channipa et al. (2017) for the selection of highly fermentative yeasts for ethanol production. Similar results were obtained by Pandey et al. (2019) who evaluated ethanol production in a synthetic medium (Sabouraud broth + 10% glucose) for the selection of robust yeast strains for ethanol production from lignocellulosic material and obtained a concentration of 49.77 ± 0.34 g.l⁻¹ after 24 h of fermentation at 30 °C with *S. cerevisiae* NGY10 as the best producer among others. This value is relatively close to that obtained with isolate LT30 (48.32 ± 3.29 g.l⁻¹) providing from Bonabérie. Furthermore, the low concentrations obtained by isolates LT1, LT16 and LT54 are justified by the fact that some yeasts do not normally produce large quantities of ethanol. However, culture conditions such as temperature, pH and the nature of the sugars could have a considerable influence. While some strains were found to have unfavourable conditions, others were found to have suitable conditions such as *Pichia kudriavzevii* kk19 and *Candida tropicalis* CTB018 (Channipa et al., 2018).

However, given that the processing of artisanal alcoholic beverages such as *Sha'a* does not include any step of destruction or filtration of the fermentation agents, it is necessary to follow the survival of its starter culture during their passage through the barriers of the gastrointestinal tract and to determine what could be their beneficial effects.

3.5. Survival of isolates to simulated gastrointestinal juices

To reach their action site, probiotics must overcome various adversities to exert their beneficial effect (Romanin et al., 2010). In this work, survival was assessed in a continuous model: after the tested isolates had been left in simulated gastric juice (SGJ) at pH 2 and 3, the cells were harvested by centrifugation and resuspended in simulated intestinal juice (SIJ). In general, the highest overall viabilities were exhibited by isolates LT16 (104.25 ± 0.19%) and LT80 (100.99 ± 7.16%) in simulated gastric juice at pH 3 and simulated intestinal juice (SGJ-pH3-SIJ) and by the same isolates LT80 (95.90 ± 7.42%) and LT66 (96.74 ± 1.60%) in simulated gastric juice at pH2 and simulated intestinal juice (SGI-pH2--SIJ); these values being significantly identical under the same conditions ($p > 0.05$). Sequentially, survival in SGJ-pH3 are higher than in SGJ-pH2 and vary from 4.12 ± 1.63 to 104.25 ± 0.19% and from 0.56 ± 0.20 to 96.74 ± 1.60% respectively. However, depending on the isolates, survival was significantly ($p < 0.05$) reduced for isolates LT1, LT76, LT4, LT16, LT70, LT54, LT22, while it increases significantly ($p < 0.05$) for

Table 2. Survival percentage of isolates at different pH of simulated gastric juices (SGJ) and intestinal juices (SIJ).

Isolates	Viable count (log CFU/ml)			Overall survival (%)			SGJ, pH 2			SIJ, pH 7.4			Overall survival (%)		
	SGJ, pH 3			Survival (%)			0 min			180 min			Survival (%)		
	0 min	120 min	Survival (%)	180 min	Survival (%)	120 min	Survival (%)	180 min	Survival (%)	180 min	Survival (%)	180 min	Survival (%)	180 min	Survival (%)
LT1	7.14 ± 0.00	6.74 ± 0.00	39.39 ± 0.08 ^f	6.31 ± 0.01	37.34 ± 0.55 ^g	6.18 ± 0.02	12.91 ± 0.87 ^g	4.80 ± 0.14	4.31 ± 1.25 ^h	6.18 ± 0.02	12.91 ± 0.87 ^g	4.80 ± 0.14	4.31 ± 1.25 ^h	0.56 ± 0.20 ^{hh}	
LT3	7.08 ± 0.00	6.98 ± 0.02	81.21 ± 2.07 ^{bc}	6.95 ± 0.00	91.49 ± 4.50 ^{cd}	7.23 ± 0.00	92.26 ± 3.24 ^{ab}	7.15 ± 0.03	84.08 ± 4.40 ^{bc}	7.23 ± 0.00	92.26 ± 3.24 ^{ab}	7.15 ± 0.03	84.08 ± 4.40 ^{bc}	77.55 ± 6.78 ^{bca}	
LT4	7.06 ± 0.00	7.05 ± 0.00	97.64 ± 0.02 ^a	6.68 ± 0.01	42.60 ± 0.18 ^h	7.15 ± 0.00	95.58 ± 2.64 ^a	5.48 ± 0.05	2.17 ± 0.24 ^h	7.15 ± 0.00	95.58 ± 2.64 ^a	5.48 ± 0.05	2.17 ± 0.24 ^h	2.07 ± 0.29 ^{hh}	
LT11	6.97 ± 0.00	6.87 ± 0.03	79.87 ± 3.75 ^c	6.17 ± 0.03	20.01 ± 2.69 ^h	6.65 ± 0.05	81.22 ± 7.36 ^c	5.85 ± 0.02	15.92 ± 1.18 ^g	6.65 ± 0.05	81.22 ± 7.36 ^c	5.85 ± 0.02	15.92 ± 1.18 ^g	12.89 ± 0.22 ^{ga}	
LT16	6.97 ± 0.00	6.96 ± 0.00	98.98 ± 0.08 ^a	6.99 ± 0.00	105.33 ± 0.10 ^a	7.10 ± 0.01	91.03 ± 5.18 ^{ab}	7.08 ± 0.00	95.45 ± 2.80 ^a	7.10 ± 0.01	91.03 ± 5.18 ^{ab}	7.08 ± 0.00	95.45 ± 2.80 ^a	86.78 ± 2.40 ^{1b}	
LT22	7.08 ± 0.00	6.99 ± 0.00	81.01 ± 1.09 ^{bc}	6.95 ± 0.01	92.21 ± 1.90 ^{bcd}	6.93 ± 0.04	79.13 ± 4.36 ^c	6.80 ± 0.01	72.70 ± 6.08 ^{de}	6.93 ± 0.04	79.13 ± 4.36 ^c	6.80 ± 0.01	72.70 ± 6.08 ^{de}	57.35 ± 1.65 ^{1b}	
LT25	7.07 ± 0.00	7.06 ± 0.00	98.68 ± 0.06 ^a	7.03 ± 0.01	92.25 ± 2.42 ^{bcd}	7.06 ± 0.00	96.66 ± 0.44 ^a	7.02 ± 0.01	90.14 ± 0.78 ^{ab}	7.06 ± 0.00	96.66 ± 0.44 ^a	7.02 ± 0.01	90.14 ± 0.78 ^{ab}	87.12 ± 0.36 ^{1a}	
LT30	7.08 ± 0.01	7.06 ± 0.00	96.33 ± 1.85 ^a	7.03 ± 0.00	93.10 ± 0.82 ^{bcd}	6.68 ± 0.03	95.39 ± 1.66 ^a	6.57 ± 0.05	78.49 ± 14.30 ^{cd}	6.68 ± 0.03	95.39 ± 1.66 ^a	6.57 ± 0.05	78.49 ± 14.30 ^{cd}	74.40 ± 12.33 ^{ca}	
LT53	7.00 ± 0.00	6.79 ± 0.01	61.00 ± 1.63 ^d	6.55 ± 0.06	58.65 ± 6.36 ^f	6.78 ± 0.01	60.41 ± 0.35 ^d	6.58 ± 0.01	64.06 ± 2.33 ^e	6.78 ± 0.01	60.41 ± 0.35 ^d	6.58 ± 0.01	64.06 ± 2.33 ^e	38.69 ± 1.63 ^{ca}	
LT54	7.06 ± 0.00	6.07 ± 0.02	10.08 ± 0.62 ^g	6.00 ± 0.05	16.11 ± 5.61 ⁱ	5.22 ± 0.15	2.69 ± 0.97 ^h	0.00 ± 0.00	0.00 ± 0.00 ^h	5.22 ± 0.15	2.69 ± 0.97 ^h	0.00 ± 0.00	0.00 ± 0.00 ^h	0.00 ± 0.00 ^{1b}	
LT62	7.06 ± 0.00	6.99 ± 0.00	85.15 ± 0.41 ^b	6.95 ± 0.01	90.43 ± 0.84 ^d	6.85 ± 0.00	86.13 ± 2.95 ^{bc}	6.83 ± 0.00	94.49 ± 0.23 ^a	6.85 ± 0.00	86.13 ± 2.95 ^{bc}	6.83 ± 0.00	94.49 ± 0.23 ^a	81.35 ± 2.98 ^{bca}	
LT66	7.30 ± 0.00	7.22 ± 0.02	83.43 ± 4.10 ^{bc}	7.22 ± 0.00	100.36 ± 4.52 ^{bcd}	7.28 ± 0.00	97.54 ± 1.43 ^a	7.27 ± 0.00	99.18 ± 0.19 ^a	7.28 ± 0.00	97.54 ± 1.43 ^a	7.27 ± 0.00	99.18 ± 0.19 ^a	96.74 ± 1.60 ^{1a}	
LT70	7.13 ± 0.01	6.87 ± 0.00	55.61 ± 4.26 ^e	6.71 ± 0.00	69.07 ± 0.15 ^e	6.37 ± 0.01	40.92 ± 2.56 ^f	6.09 ± 0.03	51.78 ± 1.29 ^f	6.37 ± 0.01	40.92 ± 2.56 ^f	6.09 ± 0.03	51.78 ± 1.29 ^f	21.18 ± 1.85 ^{1b}	
LT72	7.04 ± 0.01	6.62 ± 0.01	38.89 ± 1.66 ^f	5.63 ± 0.16	10.59 ± 3.75 ^f	5.06 ± 0.08	1.62 ± 0.14 ^b	3.55 ± 0.21	3.17 ± 0.95 ^h	5.06 ± 0.08	1.62 ± 0.14 ^b	3.55 ± 0.21	3.17 ± 0.95 ^h	0.05 ± 0.02 ^{1a}	
LT76	6.95 ± 0.00	6.76 ± 0.01	64.23 ± 1.53 ^d	6.47 ± 0.00	52.38 ± 1.41 ^f	6.67 ± 0.01	49.06 ± 1.33 ^e	5.97 ± 0.05	20.02 ± 1.99 ^g	6.67 ± 0.01	49.06 ± 1.33 ^e	5.97 ± 0.05	20.02 ± 1.99 ^g	9.83 ± 1.24 ^{gh}	
LT80	7.03 ± 0.01	7.03 ± 0.03	100.22 ± 2.51 ^a	7.03 ± 0.01	101.05 ± 9.68 ^{ab}	7.01 ± 0.00	97.20 ± 7.35 ^a	7.00 ± 0.00	98.92 ± 0.15 ^a	7.01 ± 0.00	97.20 ± 7.35 ^a	7.00 ± 0.00	98.92 ± 0.15 ^a	95.90 ± 7.42 ^{ga}	

^{a-i} On the same column, values with lower case letters differ significantly (p < 0.05); ^{A-B} On the same row, values with lower case letters differ significantly (p < 0.05); CFU: Colony forming unit; SGJ: Simulated gastric juice; SIJ: Simulated intestinal juice.

isolates LT53, LT66 when pH is decreased (Table 2). It should also be noted that these results are partially in agreement with those of Sourabh (2012) who demonstrated that simulated gastric juices have a severe negative effect on yeast isolates, inducing a reduction in viability from 2.71 to 4.12 log CFU/ml compared to 0.88 to 3.06 log CFU/ml after 4 h at pH 2 and 3. Furthermore, Syal and Vohra (2013) showed that the viability of yeast was slightly reduced and could remain unchanged even at pH 2 to 3. In addition, according to Amorim et al. (2018), the acidity of the medium does not always act directly on the cells as it may make pepsin more active at pH 2 which in turn will attack the cell membrane proteins. Although acidity negatively affects the viability of some isolates, bile salts could also be responsible for this effect while in the simulated intestinal juice.

The isolates that were significantly inhibited by the simulated intestinal juice were LT1, LT4, LT11, LT54, LT72 and LT76. These results could be explained by the chemical property of bile salts which dissolves fatty bodies such as lipidic constituents of yeast membranes. To this effect, bile salts use several microbial inhibition mechanisms. At high concentrations, they can dissolve and rupture the cell membrane (Coleman et al., 1980) while at low concentrations they cause a modification of the membrane fluidity, increase the flux of divalent cations or modify the structure of membrane proteins (Gómez et al., 2002). Therefore, the increase in viability of isolates in the intestinal juice observed in 10 of the 16 isolates suggests a mechanism that allows them to cancel the effect of bile salts.

3.6. Cell surface adhesion/hydrophobicity

A probiotic strain after surviving gastric juices must be able to colonise and persist in the gut due to its ability to adhere, assessed in this case by self-aggregation, hydrophobicity and co-aggregation of isolates. Self-aggregation provides information on the ability of isolates to adhere between cells of the same strain. The results obtained in the context of our study show that the self-aggregation percentages vary between 71.64 ± 0.87 and 94.72 ± 0.35% after 2 h and from 87.55 ± 0.46 to 94.53 ± 0.03% after 4 h (Table 3). All yeasts isolates showed self-aggregation above 70% and 80% after 2 h and 4 h respectively. According to Pizzolitto et al. (2012), self-aggregation is strong when its percentage is higher than 80%, indicating that all these isolates have a strong self-aggregation after 4 h. Similar observations were reported by Batra et al. (2019), Syal and Vohra (2013) who in their studies obtained values ranging from about 50% to 85% and from 47% to 97.42% after 3 h respectively. Nonetheless, the highest values at 2 h and 4 h do not show a significant difference (p > 0.05) and are attributed to isolates LT11, LT16, LT76. These interactions could be explained by self-recognised surface structures, usually proteins and exopolysaccharides (Willaert, 2018). This yeast-yeast adhesion would eliminate the interstices and thus prevent the attachment of pathogens to intestinal cells.

The competitive effect of invasive and/or pathogenic microorganisms can be regulated by their ability to adhere to the cells of another strain, also called co-aggregation. Table 3 shows the co-aggregation percentages against *E. coli* ATCC 11775; the most important performance (58.24 ± 0.16%) was recorded by isolate LT80. This value was significantly higher (p<0.05) than those of other yeast isolates. These results are very similar to those of Chelliah et al. (2021) who obtained a co-aggregation percentage of 47% with the *S. boulardii* strain KT000032 against *E. coli*. This is an antagonistic mechanism whereby yeast mimics the flocculation that usually takes place between yeast to fix and inhibit bacterial growth. In the course of their study, Tiago et al. (2012) showed that the yeast strain *S. boulardii* and *S. cerevisiae* UFMG 905 can reduce the bacterial population of *Salmonella* Typhi by 10-fold. This coaggregation is made possible by the surface molecules of the pathogen species binding to mannans and polysaccharides on the outer surface of the yeast wall (Xie et al., 2012).

The hydrophobicity percentages of the yeast isolates are reported in Table 3. In general, the adhesion percentages of the isolates are

Table 3. Percentage of adherence to hydrocarbons, self-aggregation and co-aggregation of pre-selected yeast isolates.

Isolates	Auto-aggregation 2h (%)	Auto-aggregation 4h (%)	Coaggregation (%)	Hex (%)	Chl (%)
LT1	93.44 ± 0.16 ^{bcA}	93.48 ± 1.01 ^{bA}	46.12 ± 1.81 ^g	2.05 ± 0.32 ^{kb}	26.37 ± 0.54 ^{lA}
LT3	90.29 ± 0.32 ^{efA}	89.69 ± 0.33 ^{gA}	47.78 ± 0.21 ^f	4.68 ± 0.30 ^{ib}	86.36 ± 0.15 ^{5aA}
LT4	92.61 ± 0.26 ^{cdA}	92.56 ± 0.09 ^{cdA}	44.95 ± 0.57 ^g	19.91 ± 0.42 ^{fA}	19.76 ± 1.05 ^{lA}
LT11	93.81 ± 0.27 ^{abA}	93.76 ± 0.46 ^{abA}	48.47 ± 0.28 ^{ef}	27.87 ± 0.45 ^{dA}	21.66 ± 0.23 ^{hb}
LT16	94.72 ± 0.35 ^{aA}	94.53 ± 0.03 ^{aA}	47.97 ± 0.40 ^f	36.70 ± 0.69 ^{ab}	86.06 ± 0.42 ^{aA}
LT22	92.24 ± 0.43 ^{dA}	92.43 ± 0.29 ^{deA}	54.89 ± 0.13 ^b	15.98 ± 0.90 ^{gB}	72.96 ± 0.82 ^{eA}
LT25	86.93 ± 0.24 ^{gB}	93.42 ± 0.19 ^{bA}	50.54 ± 0.55 ^d	21.49 ± 0.21 ^{eb}	79.33 ± 0.53 ^{bA}
LT30	86.06 ± 0.18 ^{gB}	92.20 ± 0.19 ^{deA}	49.26 ± 0.06 ^e	18.72 ± 0.21 ^{fA}	11.44 ± 1.05 ^{mB}
LT53	92.53 ± 0.17 ^{cdA}	91.64 ± 0.24 ^{efA}	38.18 ± 0.17 ⁱ	9.82 ± 0.19 ^{iAB}	74.83 ± 1.06 ^{dA}
LT54	77.43 ± 1.13 ^{ib}	90.26 ± 0.14 ^{gA}	52.90 ± 0.07 ^c	10.90 ± 0.80 ^{hb}	77.26 ± 0.00 ^{cA}
LT62	71.64 ± 0.87 ^{kb}	90.31 ± 0.49 ^{gA}	50.93 ± 0.37 ^d	28.23 ± 1.26 ^{dB}	66.94 ± 0.95 ^{fA}
LT66	73.09 ± 0.40 ^{ib}	90.13 ± 0.20 ^{gA}	47.79 ± 0.12 ^f	31.58 ± 0.51 ^{eb}	39.45 ± 0.82 ^{hA}
LT70	89.62 ± 0.74 ^{fb}	91.34 ± 0.19 ^{efA}	45.67 ± 0.15 ^g	12.02 ± 0.00 ^{hb}	66.36 ± 0.43 ^{efA}
LT72	90.99 ± 0.05 ^{eA}	90.15 ± 0.35 ^{gB}	53.12 ± 0.31 ^c	32.95 ± 1.03 ^{bA}	28.37 ± 0.51 ^{lA}
LT76	94.11 ± 0.55 ^{abA}	93.31 ± 0.01 ^{bcA}	40.61 ± 0.77 ^h	10.27 ± 0.22 ^{bB}	41.15 ± 0.34 ^{gA}
LT80	83.05 ± 0.16 ^{hb}	87.55 ± 0.46 ^{hA}	58.24 ± 0.16 ^a	28.24 ± 0.43 ^{dA}	25.93 ± 0.14 ^{ib}

^{a-m} In the same column, values with lower case letters differ significantly ($p < 0.05$); ^{A-B} In the same row, values with lower case letters differ significantly ($p < 0.05$); Hex: hexane; Chl: chloroform.

significantly higher in chloroform than in hexane. However, significantly higher values ($p < 0.05$) were obtained by isolates LT16 ($36.70 \pm 0.69\%$) in hexane and LT3 and LT16 (86.36 ± 0.15 and $86.06 \pm 0.42\%$ respectively) in chloroform. These results are in agreement with those of Helmy et al. (2019) who demonstrated that the affinity of yeast is function of the tested solvents. In this work, the highest hydrophobicity value was 46.185% for chloroform. Although for many authors these properties allow isolates to adhere, colonise, increase their stay in the host gut due to Van Der Waals interactions and electrostatic forces between the strain and the host cell, thus reducing their elimination in the faeces via the effect of peristalsis (Beganović et al., 2013). Some authors believe that hydrophobicity can be considered as a pre-test and would be influenced by many factors such as pH, temperature, viability, growth stage and the composition of the culture medium in carbohydrates and bile salts thus making the correlation between hydrophobicity and adhesion ambiguous (Sidira et al., 2015; García-Cayuela et al., 2014; Hernandez-Hernandez et al., 2002; Tiago et al., 2012).

3.7. Principal component analysis (PCA) of probiotic properties

PCA was used to select the best isolates by considering 7 variables: overall survival to simulated gastrointestinal juices (SGJ2 and SJJ3), adhesion to chloroform (Chl) and hexane (Hex), self-aggregation at 2 h (Ag2h) and 4h (Ag4h) and co-aggregation (CoA). Two axes were chosen (F1 and F2) following the Kaiser Guttman's rules. The contribution of the different factors to the formation of these axes is shown in Table 4. It can be seen that the variables SGJ2, SGJ3, CoA contribute mainly to the formation of the F1 axis (explains 41.92% of the variability) while Ag2h, CoA and Chl contribute more to the formation of the F2 axis (explains 20.65%

of the variability). Thus, the F1 axis is strongly correlated with survival, co-aggregation and cell adhesion to hexane while the F2 axis is better correlated with cell adhesion to hexane and self-aggregation. Following this analysis, the Biplot representation (Figure 1) allows grouping the isolates into 4. Group I, consisting of LT16, LT25, LT3 and LT22 considered as those with good survival to SGJ2 and SJJ-pH 3 and Chl; group II, consisting of LT30, LT62 and LT66 characterised by high CoA and Hex values; group III with good Ag2h and Ag4h and represented by isolates LT53, LT70 and LT76; finally, group IV consisting of LT1, LT4, LT54 and LT72. The desired characteristics are isolates with good survival and persistence. For this reason, LT80, although located in group II, has good adhesion and persistence. Given the above arguments, only isolates from group I were selected, considering LT80 for the rest of the work.

3.8. Safety of isolates and their susceptibility to antibiotics and antifungals

The results of the isolate's ability to produce harmful substances, sensitivity to antibiotics and antifungals are presented in Table 5. None of the isolates produced biogenic amines or gelatinase. Moreover, they are all resistant to antibiotics and sensitive to the antifungals used. Indeed, bacterial infections are very short-lived and very often involve antibiotic therapy, hence the importance of the non-susceptibility of yeast strains to antibiotics. However, these strains can be controlled by antifungal drugs if they become pathogenic. Furthermore, isolates LT3 and LT22 showed α -haemolysis, but none of them showed β -haemolysis. These results corroborate those of Syal and Vohra (2013) and Fadda et al. (2017) who showed respectively the absence of gelatinase production and β -haemolysis in seven strains from Indian fermented foods and seven strains of the genus *Kluyveromyces* isolated from cheeses in Italy.

Table 4. Contribution of variables to PCA factors based on correlation.

	F1	F2	F3	F4	F5	F6	F7
Survival pH3	0.786	0.510	0.164	-0.175	0.219	0.038	-0.122
Survival pH2	0.864	0.432	0.041	-0.189	0.106	-0.017	0.134
Auto-aggregation 2h (%)	-0.689	0.347	0.341	0.222	0.400	-0.283	0.015
Auto-aggregation 4h (%)	-0.555	0.554	0.472	0.110	-0.161	0.351	0.017
Co-aggregation (%)	0.654	-0.413	0.099	0.521	0.285	0.197	0.010
Hexane (%)	0.572	-0.122	0.652	0.167	-0.394	-0.223	-0.009
Chloroform (%)	0.180	0.621	-0.581	0.433	-0.220	-0.088	-0.011

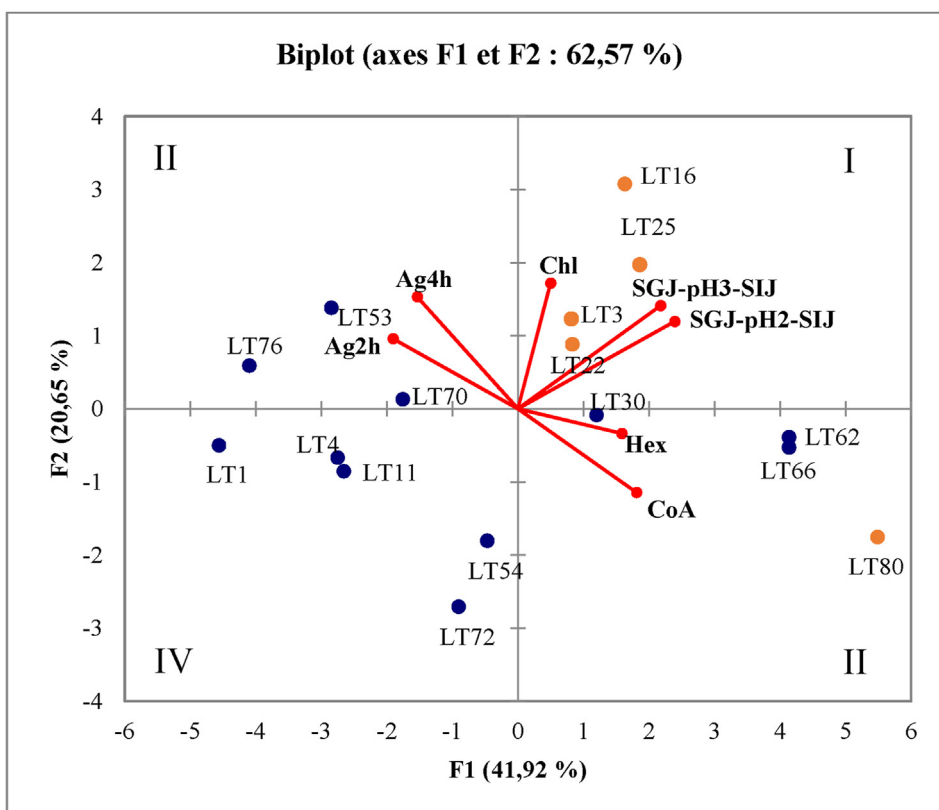


Figure 1. Projection of the variables and yeast isolates onto the plane formed by the two factors obtained by PCA. Hex: hexane; Chl: chloroform; Ag2h (4 h): auto-agrégation after 2 h (or 4 h); CoA: Co-aggregation; SGJ-pH3-SIJ: simulated gastric juice at pH3 follow by simulated intestinal juice; SGJ-pH2-SIJ: simulated gastric juice at pH 2 followed by simulated intestinal juice.

Table 5. Presence or absence of biogenic amine, hemolysin and gelatinase production and their susceptibility to antibiotics.

Isolates	Antibiotics						Antifungals			Heamolysin	
	Amox	Erhy	Cipro	Genta	Tetra	Levo	Nysta	Fluco	Itra	β-heamolysin	α-heamolysin
LT3	R	R	R	R	R	R	S	S	S	-	+
LT16	R	R	R	R	R	R	S	S	S	-	-
LT22	R	R	R	R	R	R	S	S	S	-	+
LT25	R	R	R	R	R	R	S	S	S	-	-
LT80	R	R	R	R	R	R	S	S	S	-	-

Amox: amoxicillin, Ery: erythromycin, Cipro: ciprofloxacin, Genta: gentamicin, Tetra: tetracyclin, Amp: ampicillin, Levo: levofloxacin, Fluco: fluconazol, Itra: itraconazol, Fluoro: fluorocytosin, Anud: anudilafungin, Ampho: amphotericin B, Nyst: nystatin.

3.9. Bile salt hydrolase (BSH) activity

Base on ACP, three isolates were selected and all were capable of hydrolysing bile salts. Two types of halo around the spots were observed (Figure 2): LT16, LT25, LT80 show clear and opaque halos around the spots; this would reflect the ability of these isolates to deconjugate glyco and tauroconjugated bile salts (Fadda et al., 2017).

Table 6 summarises the BSH activities of the three isolates selected for further investigation. It can be seen that all the yeast isolates show an ability to deconjugate bile salts and is dependent on the compartment tested. The highest amount of amino acids released was provided by LT25 (41.99 ± 0.00%) and LT16 (60.54 ± 0.12%) in the supernatants and intracellular contents respectively. Although this type of result is still poorly described, it could be explained by the ability of the isolates to produce BSH that hydrolyse bile salts allowing the isolates to better adapt to the intestinal conditions of their host. Very few authors have obtained BSH activities. While bile salt deconjugation is quite common in bacteria of the genus *Lactobacillus* (Foko et al., 2018) and *Bifidobacterium* (Jarocki

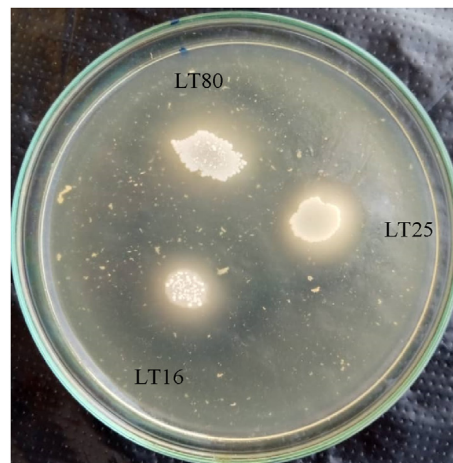


Figure 2. Hydrolysis of bile salts on Yeast extract Peptone Glucose agar plate presenting clear and opaque areas around the spots.

Table 6. Bile salt hydrolase and antiradical activities of supernatants and intracellular contents of selected isolates.

Isolates	Supernatant		ICC		Reduction of DPPH [•] (%) of supernatants	Reduction of DPPH [•] (%) of ICC	Reduction of DPPH [•] (%) of cells
	Enzymatic activity (µg of bile salt/ml/min)	Amino acid (%)	Enzymatic activity (µg of bile salt/ml/min)	Amino acid (%)			
LT16	1.02 ± 0.00 ^{CB}	20.36 ± 0.12 ^{CB}	3.06 ± 0.02 ^{AA}	60.54 ± 0.12 ^{AA}	46.58 ± 1.42 ^{BB}	13.02 ± 0.55 ^{CC}	0.00 ± 0.00 ^{CC}
LT25	2.12 ± 0.00 ^{AA}	41.99 ± 0 ^{AA}	0 ± 0.00 ^{BB}	0 ± 0.00 ^{BB}	55.94 ± 1.14 ^{AA}	24.26 ± 0.49 ^{AA}	12.64 ± 0.02 ^{AA}
LT80	1.84 ± 0.00 ^{BA}	38.54 ± 1.8 ^{BA}	0 ± 0.00 ^{BB}	0 ± 0.00 ^{BB}	34.56 ± 1.07 ^{CC}	22.86 ± 1.24 ^{BB}	11.30 ± 0.04 ^{BB}

^{a-c} On the same column, values with lower case letters differ significantly ($p < 0.05$); ^{A-C} On the same row for a given activity, values with higher case letters differ significantly ($p < 0.05$); ICC: IntraCellular Content.

and Targonski, 2013) isolated from spontaneous fermentations, some authors have shown that very few yeasts possess the ability to deconjugate bile salts, although this mechanism has not been sufficiently explored. Hernández-Gómez et al. (2021) demonstrated for the first time that the *S. cerevisiae* var. *boulardii* CNCM I-745 strain has a good ability to hydrolyse bile salts and had a closer affinity towards glycodeoxycholic bile salts, even though this was not detected in Petri dishes. When *S. boulardii* CNCM I-745 is administered to humans, an anti-hypercholesterolemic effect is observed (Briand et al., 2019). In fact, the hydrolysis of bile salt in the intestine leads to the breaking of the enterohepatic cycle and the elimination of the products of this hydrolysis in the faeces. The organism is thus forced to draw cholesterol from its stock to renew the bile salt pool and consequently reduce the endogenous cholesterol level. Furthermore, micelles provide a solvent vehicle for water-insoluble compounds, promoting their absorption along the small intestine (Ridlon et al., 2016). Therefore, an increase in BSH activity in the small intestine disrupts the formation of micelles and thus the absorption of cholesterol and lipids. However, BSH activity is not unique to *Saccharomyces* as it has already been demonstrated in seven strains of the genus *Kluyveromyces* (Fadda et al., 2017).

3.10. DPPH[•] radical scavenging

Free radical scavenging activities of the supernatants and intracellular contents of the isolates relating to their ability to scavenge DPPH[•] radicals are recorded in Table 6. This property is isolate dependent and depends on the compartment tested. The highest activities were 55.94 ± 1.14 with LT25 supernatant, 34.56 ± 1.07 with LT80 intracellular content and 12.64 ± 0.02% with LT25 whole cells. According to Gil-Rodríguez et al. (2015), the antiradical activities are classified into five groups according to their percentage of DPPH[•] inhibition: very poor (<20%), poor (20–30%), good (30–40%), very good (40–50%) and finally excellent (>50%). It follows from this classification that our yeast isolates have lower free radical scavenging activities obtained from cells than those obtained from supernatants where LT25 had an excellent activity (55.94 ± 1.14%). These results are contrary to those of Fakruddin et al. (2017) who demonstrated that *S. cerevisiae* IFST062013 cells show excellent DPPH[•] radical inhibition. Indeed, during their growth, yeasts produce a wide range of metabolites (ascorbic acid, polyphenols, glutathione, thioredoxin, melatonin...) with the capacity to inhibit free radicals (Vázquez et al., 2017; Abegg et al., 2012). They are the first line of defence against free radicals. In the human body, antioxidants, including anti-free radical compounds, reduce the pool of free radicals by converting hydroperoxides and hydrogen peroxide into alcohols and water respectively, without generating free radicals, thus reducing cellular aggression. Thus, a regular and adequate intake of antioxidants through the diet could reduce the risk of many chronic diseases such as diabetes, atherosclerosis, cancer and Alzheimer's disease (Rani et al., 2021; Rani, 2017).

3.11. Identification of the selected yeasts

Following PCA and safety testing three of the five isolates were selected and sequencing of the 28S rRNA of LT25 and LT80 portion and 5.8S of LT16 suggests that LT25 and LT16 correspond to *Saccharomyces*

cerevisiae and LT80 to *Nakaseomyces delphensis*, their accession numbers are respectively ON142612, ON149750, and ON142613. Unlike *S. cerevisiae* which is widely present in fermented starchy beverages, this is the first time that *N. delphensis* has been isolated from a fermented beverage (*Sha'a*). The fact that malted beverages are sweetened is favourable for the development of *S. cerevisiae* while the presence of *N. delphensis* can be associated with the storage conditions since it has been shown that *N. delphensis* is generally found in *Drosophila* and other flying insects (Morais et al., 1992) highly present in the *Sha'a* consumption area. *N. delphensis* has been subjected to very poor functional characterisation but has already been isolated from sweet dried fig waste (Davide et al., 2018). While belonging to the *Nakaseomyces* clade, *N. delphensis* is non-pathogenic as *N. bacillisporus* (Kurtzman et al., 2011).

4. Conclusion

The yeast flora of *Sha'a* is made up of a multitude of yeasts with varied potential. The 16 isolates pre-selected in this work showed a good ability to ferment glucose, assimilate maltose (the main sugars in germinated cereals) and to tolerate alcoholic, osmotic and acid stress during fermentation. In addition, five isolates with the best fermentation skills showed the ability to survive the barriers of the gastrointestinal tract. Three of these isolates, identified as *Saccharomyces cerevisiae* LT25 and LT16 and *Nakaseomyces delphensis* LT80, were the most promising and can exert a probiotic effect by producing antioxidants and a hypocholesterolemic effect through bile salt hydrolases beneficial for the prevention of cardiovascular disease. These strains can therefore be used to standardise the fermentation of starchy beverages such as *Sha'a*. However, *N. delphensis* LT80 requires a particular reserve. Future studies will consist of obtaining active dehydrated yeasts and evaluating their viability and fermentation performance.

Declarations

Author contribution statement

Laverdure Tchamani Piame, Pierre Marie Kaktcham, Edith Marius Kouam Foko, Ulrich Daquain Fotso Techeu, Joel Romial Ngouenam, François Zambou Ngoufack: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Data availability statement

Data associated with this study has been deposited at LT25 and LT16 correspond to *Saccharomyces cerevisiae* and LT80 to *Nakaseomyces delphensis* under the accession numbers ON142612, ON149750, and ON142613 respectively.

Declaration of interest's statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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