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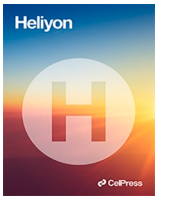

Research article

Isolation of yeast from some Ethiopian traditional fermented beverages and *in vitro* evaluation for probiotic traits

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ABSTRACT

Traditional fermented foods and beverages are important sources of probiotic microbes. The purpose of this study was to isolate yeast from Ethiopian fermented beverages and assess their probiotic activity in an *in vitro* setting. Yeast isolation, identification, and *in vitro* probiotic trait screening were conducted in accordance with established protocols. Eleven isolates were obtained. Of them, GB1D5, RTj3D3 and DMTD2 were low hydrogen sulfide producers and were selected. The D1/2 genotyping of selected isolates revealed that they were strains of *Saccharomyces cerevisiae*. All strains grew well at low pH, body temperature, bile salt concentrations (0.3–0.6 (w/v)) and survived at simulated gastrointestinal conditions with survival percentages of 12.8 ± 4.9 to 14.4 ± 5.0 % and 5.3 ± 1.7–5.9 ± 1.8 %, respectively. They demonstrated surface hydrophobicity ranging from 61.3 to 68.7 %; and 80.7–86 % auto-aggregation percentages after 24 h of incubation. They also showed hydroxyl radical scavenging activity ranging between 91.6 and 92.3 % and mild inhibitory activity against *Escherichia coli* (ATCC 893614) and *Staphylococcus aureus* (ATCC 892760). The PCA revealed that two strains (DMTD2 and RTj3D3) have a strong association with most probiotic properties, which affirms their promising candidacy. Safety assessments indicated that they were resistant to antibacterial antibiotics, susceptible to antifungals, and negative for protease, gelatinase, biogenic amine production, and hemolytic activity. All these suggest that they are promising candidates for the production of food containing probiotics. Examining their performance in vivo circumstances is recommended.

1. Introduction

The report in [1] indicated that the global population was 7.7 billion in 2019, and this number is expected to rise to 8.5 billion by 2030, 9.7 billion by 2050, and 10.9 billion by 2100. This demands food availability that will be 60 % greater than what is available today. It is not only about food availability; the types of foods needed and their consumption patterns and contributions as a diet will also change [1].

Of all food types, protein demand is growing alarmingly due to socio-economic shifts such as economic development, increased urbanization, and growing awareness of the health value of protein [2]. In recent years, people have preferred to consume functional

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foods such as probiotics, which they recognize as rational alternatives to antimicrobial compounds to fight infectious diseases beyond the basic nutrition value they provide [3–5].

Probiotics are referred to live microorganisms that, when taken in sufficient quantities, improve the host's health [6]. Probiotics boost the host immune system [7], balance micro-flora homeostasis in the gut [8], reduce the risk of various cancers [9], enhance food digestion, reduce cholesterol [10], produce vitamins, provide antibacterial and antioxidant activity [11–13], and prevent cardio-vascular diseases and some allergic reactions [13]. Probiotic organisms should be non-pathogenic, non-toxic, and harmless and provoke health value in the host cell [14].

For a microbe to be classified as probiotic, it must fulfill a number of requirements. The most essential parameters are the ability to tolerate stressful gastro-intestinal environments (acidic gastric pH, alkaline intestinal pH, bile salt, and digestive enzymes) [15], attachment to epithelial cells of the host, and sensitivity to antibiotics [16]. The majority of probiotics are bacteria, and *Lactobacillus acidophilus* and *Lactobacillus rhamnosus* strain GG have the oldest history of being used as probiotics [17]. Recently, various bacterial and yeast probiotics have become available on the market [12]. Compared to bacterial probiotic candidates, yeast have a higher tolerance to low pH and they are potential probiotic candidates [18]. Yeast cells are larger in size than bacteria, which helps them to impact a steric impediment against pathogenic bacteria, and thus increase their potential to be a probiotic candidate. This has prompted a great deal of research into the selection and use of possible strains of probiotic yeast in the development and production of probiotic containing foods [17,19]. However, only a few *Saccharomyces cerevisiae* strains have been identified and marketed as probiotics for human use [20].

Traditional fermented foods and beverages are known sources of probiotic microbiota [21,22]. In different societies, there is the notion that certain fermented foods and beverages have therapeutic benefits in various communities across the world, which might be due to probiotic microorganisms [23,24]. Furthermore, reports concluded that traditional fermented foods and beverages can be exemplary for the creation and production of probiotic food [25–27].

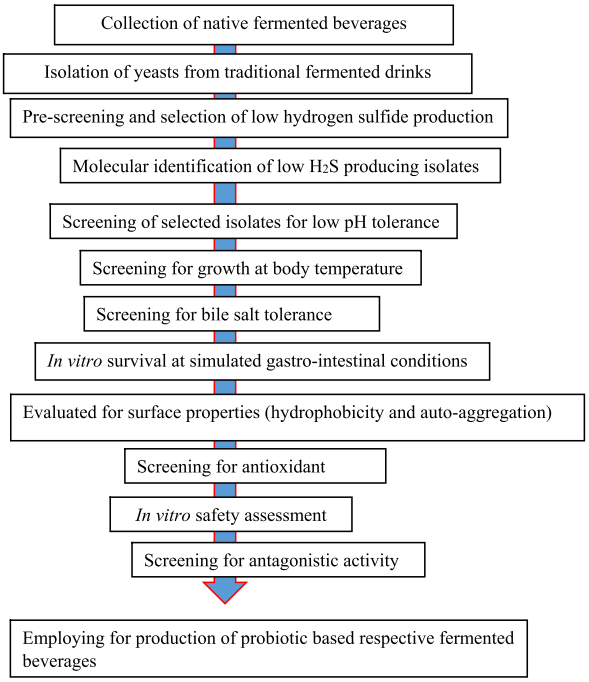
Ethiopia is a multiethnic nation, and diverse types of fermented products are prepared and consumed by different communities [28]. Among the native fermented drinks of Ethiopia, *Tella* (traditional beer), *Tej* (Ethiopian honey wine), and *Bubugne* are prepared and drunk in various areas of the country. *Tella* and *Tej* are considered alcoholic and are more popular in the central and northern regions of the country [29,30]. Whereas *Bubugne* is non-alcoholic, and it is a common drink in North Gondar communities [31]. *Tella* and *Bubugne* are cereal-based fermented beverages, whereas *Tej* is made from honey with the leaves and stems of *Rhamnus prinoides* as a bittering agent [30]. They are consumed on different occasions like holidays and wedding ceremonies and are part of the staple foods of rural households throughout the hectic farming seasons as refreshing and energy drinks [23,32].

Tella, *Tej*, and *Bubugne* are among the fermented beverages that are still prepared at the household level in uncontrolled conditions using basic tools like earthen vessels and empty oil vats. Handling and consumption of these products frequently occur in unsanitary conditions [33]. Fermentation is initiated by different groups of microorganisms, and as time goes on, some organisms sequentially become dominant. The organoleptic properties of the final product are determined by a combination of molecules produced during the course of fermentation [34]. Lactic acid bacteria (LAB) and yeasts are the major actors and contribute to the taste and favor of the fermenting material. As a result of the spontaneous fermentation processes of *Tella*, *Tej*, and *Bubugne*, there are no national standards for the final products. Therefore, it is evident that traditional beverages' nutritional qualities need to be improved in order to support their anticipated commercialization in both domestic and international markets [23].

In line with this, native fermented products are under a great deal of study to understand microbial dynamics [33], isolation of dominant microbes [35], and evaluation for probiotic traits [36,37]. *Saccharomyces cerevisiae* and related species are predominantly found in Ethiopian fermented beverages and foods [28,35,38]. However, very limited efforts have been made so far in the evaluation of yeast isolate for probiotic traits, targeting their possible application for functional food production. Thus, we herein aim to isolate yeasts from some Ethiopian traditional fermented beverages, i.e., *Tella, Tej* and *Bubugne*, and assess their probiotic potential to enhance their value, production and facilitate commercialization as functional foods.

2. Materials and methods

2.1. Conceptual framework



2.2. Sample collection and transportation

Fermented beverages, i.e., *Tella* and *Tej,* were collected respectively from Debre Markos and Robit districts of Amhara Regional State, Ethiopia. Samples of *Bubugne* were collected from Gondar district. At each sampling, 250 mL of each fermented beverage were collected using a 300 mL sterile glass bottle. Then, collected samples were brought to the Cellular and Microbial Laboratory, Institute of Biotechnology, University of Gondar, with an ice box to avoid the dynamics of microbes in the sample. Prior to processing, all samples were kept at 4 °C [39].

2.3. Isolation and morphological characterization of isolates

Each collected fermented beverage was serially diluted (10^{-1} to 10^{-5}) using peptone water as diluents, and a 50 μL sample from 10^{-4} and 10^{-5} dilution factors was spread plated onto yeast extract peptone dextrose agar (YPDA) (containing yeast extract 10 g/L (Oxoid), peptone (Himedia) 10 g/L, D-glucose (Blulux) 20 g/L, agar powder 20 g/L (Himedia) and chloramphenicol, 0.5 g/L, adjusted to pH 5). Inoculated YPDA plates were incubated at 30 °C for 48 h. Typical colonies that exhibit comparatively distinct cultural traits were subcultured onto YDPA plates to obtain the pure culture of each different likely colony. Following, each distinct colony was separately grown and kept for future characterization and probiotic performance assessment [40].

2.4. Pre-selection of yeast isolates

Considering its unpleasant taste for human consumption, hydrogen sulfide (H_2S) production was used as a pre-screening hurdle, to withdraw moderate and high H_2S-producing isolates. Hydrogen sufide production was assessed according to [41]. Each isolate was thickly streaked on bismuth sulfite agar (BSA) (containing BSA 26 g/L (Himedia), peptone 10 g/L (Himedia), yeast extract 10 g/L (Oxoid) and glucose 10 g/L (Blulux), pH 5) and incubated at 30 °C for 72 h. The degree of H_2S production was estimated by the color of the streaks, which turned from light brown to black depending on the extent of H_2S production. Non-producers remain white. According to the color of the colonies, different rankings (0–4) were assigned: 0, white; 1, light brown; 2, brown; 3, dark brown; and 4, black [41]. Those low-H_2S-producing isolates were selected and characterized for probiotic traits.

2.5. Molecular identification of selected isolates

2.5.1. DNA extraction

Low-H_2S producing isolates were pre-grown on YPDA plate, and their genomic DNA was extracted using GenElute^{TM} Fungal/ Plant Genomic DNA Miniprep Kit (Sigma Aldrich). The concentration and quality of extracted DNA were determined using NanoDrop (BIOBASE) and gel electrophoresis (BIOBASE). Extracted DNA was kept at -20 °C until processed for PCR amplification.

2.5.2. PCR amplification of D1/D2

The D1/2 region of the 26S rRNA gene was used as a marker and amplified using forward primer NL1 (5′- GCC ATA TCA ATA AGC GGA GGA AAA G-3′) and the reverse primer LS2 (5′-ATT CCC AAA CAA CTC GAC TC-3) (Sigma Company). The PCR reaction volume was 20 μL containing (5 x FIREPol® Master Mix 4 μL, forward primer 0.3 μL (10 pmol/μL), reverse primer 0.3 μL (10 pmol/μL), 3 μL (30 ng), template DNA, and 12.4 μL nuclease-free water (Solis BioDyne Data Sheet). The PCR was run for 30 cycles, with initial denaturation at 95 °C for 5 min, denaturation at 95 °C for 30 s, annealing temperature at 54 °C for 30 s, extension at 72 °C for 2 min, and final extension at 72 °C for 7 min [42]. The quality of PCR products were analyzed by loading 5 μL onto 2 % agarose gels containing 3 μL ethidium bromide and visualized under UV light. The approximate molecular sizes of the amplicons were determined using 50 bp DNA Ladder as a molecular weight marker. The PCR products were then sent to Macrogen Europe, the Netherlands for sequencing.

2.5.2.1. Sequence analysis, annotation and phylogenetic tree construction. The D1/2 sequence of each isolate was edited using the BioEdit software package and checked for similarity using the BLAST program (https:// blast. ncbi.nlm.nih.gov/Blast.cgi) against previously annotated sequences from GenBank databases. Species determination was made by considering identity percentage $\geq$ 95 %, E-value equal to or near zero, and query coverage $\geq$ 95 %. The D1/D2 sequence of close-related strains was retrieved from the GenBank, and multiple sequence alignment was done using the CLUSTAL W program. The phylogenetic tree was constructed using the neighbor-joining method [43] and the Jukes-Cantor model [44] by MEGA 11 software, considering 1000 bootstrap replication and a 50 % cut-off value [45]. Finally, the sequence of each isolate was annotated to the GenBank of the NCBI database for permanent deposit.

2.6. Screening of identified isolates for probiotic traits

2.6.1. Growth and survival at low pH

The protocol of [46] was used to assess the growth and survival of low-H_2S-producing strains at low pH values (1.5, 2, and 2.5). A 100 μL suspension (10^8 CFU/mL) of each strain was inoculated into a separate test tube containing 20 mL of yeast extract dextrose (YPD) broth (containing yeast extract, 10 g/L (Oxoid); peptone (Himedia), 10 g/L; D-glucose (Blulux), 20 g/L, adjusted to each test pH value) and incubated at 37 °C. After 72 h of incubation, the broth culture of each strain was serially diluted, and their viability was determined by spreading 20 μL of cell suspension from the 10^{-3} dilution factor onto YPDA plates (pH 4.5). The survival percentage of each strain at each pH value was determined relative to their growth in the control medium (pH 4.5, the optimum pH [40]) using equation (1).

$$\text{Survival (\%)} = \frac{CFU/mL \text{ in low pH stressed medium}}{CFU/mL \text{ from control medium}} X\ 100 \quad (1)$$

2.6.2. Growth and survival at body temperature

Likely, the growth and survival of low H_2S-producing strains at body temperatures (37 °C) and 40 °C (considering extreme conditions) were evaluated by inoculating 100 μL of each strain (10^8 CFU/mL) into a separate test tube containing 20 mL of YPD broth (pH 4.5) and incubating at 30, 37, and 40 °C for 72 h. Then broth cultures of each strain were serially diluted, and their viability was determined by spreading 20 μL of cell suspension from a 10^{-3} dilution factor onto YPDA plates (pH 4.5). The tolerance and growth of each isolate was evaluated qualitatively by observing culture turbidity [47]. The survival percentage of each strain at 37 and 40 °C was determined relative to their survival at 30 °C (the optimum temperature) [40] using equation (2).

$$Survival(\%) = \frac{CFU/mL \text{ in high temperature stressed medium}}{CFU/mL \text{ at } 30^{\circ}C} \times 100 \quad (2)$$

2.6.3. Bile salt tolerance

The bile salt tolerance of strains was evaluated according to [48], with little modification. A 100 μL cell suspension (10^8 CFU/ml) of each strain was inoculated into 20 mL of YPD broth having different concentrations (0.3 %, 0.4 %, 0.5 %, and 0.6 %, w/v) of bile salt (Sigma Aldrich) and incubated at 37 °C for 48 h. Following, cultures of each isolate were serially diluted, and their viability was checked by spreading 20 μL of cell suspension from the 10^{-3} dilution factor onto YPDA plates (pH 4.5). Pure medium without bile salt was used as a control. The growth of each strain at each bile salt concentration was assessed qualitatively by looking at culture turbidity. The survival strains at each bile salt concentration were calculated in comparison to their survival in the control broth medium according to equation (3).

$$\text{Survival (\%)} = \frac{CFU/mL \text{ in bile salt stressed medium}}{CFU/mL \text{ from control medium}} X\ 100 \quad (3)$$

2.6.4. In vitro survival test in simulated gastro-intestinal conditions

2.6.4.1. Survival in gastric juice. The survival of selected strains in gastric juice conditions was estimated by preparing simulated gastric juice according to Ref. [48] with little modification. Simulated gastric juice (containing NaCl (2.05 g/L), KH_2PO_4 (0.60 g/L),

$CaCl_2$ (0.11 g/L), and KCl (0.37 g/L), pH 2.3) was prepared and sterilized at 121 °C for 15 min. After sterilization, it was cooled until it could be touched by hand, and lysozyme (0.02 g/L) was added to the simulated gastric juice. The viability of each strain was determined according to Ref. [48]. Twenty microliters of a 24 h old culture of each strain (10^8 CFU/mL) were inoculated into 15 mL of simulated gastric juice and incubated at 37 °C. After 3 h of incubation, 20 μL of suspension was spread-plated on YPDA (pH 4.5) and incubated at 37 °C for 72 h. A phosphate buffer saline (PBS) suspension of each strain was used as a control. The viability of each strain was estimated by counting colonies from both the treatment and control groups. Finally, the survival percentage for each strain was calculated according to equation (4).

$$\text{Survival } (\%) = \frac{CFU/_{mL} \text{ in gastric juice stressed medium}}{CFU/_{mL} \text{ from control medium}} X\ 100 \tag{4}$$

2.6.4.2. Survival in intestinal tract conditions. Pancreatic digestive juice was prepared according to [49] by suspending bile salts (3.0 g/L), trypsin (0.1 g/L), Na_2HPO_4 (26.9 g/L), and NaCl (8.5 g/L) using a PBS solution (Blulux) (pH 7.4) and pH adjusted to 8.0 with HCl. After 3 h of incubation, cell suspensions from gastric juice were pelleted by centrifuging at 4000 rpm for 10 min, washed twice with PBS, and inoculated in 15 mL of pancreatic digestive juice. Inoculated tubes were incubated at 37 °C for 3 h. The viability of each strain was determined according to Ref. [48] as equation (5).

$$\text{Survival } (\%) = \frac{CFU/_{mL} \text{ in pancratic juice stressed medium}}{CFU/_{mL} \text{ from control medium}} X\ 100 \tag{5}$$

2.6.5. Cell surface hydrophobicity

Cell surface hydrophobicity of strains was evaluated on the basis their adherence to hydrocarbon compounds, according to [50]. Strains were grown in YPD broth at 37 °C for 48 h and pelleted through centrifugation at 16000 rpm for 15 min at 10 °C. The collected pellet of each strain was washed twice with PBS (pH 7.4), re-suspended with PBS, and adjusted to an initial absorbance (Ao) to an optical density (OD) of 0.50 using a UV-spectrophotometer (BIOBASE) at 600 nm. Then 3 mL of cell suspension was transferred into a new glass tube, 1 mL of xylene (ACS) was added, and the mixture was vortex-mixed for 2 min. The mixture was then incubated at 37 °C for 20 min to ensure the separation of the aqueous phase from the non-polar phase. The non-polar phase was discarded. The aqueous phase was carefully transferred into a glass cuvette, and its absorbance was determined at 600 nm. Finally, the percentage of cell surface hydrophobicity was computed according to equation (6).

$$\text{Hydrobocity } (\%) = \left(1 - A/_{AO}\right) X\ 100 \tag{6}$$

Where A0 is cell suspension initial absorbance, and A is the absorbance of the aqueous phase after mixing.

2.6.6. Auto-aggregation

The protocol of [51] was used to assess the self-aggregation characteristics of strains. Each strain was grown in YPD broth at 37 °C for 24 h and pelleted by centrifugation at 16000 rpm for 15 min at 10 °C. The collected pellet of each strain was washed twice with PBS (pH 7.4), resuspended in PBS, and adjusted to the initial absorbance (A0) OD 0.5 using a UV spectrophotometer (BIOBASE) at 600 nm. Then cell suspensions were incubated at 37 °C, and the auto-aggregation of strains was determined after 2, 4, and 24 h of incubation. Finally, the auto-aggregation percentage was computed following equation (7).

$$\text{Autoaggregation } (\%) = \left(1 - At/_{Ao}\right) X\ 100 \tag{7}$$

Where A0 is the initial absorbance of the cell suspension, and A_t is the absorbance of the cell suspension after time t.

2.6.7. Antioxidant activity

2.6.7.1. Hydroxyl radical scavenging assay. The hydroxyl radical scavenging activity (HRSA) of strains was estimated according to [49]. Strains were pre-grown in YEPD broth at 37 ^{O}C for 24 h and harvested through centrifugation at 4000 rpm for 15 min. The recovered pellets were washed three times with sterile PBS and then re-suspended in 2 mL of PBS for intracellular antioxidant analysis. Following, 1 mL of each isolate (adjusted to 10^8 CFU/mL) was inoculated into a reaction mixture containing 1 mL of Brilliant Green (0.435 mM), 2 mL of $FeSO_4$ (0.5 mM), and 1.5 mL of H_2O_2 (3.0 %, w/v), and incubated at room temperature for 20 min. The absorbance was measured at 624 nm using a UV-spectrophotometer (BIOBASE), and HRSA was calculated using equation (8).

$$\text{Survival } (\%) = \frac{As - A0}{A - A0} X\ 100 \tag{8}$$

Where *As* is the optical density of the mixture (cell + reaction mixture), *A*0 is the optical density of PBS, and *A* is the optical density of reaction mixture only.

2.6.8. Safety assessment of isolates

2.6.8.1. Protolytic activity. Proteolytic activity was evaluated by spotted-plating 20 μL of strain suspension (10^8 CFU/mL) on skim milk agar medium. The inoculated plates were incubated at 37 ^{O}C for 72 h. Strains that showed a clear zone apart from their growth were considered positive for proteolytic activity [52].

2.6.8.2. Biogenic amine production. Selected *S. cerevisiae* strains were evaluated for biogenic amine production, according to [53]. The tested strains were grown on YPDA for 24 h and then spotted onto a test YPDA medium (yeast extract 1 % (Oxoid), peptone 2 % (Himedia), dextrose 2 % (Blulux), agar 2 % (Himedia), arginine 2 % (Blulux), histidine 2 % (Blulux), bromocresolpurple 0.5 % (Blulux), w/v and pH adjusted to 5.4) separately. The negative control was constituted with YPDA components and bromocresol purple and without amino acids. The inoculated plates were incubated at 26 °C for 72 h. The appearance of a purple halo around their culture, while yellow in the control medium, was considered biogenic amine (histamine and putrescine) production.

2.6.8.3. L-arginase production. Strains were screened for biogenic amine production by streaking on media constituting (KCl 5 g/L, MgSO4 5 g/L, KH_2PO_4 10 g/L, $FeSO_4$ 1 g/L, $ZnSO_4$, 1 g/L and arginine 10 g/L (Blulux)). A 1 mL of 20 % (w/v) phenol red (ACS) dissolved in 70 % ethanol was used as an indicator. The inoculated plates were incubated for 72 h at 30 °C. A medium containing all other components but not arginine was used as a negative control. The strain that turned the medium from yellow to pink were considered positive for arginase production [54].

2.6.8.4. Gelatinase activity. Gelatinase production by strains was studied by using trypetone-neopeptone-dextrose (TND) agar containing (trypetone 17 g/L (Himedia), neopeptone 3 g/L (Himedia), dextrose 2.5 g/L (Himedia), NaCl 5 g/L, K_2HPO_4 2.5 g/L, agar 20 g/L (Himedia) and gelatin powder 4 g/L). A dried medium was spot-inoculated with loopful of strains and incubated at 37 °C for 48 h. The Petri plates were then flooded with a saturated ammonium sulfate solution. The development of clear zones around the spots indicated gelatinase activity. Medium without gelatin was used as a negative control [55].

2.6.8.5. Hemolytic activity. The hemolytic activity was assessed to evaluate the pathogenicity of the strains. Each strain was spotted on blood agar (containing 7 % v/v human blood) and incubated at 37 ^{O}C for 48 h. Hemolytic activity was assessed by observing lyse zones around the colonies. *Staphylococcus aureus* (ATCC 892760) was used as a positive control [56].

2.6.8.6. Susceptibility to antibacterial and antifungals. The responses of strains to antibacterial and antifungal antibiotics were investigated using disc diffusion and agar well dilution assays, respectively. Each strain was grown in a YPDA (pH 4.5) plate and adjusted to 10^8 CFU/mL of saline water using the McFarland standard of 0.5. A 20 μL cell suspension of each strain was inoculated on Muller-Hinton agar (MHA) (Himedia) supplemented with 2 % (w/v) glucose. Antibacterial (ampicillin (30 μg), chloramphenicol (30 μg), erythromycin (15 μg), gentamicin (30 μg), tetracycline (30 μg), and vancomycin (30 μg)) discs were placed on pre-inoculated MHA plates and incubated at 37 °C for 48 h. A 200 mg of each antifungal were diluted with dimethyl sulfoxide (amphotericin B and ketoconazole) and water (fluconazole) into 12.5 μg/mL, which is within the established minimum inhibitory concentration (MIC) breakpoint of 0.0313–16 μg/mL. Then, 100 μL of each antimycotic agent was pipetted into a 6 mm well on MHA plates. These antimycotic agents were selected based on their availability in Pharmacies in the study area. Inhibition zones were measured, and isolates were categorized into susceptible, intermediate and resistant [57].

2.6.8.7. Antibacterial activity of isolates against selected pathogens. The antibacterial activity of the whole cell and cell-free supernatant of probiotic yeast isolates was evaluated against selected human pathogens, i.e., *E. coli* (ATCC 893614), *S. aureus* (ATCC 892760), *Salmonella Typhi* (ATCC 14028), and *Pseudomonas aeroginosa* (ATCC 27853). The whole-cell antagonistic property was evaluated using the agar overlay protocol as described by [49]. Whereas, the antibacterial properties of cell-free extracts were evaluated using the agar-well diffusion method. Probiotic strains were grown in YPD broth at 37 °C for 48 h. The cell-free supernatant of each strain was separated by centrifuging at 4000 rpm for 10 min. The supernatant was mixed with ammonium sulfate (60 % (w/v)) and kept overnight in the refrigerator for partial extraction of extracellular bioactive (killer) protein. Then, the mixture was centrifuged, and the pelleted protein was diluted with sterile saline water. Finally, 100 μL of extracted protein was loaded onto 6 mm wells and incubated at 37 °C for 48 h. The antagonistic activity of both the whole cell and the supernatant of isolates was assessed through observation and measurement of the clear zone around the well in mm. Isolates were categorized as negative, mild, strong, and very strong inhibitors, respectively, if < 5 mm, 5–10 mm, 10–20 mm, and >20 mm [58].

2.7. Quality control and statistical analysis

Standard and previously developed protocols were adopted to screen tested strains for potential probiotic properties. Negative controls were used in most of the experiments. All experimental data were generated in triplicate and documented on an Excel sheet. Recorded data sets were analyzed using one-way ANOVA analysis using SPSS version 23. Tukey post hoc multiple comparison test was used for mean comparison. The significant difference among variables was considered at $p \leq 0.05$. Principal component and clustering analysis were conducted using to Origin pro 8 version 22 to analyze the correlation between probiotic properties and yeast strains.

3. Results and discussions

3.1. Isolation and morphological characterization of yeast isolates

A total of 11 yeast isolates were isolated from *Tella* (2), *Bubugne* (5) and T*ej* (4). Morphological characterization of isolates showed variation in their colony margin, elevation, texture, and cell shape (Table 1). The majority of isolates were white in color, had smooth surface texture, and circular colony shape as shown in typical Fig. 1(A–C). Microscopic characterization demonstrated that most isolates are oval in their cell shapes. All are budding yeasts, with most budging at one pole, as indicated in typical Fig. 2(D–F). Isolates were designated as GB1D1, GB1D2, GB1D3, GB1D4 and GB1D5 (for *Bubugne*), RTj3D1, RTj3D2, RTj3D3 and RTj3D4 (for *Tej*) and DMTD1 and DMTD2 (for *Tella*) taking into account the sampling location, kind of beverage isolated from, and sample number.

3.2. Hydrogen sulfide production

All isolates produce H_2S, but the degree of colony coloration was quite different among isolates. Three isolates (DMTD2, GB1D5 and RTj3D3) were light brown, i.e., they are low H_2S producers (Fig. 3(B–D)). The remaining isolates appeared brown, which means they produce a moderate amount of H_2S. According to the study of [59], among 16 *S. cerevisiae* strains, a few were high producers, some were low producers, and some were H_2S-free. Research has revealed that the final H_2S concentration during natural fermentation of food and beverages is strain-dependent, involvement of multi-genes and medium nutritional composition [59–61].

Hydrogen sulfide is a pungent-tasting off-flavor compound produced by yeasts during fermentation. Traces of H_2S can change the organoleptic properties of fermented food products due to its high volatility and low sensory threshold value [62,63]. Its high concentration in the intestinal tract also causes cell toxicity and lowers the pH of the environment, which in turn interferes with the hemostasis of the gut microbiota [64]. In view of this, low-producer isolates, i.e., DMTD2, GB1D5 and RTj3D3 could be potential candidates for the production of probiotic-based beverages.

3.3. Molecular identification of low-H_2S producer isolates

As shown from Table 2, each isolate showed a reasonably high identity similarity percentage with the respective top hit strains previously annotated in the GenBank database. They were therefore recognized as strains of *S. cerevisiae*. The sequences of isolates were annotated in the GenBank, and the respective accession numbers were obtained as shown in Fig. 4.

Regarding the evolutionary relationship of the investigated strains alone and compared to the related strains, the tree suggests that the tested strains are closely related as compared with the reference strains. *Saccharomyces cerevisiae* DMTD2 and *Saccharomyces cerevisiae* GB1D5 are laid in the same clad, which implies they are more related than *Saccharomyces cerevisiae* RTj3D3.

3.4. Screening of isolates for probiotic traits

3.4.1. Growth and survival of S. cerevisiae strains at low pH

The investigation of the low pH tolerance of the tested strains revealed that all strains grew well and showed considerable survival at pH 2 and 2.5. Their survival ranged between 62.5 and 65.7 % and 70.3 and 78.5 %, respectively, at pH 2 and 2.5. But none of the strains were able to tolerate pH 1.5, as indicated in Fig. 5(A).

The result of the present study is in agreement with reports examining the probiotic properties of yeast isolates from different food sources [49,65,66]. However, it disagrees with [17,67], found yeast strains capable of surviving at pH 1.5. This difference could be attributed to species variation, source of isolation, cell wall composition [68], cell membrane lipid content, and genetic constituents responsible for pH tolerance in general [69].

Table 1
Morphological characterization of yeast isolates.

Isolate	Colony Characteristics						Microscopic Characteristics	
	Shape	Margin	Elevation	Size	Color	Surface texture	Shape	Budding polarity
GB1D1	Circular	Entire	Umbonate	Med.(7 mm)	White	Smooth	Circular	Dipolar
GB1D2	Circular	Entire	Raised	Med.(6 mm)	White	Smooth	Circular	Unipolar
GB1D3	Circular	Undulate	Umbonate	Med.(6 mm)	White White	Rough	Oval	Unipolar
GB1D4	Circular	Entire Entire	Umbonate	Med.(7 mm)	White White	Smooth	Oval	Unipolar
GB1D5	Circular	Undulate	Umbonate	Med.(7 mm)	White	Smooth Rough	Circular	Unipolar
RTj3D1	Circular	Entire Entire	Flat	Large (9 mm)	Yellow	Rough Smooth	Oval	Unipolar
RTj3D2	Circular	Entire Entire	Flat	Large.(8 mm)	White	Smooth	Oval	Unipolar
RTj3D3	Circular	Entire	Umbonate	Med.(7 mm)	White	Smooth	Oval	Unipolar
RTj3D4	Circular		Raised Umbonate	Med.(6 mm)Med.(5.8 mm)	White	Smooth	Oval	Unipolar
DMTD1	Circular		Raised	Med.(6.5 mm)			Oval	Dipolar
DMTD2	Circular						Oval	Unipolar

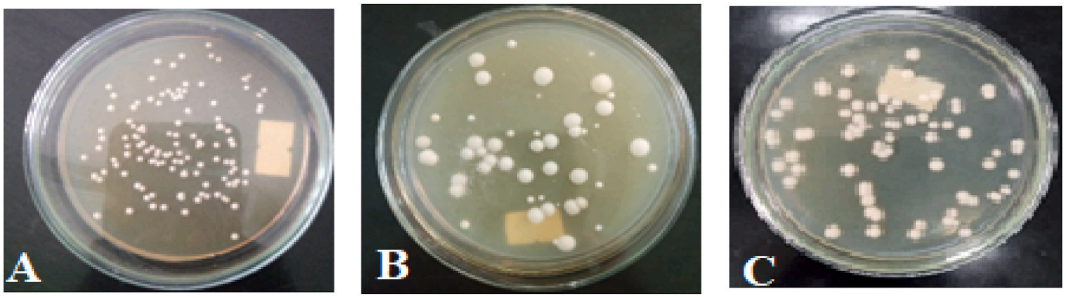


Fig. 1. Typical colony picture taken during isolation of GB1D5 (A), RTj3D3 (B) and DMTD2 (C).

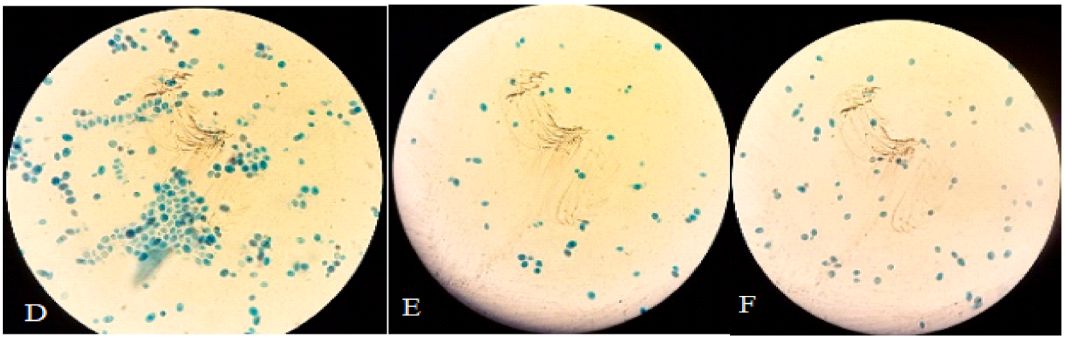


Fig. 2. Typical microscopic image for GB1D5 (D), DMTD2 (E) and RTj3D3 (F).

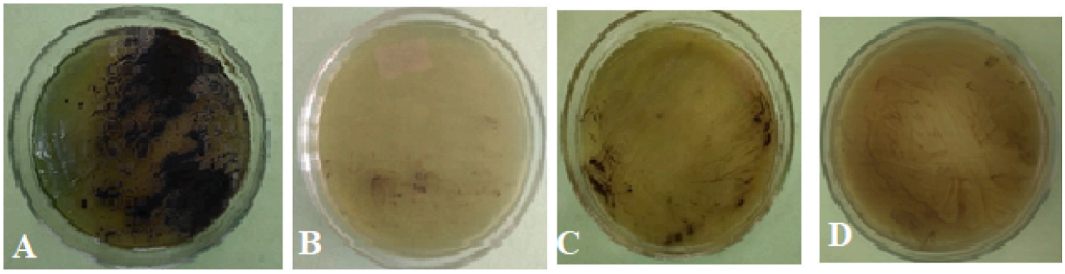


Fig. 3. Hydrogen sulfide production test. Control (high H_2S production) (A), DMTD2 (B), GB1D5 (C), RTj3D3 (D).

Table 2
Species level determination through BLAST hit score against annotated sequences in the GenBank.

Isolates	Top hit strain for each isolate	Identity percentage	E-value	Acc. number
DMTD2	*S. cerevisiae*isolate NP-7-5	99.01	2e-97	MN736547
GB1D5	*S. cerevisiae*strain WQY-17	99.49	7e-97	OR786928
RTj3D3	*S. cerevisiae* var. boulardii (nom. inval.) strain H11	98.98	4e-94	MT449164

Key: Acc. number (Accession number).

The *in vitro* screening of isolates for defined probiotic traits is considered a primary requirement to define and use probiotics [16]. The ability to prevail under stress conditions in the human body, such as low pH, high temperature, bile salt, gastric, and intestinal conditions, are the basic features of probiotic microorganisms [70]. The pH of the stomach varies between 2 and 3, and it is regarded as a strong chemical hurdle for probiotic microorganisms [71]. The survival of the tested strains at low pH conditions (pH 2 and 2.5) ensures their survival during passage in the gastric tract and their probiotic potential.

3.4.2. Growth and survival at body temperature

Screening for temperature tolerance indicated that tested strains survived with survival rates ranging from 55.4 to 57.5 % and 10.2–13.4 %, respectively, at 37 and 40 °C (Fig. 5(B), Sd Table 1). Their survival percentage was significantly reduced at 40 °C as

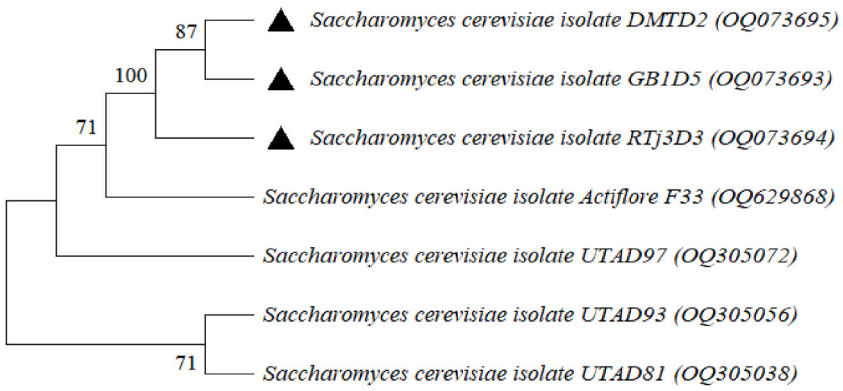


Fig. 4. Phylogenetic tree of investigated *S. cerevisiae* strains (pyramid labeled) and selected closely related strains.

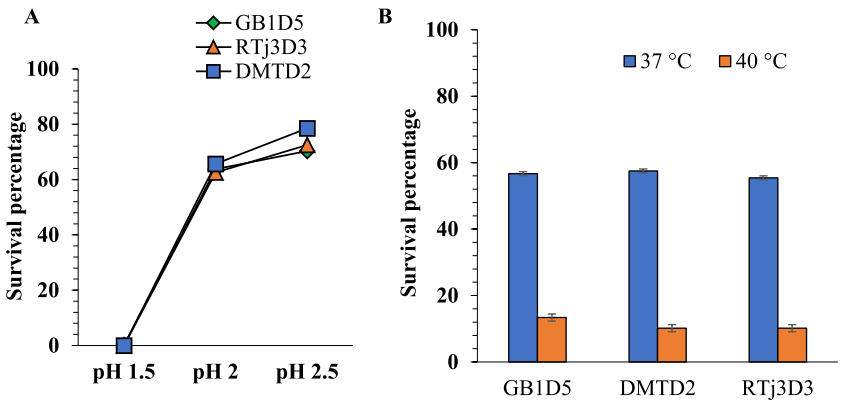


Fig. 5. Low pH survival percentage of *S. cerevisiae* strains **(A), s**urvival percentage of strains at body temperature and 40 ^{O}C **(B).** GB1D5 (*S. cerevisiae* GB1D5), RTj3D3 (*S. cerevisiae* RTj3D3) and DMTD2 (*S. cerevisiae* DMTD2).

compared to their viability at 37 °C. This was in line with the study by [26], which described that nine isolates among the 16 tested isolates demonstrated good viability at 37 °C.

Animal body temperature is 37 °C; the extreme range is up to 42 °C [12]. Evaluation of probiotic microbes for their ability to

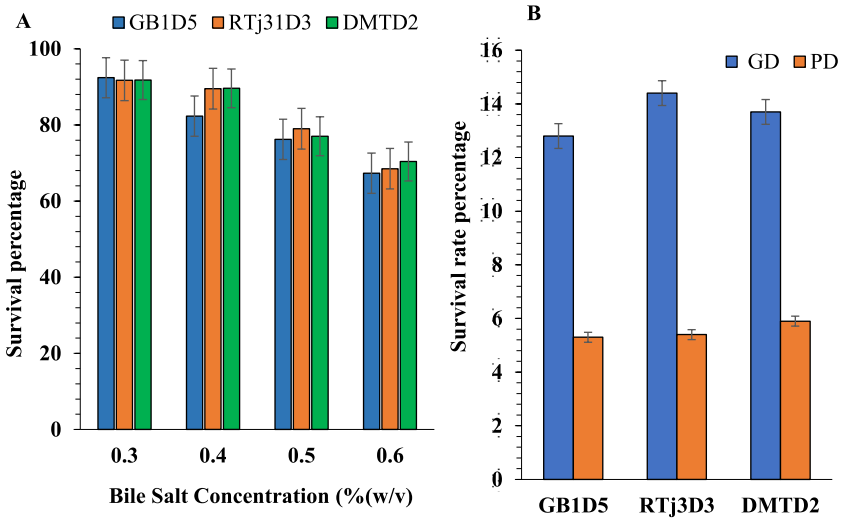


Fig. 6. Bile salt tolerance of selected isolates (**A**) and *in vitro* survival of isolates in simulated gastric and intestinal conditions (overall survival) (**B**). GD (Gastric Digestive), PD (Pancreatic Digestive).**)**. GB1D5 *(S. cerevisiae* GB1D5), RTj3D3 (*S. cerevisiae* RTj3D3) and DMTD2 (*S. cerevisiae* DMTD2).

survive and grow at 37 °C is an important parameter to ensure the exertion of their probiotic effect on the host cell [72]. In this regard, the reasonable growth and survival of the tested *S. cerevisiae* strains suggest that they can survive at host body temperature and impact its positive effect on the host cell.

3.4.3. Bile salt tolerance

Regarding bile salt tolerance, *S. cerevisiae* strains survived at all bile salt concentrations, with a survival percentage ranging from 91.7 to 92.4 % at a 0.3 % bile salt concentration. Their survival, however, was significantly reduced at 0.6 %, which declined between 67.3 and 70.4 % (Fig. 6(A), Sd Table 1**)**. Results of the current study are in agreement with the findings of [12,73], which stated the good survival of isolates at higher bile salt concentrations, which is one of the obstacles to probiotic survival in the gastrointestinal tract. Bile concentrations in the human gut range between 0.3 and 0.6 % [74,75]. Though the survival of tested strains at higher bile salt concentrations was significantly ($p \leq 0.05$) declined, their survival is still reasonable. The *in vitro* survival of strains at tested bile salt concentrations indicates their tolerance of bile salt in the intestinal tract and survival in the intestine, which is the action site. This demonstrated the potential of these strains to be considered probiotics.

3.4.4. Survival in simulated gastro-intestinal tract (GIT) conditions

The survival of tested strains was significantly reduced at gastro-intestinal conditions with survival percentages ranging from 12.8 ± 4.9 to 14.4 ± 5.0% and 5.3 ± 1.7 to 5.9 ± 1.8 %, respectively, at simulated gastric and intestinal conditions (Fig. 6(B), Sd Table 2). This was significantly lower as compared with their survival at low pH, body temperature and bile salt concentrations alone**.**

The current study's findings are consistent with the study of [14], declared that the survival percentage of yeast isolate was 1.78–75.8 and < 0.08 to 6.92 at simulated gastric and intestinal conditions, respectively. On the contrary, the survival of the tested strains was lower as compared with previous reports [50,54,76], as some probiotic *S. cerevisiae* strains demonstrated considerable survival, though some did not. This might be attributed to species and/or strain variations.

The tolerance of probiotic organisms in simulated GIT conditions is critically important to their being viable during passage through the GIT, where they are expected to reside. But the overall survival of the tested strains in GIT conditions was low. However, this issue can be partially offset by their frequent consumption as a result of their excretion with feces [77] and dose dependent effect [16].

3.4.5. Cell surface hydrophobicity and auto-aggregation assay

The evaluation of strains for cell surface hydrophobicity revealed that all strains showed good hydrophobicity, ranging from 61.3 ± 3.0 to 68.7 ± 3.5 % toward the organic compound xylene (Fig. 7(A), Sd Table 3). The highest hydrophobicity was demonstrated by *S. cerevisiae* RTj3D3, followed by *S. cerevisiae* DMTD2, which exhibits 66.7 % hydrophobicity. This is consistent with [76], which described a hydrophobicity percentage range of 40–72 % with the same organic solvent. The report [78] affirmed that probiotic strains with a hydrophobicity percentage > 40 % are hydrophobic. This implies that the tested strains are hydrophobic and could attach to the host's epithelial cells.

Similarly, the test strains demonstrated auto-aggregation activity ranging between 38.0 ± 10.58 % and 44.7 ± 9.8 % after 2 h of incubation at 37 °C. The auto-aggregation of strains increased as the incubation period was extended to 24 h, and it was between 80.7 ± 4.2 and 86.0 ± 2.0 % (Fig. 7(B)). The highest was recorded from *S. cerevisiae* RTj3D3. This was in agreement with the reports of [49], found self-aggregation of yeast isolates ranged from 64.9 to 82 %. But it is lower as compared with [54], who found that auto-aggregation percentages differ from 71.64 ± 0.87 to 94.72 ± 0.35 % after 2 h and from 87.55 ± 0.46 to 94.53 ± 0.03 % after 4 h of incubation. According to [79], probiotic microbes must have an auto-aggregation capability greater than 40 % so as to form a strong

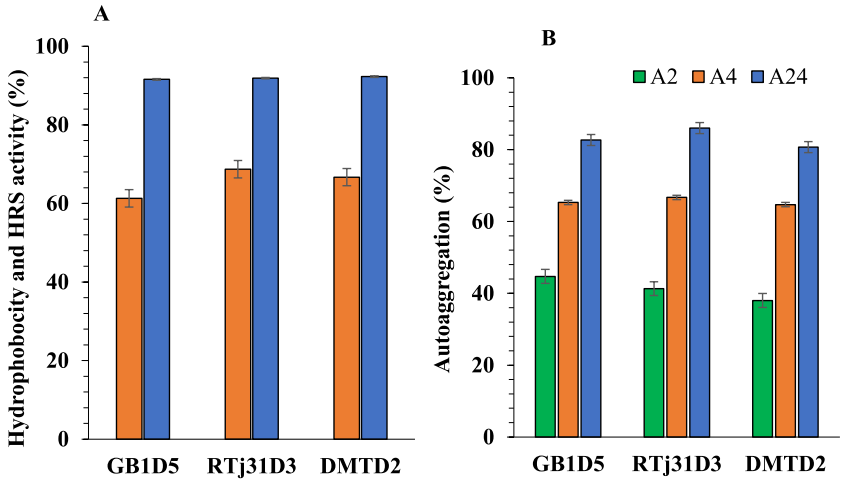


Fig. 7. Hydrophobicity and antioxidant activity **(A)**, and auto-aggregation of strains**(B)**. Auto-aggregation percentage A2 (after 2hrs), A4 (after 4hrs) and A24 (after 24hrs). GB1D5 *(S. cerevisiae* GB1D5), RTj3D3 (*S. cerevisiae* RTj3D3) and DMTD2 (*S. cerevisiae* DMTD2).

Table 3
The antibacterial activity of cell-free extract and susceptibility of isolate for antifungal antibiotics (inhibition zones in mm).

Strains	Pathogens				Antifungal Antibiotics		
	E. coli	*S. Typhi*	*P. aeroginosa*	*S. aureus*	Amp. B	Ketoconazole	Fluconazole
GB1D5	7.2 ± 0.8	2.3 ± 0.4	2.0 ± 0.3	18.0 ± 3.0	19.3 ± 3.2	17.0 ± 1.7	19.3 ± 1.2
RTj3D3	7.0 ± 1.7	2.1 ± 0.4	0.9 ± 0.1	11.3 ± 3.0	18.7 ± 3.1	20.0 ± 2.0	19.7 ± 4.2
DMTD2	6.7 ± 1.5	0.9 ± 0.2	1.0 ± 0.3	11.3 ± 1.5	19.3 ± 2.1	17.3 ± 1.5	19.4 ± 3.0

Key: Amphotericin B (Amp. B). GB1D5 *(S. cerevisiae* GB1D5), RTj3D3 (*S. cerevisiae* RTj3D3) and DMTD2 (*S. cerevisiae* DMTD2).

biofilm on the epithelial layer. Thus, the tested strains would establish a robust biofilm on the epithelial layer, thereby preventing pathogen attachment.

Since the GIT is always in a mobile phase, cell surface hydrophobicity and auto-aggregation are important traits of probiotic microbes to resist the peristaltic movement and colonize the epithelial cells of the GIT [56]. Hydrophobicity denotes probiotic strain cell surface hydrophobicity [80] whereas auto-aggregation is about the ability to film on the host cell [81]. Having these features increases the competition of probiotic strains for space with pathogens and ultimately excludes pathogen colonization of the intestinal tract. This suggests that all tested strains are able to attach to the epithelial surface of the host cell.

3.4.6. Antioxidant assay

Investigation of internal cellular antioxidant activity revealed that tested strains had significant hydroxyl radical scavenging activity ranging between 91.6 ± 0.9 and 92.3 ± 2.3 % (Fig. 7, Sd Table 3**)**. This is in agreement with [82] stating an excellent HRS activity of *S. cerevisiae* IFST 062013 against toxic radicals. However, it is higher than the study of [49], which reported HRS activity ranging of 51 % for *C. tropicalis* 12a to 57 % for *C. tropicalis* 33d. Antioxidant activity is a species-dependent property, so the variation might be due to their taxonomic variation. The study of [72] classified probiotic antioxidant activities into five groups based on 2, 2-Diphenyl-1-picryl-hydrazyl (DPPH) free radical scavenging percentage: very poor (<20 %), poor (20–30 %), good (30–40 %), very good (40–50 %) and finally excellent (>50 %). On the basis of this category, the tested strains have excellent free radical scavenging activities.

Antioxidant activity is another important property of probiotic microbes. Probiotics have been shown to have excellent antioxidant properties against oxygen radicals [11,83]. Thus, the excellent hydroxyl radical scavenging ability implies that tested strains are potential probiotic candidates.

3.4.7. Principal component analysis (PCA)

The PCA was used to estimate the correlations between the yeast strains and the tested probiotic properties, including hydrophobicity, auto-aggregation, antioxidant activity, bile salt concentrations and simulated gastrointestinal conditions, tolerance to low pH, and body temperature. As shown from the PCA biplots, the first two principal components accounted for 100 % of the total variance in the probiotic characteristics of the tested yeast strains (Fig. 8).

The principal component (PC1) explained 55.55 %, and PC2 accounted for 44.45 % of total probiotic trait variances for the *S. cerevisiae* strains, respectively. The *S. cerevisiae* strains were distributed in three different quadrants. *Saccharomyces cerevisiae* strain DMTD2 located in quadrant I (top right), showed a high correlation with tolerance to bile salt concentrations (BS 0.4 and 0.6 %), pH (2 and 2.5) and pancreatic digestive juice (PD). Strain GB1D5 was laid in quadrant III (bottom left) and showed correlation with temperature (40 ^{O}C) and auto-aggregation after 2 h. *Saccharomyces cerevisiae* strain RTj3D3 present in quadrant IV (bottom right) and strongly related to hydrophobicity, gastric digestive juice (GD), bile salt (BS%) and auto-aggregation after 4 and 24 h any of the probiotic traits and yeast strains, which implies this is an outlier and less important to be considered as a probiotic. *Saccharomyces cerevisiae* GB1D5 was laid in quadrant IV (bottom right) and related to simulated gastrointestinal conditions (GD and PD), body temperature (37 and 40 °C), and low pH tolerance at pH 2. *Saccharomyces cerevisiae* DMTD2 and RTj3D3 exhibited a stronger correlation among probiotic properties to PC1 than GB1D5. Based on the PCA biplots, these strains (DMTD2 and RTj3D3) were identified as the most promising probiotics.

3.4.8. Safety assessment

Safety assessments of tested *S. cerevisiae* strains revealed that they are negative for hemolytic, gelatinase, and biogenic amine production (histamine and putrescine). These results are consistent with previous reports [11,54,66,84], which stated that probiotic yeasts are negative for the aforementioned safety assessment tests. This shows the lower likelihood that these strains are pathogenic, which implies their possibility of being considered probiotics.

The antibacterial and antifungal susceptibility tests demonstrated that these strains were resistant to tested antibacterial antibiotics, but they were susceptible to antifungals, with inhibition zones ranging from 18.7 ± 3.1 to 19.7 ± 4.2 mm. This is in agreement with [54,67], which reported the susceptibility and resistance profiles of probiotic *S. cerevisiae* strains to antifungals and antibacterial antibiotics, respectively.

Safety is a big concern in the development and production of probiotics. Antibiotic resistance is one of the issues with using probiotics in the food business [85]. The negative responses of investigated strains for hemolytic and gelatinase activity, production of biogenic amines, resistance to antibacterial agents, and susceptibility to antimycotic antibiotics entail their safety. This implies that these strains remain unaffected by antibacterial antibiotics that could be taken during bacterial infections. Moreover, their

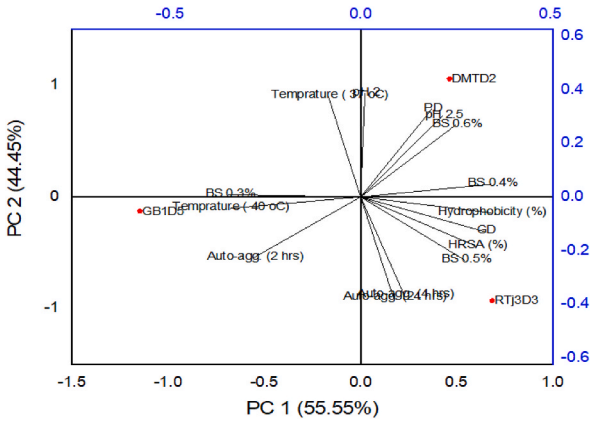


Fig. 8. Principal component analysis (PCA) biplot of probiotic characteristics of *S. cerevisiae* strains. GB1D5 *(S. cerevisiae* GB1D5), RTj3D3 (*S. cerevisiae* RTj3D3) and DMTD2 (*S. cerevisiae* DMTD2).

susceptibility to antifungals indicated that they could be managed if they imposed infection.

3.4.8.1. Antibacterial activity and susceptibility to antimycotic agents. The evaluation of the antagonistic activity of identified *S. cerevisiae* strains showed that the cell-free extract showed mild to strong antibacterial activity, with an inhibition zone ranging from 6.7 ± 1.5 to 18.0 ± 3.0 (Table 3). They showed mild antibacterial activity against *E. coli* (ATCC 893614) and *S. aureus* (ATCC 892760). But they haven't shown antagonistic activity against *S. Typhi* (ATCC 14028) and *P. aeroginosa* (ATCC 27853). This is in agreement with the study of [86] that investigated the antibacterial activity of *S. cerevisiae* against some selected food-borne pathogens.

But the whole cell did not show antagonistic activity against the tested pathogens. This is consistent with the study by [66], which stated none of the eight probiotic yeasts isolated from the fermented nectar of toddy palm showed inhibitory activity against selected human pathogens. The antibacterial activity of the supernatant but not the cell suggests that the inhibitory activity is due to the synthesis of antimicrobial metabolites.

The capability of having and/or producing antimicrobial substances is one of the essential features of probiotic microbes to avoid pathogens in the GIT, aside from competitive exclusion [11].The antibacterial properties of the studied strains suggest their potential to avoid pathogens in the GIT.

4. Conclusion

A total of 11 isolates were obtained, of which three isolates (DMTD2, GB1D5, and RTj3D3) were found to be low-H_2S producers. Molecular characterization of these isolates revealed that they are strains of *S. cerevisiae.* The survival under stressor conditions that existed in the host cell, such as low pH, body temperature, bile salt, and simulated gastro-intestinal conditions, revealed that all tested *S. cerevisiae* strains are promising probiotic candidates. Moreover, surface hydrophobicity, aggregation, antioxidant, and antagonistic activity confirmed that they are potential probiotics. Furthermore, the safety assessment result indicated that they are non-pathogens, resistant to antibacterial antibiotics, and susceptible to antifungals, which implies they can be used as probiotics. The PCA biplot showed that *S. cerevisiae* DMTD2 and RTj3D3 showed strong correlations to important probiotic traits, which implies they are potential candidates. The result of this study also proved that traditional fermented beverages are an important source of probiotic yeasts. These strains can be used for the production of probiotic-based fermented beverages. A future study is recommended to evaluate the probiotic potential of isolates under in *vivo* conditions.

CRediT authorship contribution statement

Dagnew Bitew: Writing – original draft, Formal analysis, Data curation, Conceptualization. **Bogale Damtew:** Writing – original draft, Formal analysis, Data curation, Conceptualization. **Anteneh Tesfaye:** Writing – review & editing, Validation, Supervision. **Berhanu Andualem:** Writing – review & editing, Validation, Supervision.

Declaration of funding issue

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Ethical statement

This study does not involve any human or animal subjects, and it is in accordance with research ethical standards.

Data availability statement

The data associated with the present study is not deposited into a publicly available repository yet. Data will be made available on principal author request.

Declaration of competing interest

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

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e40520.

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