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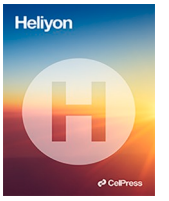

Research article

Probiotic yeast characterization and fungal amplicon metagenomics analysis of fermented bamboo shoot products from Arunachal Pradesh, northeast India

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ABSTRACT

This study investigates the diverse fungal community and their probiotic functions present in ethnic fermented bamboo shoots of Arunachal Pradesh. Among 95 yeast isolates, 13 demonstrated notable probiotic attributes. These included growth at pH 3, bile tolerance, autoaggregation, co-aggregation, hydrophobicity, lysozyme tolerance and antimicrobial activity. Confirmation of some of the probiotic properties through specific primers enabled the detection of genes associated with acid and bile tolerance, antimicrobial activity, and adhesion. Probiotic yeasts were finally identified based on D1 and D2 sequences of large ribosomal subunit as *Meyerozyma guilliermondii* (BEP1, KGM1_3, NHR3), *Meyerozyma caribbica* (GEP7), *Candida orthopsilopsis* (ES1_2, EB1_2, EEGM2_4, GEP2, NEK9), *Candida parasilopsis* (HD1_1), *Pichia kudriavzevii* (NHR12), *Pichia fermentans* (BEP2), and *Saccharomyces cerevisiae* (NEP2). Fungal amplicon sequencing highlighted the predominance of Ascomycetes, particularly *Pestalotiopsis* and *Penicillium* genera. In this study we have perfomed a culture dependent isolation and probiotic study of yeasts and culture independent analysis of the fungal community present during the fermentation of bamboo shoots of Arunachal Pradesh which provides information about the beneficial properties of bamboo shoots as the reservoir of probiotic microorganisms.

1. Introduction

The native people of Arunachal Pradesh have a culinary tradition that utilizes these local resources employing traditional fermentation, which adds a unique flavour and aroma to their dishes [1]. The ethnic fermented bamboo shoots (FBS) in Arunachal Pradesh, are popularly known as *ekung*, *hirring*, *eup or eepe*, as per the local dialect of the ethnic tribes and each product has specific methods of fermentation [2]. *Ekung* is prepared by packing the young bamboo shoots in bamboo baskets near a water source for 1–3 months [2]. The tender tip portion of the shoot is cut into 2–3 pieces or flattened, placed in bamboo baskets, and then fermented for 1–3 months to prepare *hirring*. *Eup* or *eepe* is fermented like *ekung*, completing the process within 1–3 months after which it is dried in the sun for 5–10 days. *Eepe*, a dark brown colour with a stronger flavour, has an extended shelf life. *Kupe* is made by cleaning bamboo

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shoots, separating tender tips, fermenting them, and sun-drying them for 7–10 days [3].

Yeast is commonly found in fermented vegetables, such as *sauerkraut*, *kimchi*, fermented olives, fermented cucumber, etc. contributing to their unique flavours and textures [4–8]. For instance, *Pichia fermentans*, has been associated with the development of aroma in cocoa beans [9] while it has been implicated in the significant production of volatile compounds, particularly esters during sauerkraut fermentation [6]. During the fermentation process of kimchi, various genera and species of yeasts have been identified, including *Saccharomyces, Tolulopsis, Debaryomyces, Pichia, Rhodotorula, Endomycopsis, Kluyveromyces, Cryptococcus, Trichospora* among which *Saccharomyces* is the most predominant [10]. In the later stages, mould species such as *Aspergillus, Penicillium,* and *Scopulariopsis* have also been isolated and identified in kimchi fermentation [11]. Different members from the fungal communities have also been

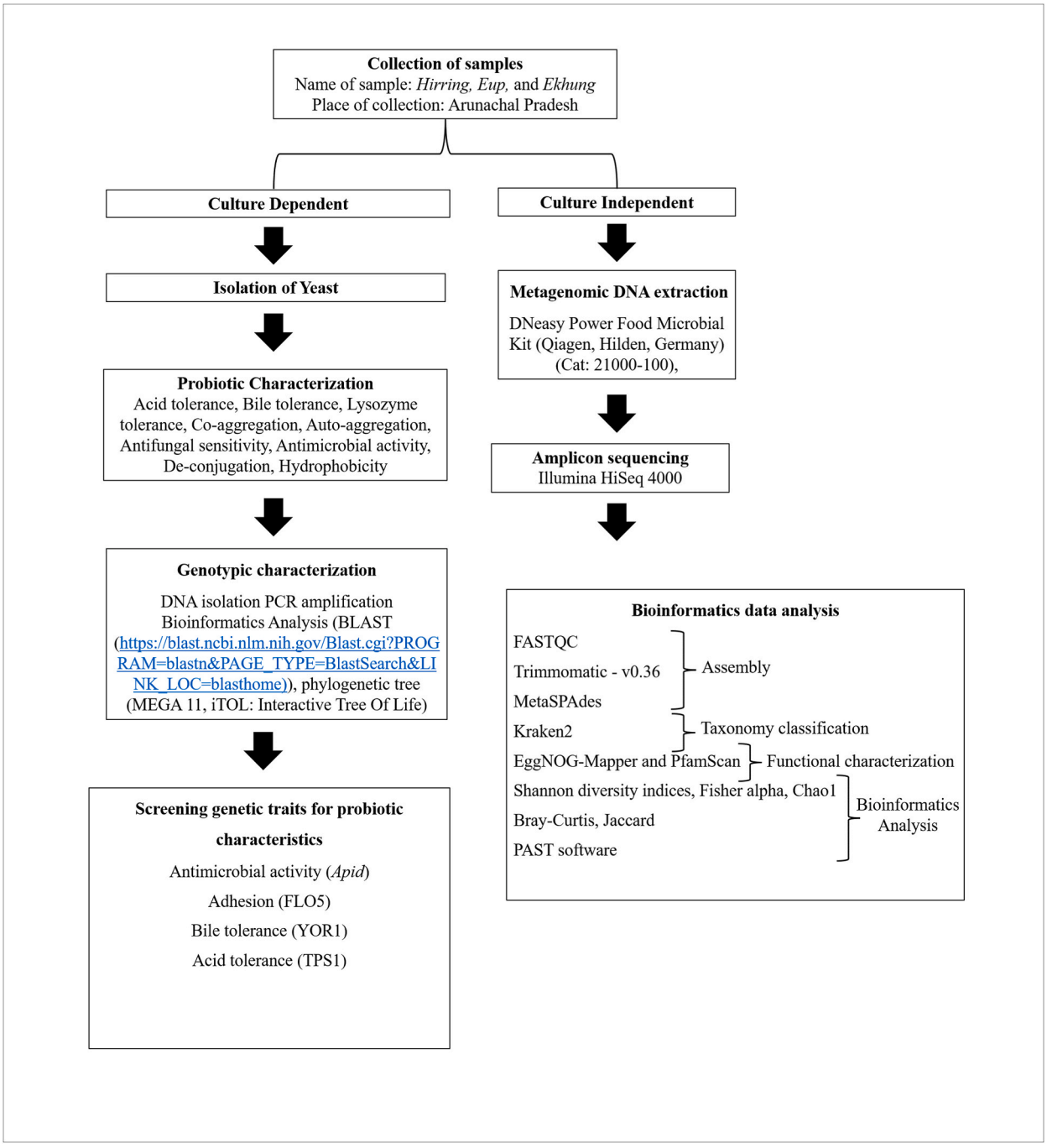

Fig. 1. Flow chart representing the methodology involved in the culture dependent and culture independent study performed in the FBS of Arunchal Pradesh.

reported from sauerkraut fermentation [6] and Isabella grapes fermentation [12].

Research on the yeast microbiology of fermented plant-based products has been limited. However, *Pichia kudriavzevii*, which was isolated from African fermented cereal foods, has the potential for improving human digestion as well as sticking to Caco-2 cells [13]. An in vitro experiment on the probiotic properties of *Saccharomyces cerevisiae*, which was isolated from *ikan budu*, has shown its ability to survive in both gastric juice and bile acid, has good hydrophobicity, and has antimicrobial activity [14] Additionally, *S. cerevisiae, Candida tropicalis,* and *Pichia manschurica* isolate from Indian idli demonstrated multiple probiotic properties [15]. In the brewing industry, a common practice is to use a combination of the probiotic strain *S. cerevisiae* var. *boulardii* and a commercial *S. cerevisiae* strain as a starter to enhance the probiotic characteristics of the beer [16]. Several desirable traits were demonstrated in isolates of *S. cerevisiae* retrieved from *tibicos*, which is a traditional Mexican fermented drink; some of these traits consisted of tolerance to pH of 2.0 and bile salts; formation of cellular aggregates and finally, these isolates showed antioxidant activity [17]. The potential functional ingredient in *Meyerozyma caribbica* has been found to exhibit probiotic properties when extracted from pineapple peel, which makes this yeast significant for production of pineapple-fermented beverages [18].

The population of fungal communities in FBS is the least studied from products of North East region of India. Metagenomic shotgun analysis of FBS products of Tripura showed the presence of yeasts where *Schizosaccharomyces pombe* is the most abundant species [19]. The study was done to evaluate the probiotic potential of yeasts present in fermented bamboo shoots of Arunachal Pradesh and also culture-independent approaches were used to elucidate fungal populations in these indigenous fermented products (Fig. 1). This research will therefore delve into the probiotic characteristics of yeasts in fermented bamboo shoots which will provide knowledge about the positive impacts of eating these local diets on human health.

2. Methodology

2.1. Sample collection and site description

Three sample of each *eup, ekung,* and *hirring* were collected from different market places in Arunachal Pradesh. *Eup, ekung,* and *hirring* were collected from Basar market (27.98° N, 94.68° E), Naharlagun Sunday market (27.11° N, 93.69° E), and Ganga market (27.10° N, 93.69° E). All collected samples were kept in an ice box carrier and immediately transported within 24 h, where they were stored at −20 °C for further analysis. The samples coded for *eup, ekung,* and h*irring* are represented in tabular form in Supplementary Table 1.

2.2. Isolation of yeast

Briefly, the samples were first homogenized using a stomacher, in stomacher bags containing the sample and physiological solution (0.85 % NaCl) in a ratio of 10:100 w/v, the pour plate method was employed, where 1 mL of the homogenized sample was transferred onto yeast malt (YM) agar plates (HiMedia, cat.no.: M424) containing chloramphenicol 34 mg/ml (w/v) (HiMedia, cat.no.: PCT1117). Randomly selected colonies that appeared on the YM plates were counted as colony-forming units (CFU/ml). To ensure the purity of the isolates, they were streaked again on fresh YM plates and examined under a phase-contrast microscope. The isolates were preserved by storing them in 20 % glycerol at −80 °C for further analysis.

2.3. In vitro screening of probiotic properties

2.3.1. Acid and bile tolerance test

Altogether, 95 isolates were tested for acid tolerance was performed using the method described by Ref. [20]. Overnight cultures were inoculated into YM broth (HiMedia, cat.no.: M425) adjusted to pH 2.0, 3.0, and 6.8, and growth was measured at 620 nm. The yeast isolate showing higest acid tolerance at pH 3 was subjected to colony count after 3 h. Following incubation, tenfold serial dilutions up to 10^{-7} for each bacterial strain were prepared with PBS. Then 100 μL of 10^{-4} to 10^{-7} dilution from each sample was spread-plated on MRS agar and incubated anaerobically at 37 °C for 24 h. After incubation, colonies on the plates were counted and enumerated as CFU/mL. The top 13 acid-tolerant yeast isolates were then subjected to bile tolerance by inoculating them into YM broth supplemented with ox gall, taurocholate, or cholate at concentrations of 0.3 %, 0.5 %, and 1.0 % (Sisco Research Laboratories Pvt. Ltd., cat.no.: 99455, 50372, and 53645), incubating at 30 °C for 3 h, and measuring OD at 600.

2.3.2. Co-aggregation

Overnight cultures were suspended in PBS after centrifugation (8000 rpm, 4 °C, 10 min). 2 mL each of yeast and bacterial cultures (*Bacillus cereus* MTCC 430*, Salmonella enterica* MTCC 3223*, Escherichia coli* MTCC 1583*,* and *Staphylococcus aureus* MTCC 740) were mixed in sterile tubes, vortexed, and incubated in PBS (pH 7.3) at 37 °C. A_{600} of supernatants was measured at 0 h and after 3 h. Coaggregation % was calculated as $\{(A_{pat} + A_{probio})/2 - A_{mix}\} \div \{(A_{pat} + A_{probio})/2\} \times 100$, where A_{pat} and A_{probio} are A_{600} of separate bacterial suspensions, and A_{mix} is A_{600} of mixed bacterial culture at specified time points [21].

2.3.3. Auto-aggregation and hydrophobicity

For autoaggregation assessment, yeast overnight cultures were suspended in PBS, centrifuged, and absorbance at 600 nm measured at 0, 1, 2, and 3 h post-incubation at 30 °C. Autoaggregation percentage was calculated using formula $[1 - (A_{time}/A_0) \times 100]$ [22]. For hydrophobicity testing, cultures were cleaned with Ringer's solution, combined with n-hexadecane, xylene, or chloroform, vortexed,

and OD600 of the aqueous phase measured after phase separation. Hydrophobicity was calculated as % decrease in OD600: % Hydrophobicity = $(A_0 - A_t)/A_0 \times 100$, where A_0 is initial OD600 and A_t is OD600 after hydrocarbon extraction [23].

2.3.4. Tolerance to lysozyme and antifungal sensitivity test

Actively growing yeast isolates were centrifuged (7000 rpm, 10 min, 4 °C), washed in PBS, and resuspended in the same solution. A 10 μl suspension was mixed with 10 mg/l lysozyme in a $CaCl_2$-NaCl-KCl-$NaHCO_3$ electrolyte solution. Tolerance was tested over 3 h at 37 °C [23]. The antifungal sensitivity of the yeast isolates was evaluated as per the method of Ruggirello et al. [24]. The antifungals were obtained commercially, itraconazole (1 mg/ml) (Merck, cat.no.: I6657), Fluconazole (150 mg/ml) (Merck, cat.no.: BP1174), Griseofulvin (1.25 mg/ml) (Merck, cat.no.: BP180), Terbinafine (2 mg/ml) (Merck, cat.no.: BP1159), and Clotrimazole (10 mg/ml) (10 mg/ml) (Merck, cat.no.: BP379) and used against the yeast isolates from fermented bamboo shoot and incubated at 28 °C for 24 h.

2.3.5. Deconjugation activity

The ability of strains to deconjugate bile salts was determined according to Hernandez-Gomez et., (2021) [25]. Bile salt plates were prepared by adding 0.5 % (w/v) sodium salts of taurocholate and glycocholate to YM agar. Overnight liquid cultures of strains 10 μl were spotted on agar plates and incubated for 72 h. The presence of precipitated bile acid around colonies (opaque halo) was considered a positive result and was measured using an antibiotic zone measurement scale.

2.3.6. Antimicrobial activity

Antimicrobial assay of yeast isolates was performed by agar well diffusion method in Mueller Hinton Agar (MHA) plates proposed by Diguță et al. (2023) [26]. The test organisms were inoculated in YM broth and incubated overnight at 28 °C to adjust the turbidity to 0.5 McFarland standards giving a final inoculum of 1.5×10^8 CFU/ml. MHA plate was lawn cultured with standardized microbial culture broth containing *Bacillus cereus* MTCC 430*, Salmonella enterica* MTCC 3223*, Escherichia coli* MTCC 1583*, and Staphylococcus aureus* MTCC 740. Wells of 6 mm were bored in the inoculated media with the help of sterile cork-borer (6 mm). Each well was filled with 50 μl of yeast culture It was allowed to diffuse for about 30 min at room temperature and incubated for 18–24 h at 37 °C. After incubation, plates were observed for the formation of a clear zone around the well which corresponds to the antimicrobial activity of tested compounds. The zone of inhibition (ZOI) was observed and measured in mm.

2.4. Genotypic characterization

2.4.1. DNA isolation

Genomic DNA from the yeast isolates was extracted using the method outlined by Renshaw et al. (2015) [27]. A 2 mL yeast malt broth culture grown at 28 °C was centrifuged at 12,000×*g* rpm for 10 min. The pellet underwent two washes with sterile 0.5 M NaCl before suspension in 400 μL lysis buffer (Tris-HCl pH 8.0, 5 M NaCl, 0.5 M EDTA pH 8.0, 10 % SDS). RNase A (20 mg/mL) was added, followed by incubation at 65 °C for 30 min. Proteinase K was added and incubated for another 30 min at 65 °C. After centrifugation, the supernatant was transferred, phenol: chloroform: isoamyl-alcohol added, and the upper aqueous layer was separated by centrifugation. Isopropanol precipitated DNA, which was collected, washed with ethanol, and dried, then dissolved in nuclease-free water. DNA quality was assessed, and purified DNA was stored at −20 °C, with a purity range of 1.8–2.2 used for PCR reactions.

2.4.2. PCR amplification

Yeast isolates were identified by amplifying the D1/D2 domains (D1 (5′ GCA TAT CAA TAA GCG GAG GA), D2 (5′ TTG GTC CGT GTT TCA AGA CG)) of the large ribosomal subunit [28]. The PCR reaction was performed in a 50 μL volume using GoTaq® Green Master Mix, NL1 and NL4 primers, and approximately 10–20 ng of DNA template. PCR amplification was conducted in a SimpliAmp™ Thermal Cycler under the following conditions: initial denaturation at 94 °C for 1 min, followed by 35 amplification cycles of 30 s at 94 °C, 30 s at 58 °C, and 30 s at 72 °C. A final extension step was performed at 72 °C for 5 min. The presence of amplicons was confirmed by electrophoresis on a 1% agarose gel and visualized using a Gel DocTM EZ system.

2.4.3. Bioinformatic analysis

Raw sequence data were analyzed as per the method described by palla et al. (2020) [29]. The quality of the raw sequences was initially checked using Sequence Scanner v2.0 (ABI 3730XL Capillary Sequencers, Applied Biosystems, Foster City, CA, USA) and Bio-edit v7.2 to assemble the good quality sequences. Sequences were analyzed using Basic local alignment search tool (BLAST) on the National Center for Biotechnology Information (NCBI) web (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome). Muscle was used to align the identified sequences for analyzing phylogenetic relationship, and a phylogenetic tree was constructed using the maximun-likelihood method based on the Tamura 3-parameter by Molecular Evolutionary Genetics Analysis software, version 11 (MEGA v11.0.13) [30]. The sequences were submitted to the NCBI GenBank for accession numbers.

2.4.4. Screening genetic traits for probiotic characteristics

The PCR reactions aimed to identify probiotic genes in yeasts. Antimicrobial activity (*Apid*), amplicon size 752bp (F-ATGAAGAATTTTATCTTCGCTATT, R-TCAGTAATATAATTCCTCATCAGC) [31]. Adhesion (FLO5), amplicon size 423bp (F- GACAATTGCACACCACTGC, R- CCTGTCATTTCTAGGGTTACG) [32]. Bile tolerance (YOR1), amplicon size 68bp (F-CCATCGGTGCTTGTGTAATGTTA, R- TTGAGAGGCGTGGAAAAAATG) [33]. Acid tolerance (TPS1), amplicon size 1600bp (F-

ATGACTACGGATAACG, R- TCAGTTTTTGGTGGCAGAGG) [34]. Each reaction contained 6μL of GoTaq® Green Master Mix, 0.6μL of forward primer, 0.6μL of reverse primer, and 1μL of template DNA, totaling 12μL. Amplification products were run on 1% agarose gel (fragments >500 bp) or 2% agarose gel (fragments <100 bp) stained with ethidium bromide. The PCR protocol included initial denaturation (95°C, 5min), followed by 40 cycles of denaturation (95°C, 30s), annealing (specific primer temperature, 10s), and extension (72°C, 15s), with a final extension step (72°C, 5min) [35].

2.5. Culture-independent analysis

2.5.1. Metagenomic DNA extraction

DNA was extracted from the bamboo shoot sample using the DNeasy Power Food Microbial Kit (Qiagen, Hilden, Germany) (Cat: 21000-100), following the manufacturer's instructions. The quality of the DNA was evaluated using a Qubit Fluorometer (Thermofisher Scientific, USA) with a detection limit of 10–100 ng/μL. For library preparation, 100ng of DNA was utilized, and the resulting library was then analyzed through 0.8% agarose gel electrophoresis.

2.5.2. Amplicon sequencing

The fungal D1/D2 region of the large subunit (LSU) ribosomal gene was amplified using universal primers F63 and LR3. PCR utilized Qiagen's HotStarTaq Plus Master Mix Kit, and amplicons were analyzed on a 2% agarose gel. Sequencing followed Illumina HiSeq 4000 manufacturer's protocol, employing a 101bp paired-end module sequencing approach.

2.5.3. Bioinformatics data analysis

After sequencing, reads obtained for each library were then subjected to quality check, trimming, and alignment. The assessment of read quality was done utilizing metaQUAST (version 3.2) [36] and FASTQC using default parameters to get high-quality (HQ) reads. HQ-filtered paired-end libraries were trimmed using a read trimming tool Trimmomatic - v0.36 using default parameters [37]. Then the reads were assembled to generate contigs using a de Bruijn graph-based assembler MetaSPAdes [38] using default parameters. All the data analysis was performed in the KBase platform, available at https://www.kbase.us/. [39].

The assembled sequences were then used to identify microbial taxonomy. Annotations, function, and composition of microbial communities were done using Omicsbox https://www.biobam.com/. Omicsbox utilizes Kraken2 to identify and count operational taxonomic units (OTUs) for microbial diversity down to strain level. The resulting representative OUTs were given taxonomic classes using the Fungi genomes (2019) and Standard Plus Protozoa and Fungi (2021) database. Kraken2 functions as a taxonomic sequence classifier, attributing taxonomic labels to short DNA reads by analyzing their k-mers [40]. It utilizes a database mapping each k-mer in its genomic library to the lowest common ancestor (LCA) in a taxonomic tree encompassing all genomes containing that k-mer.

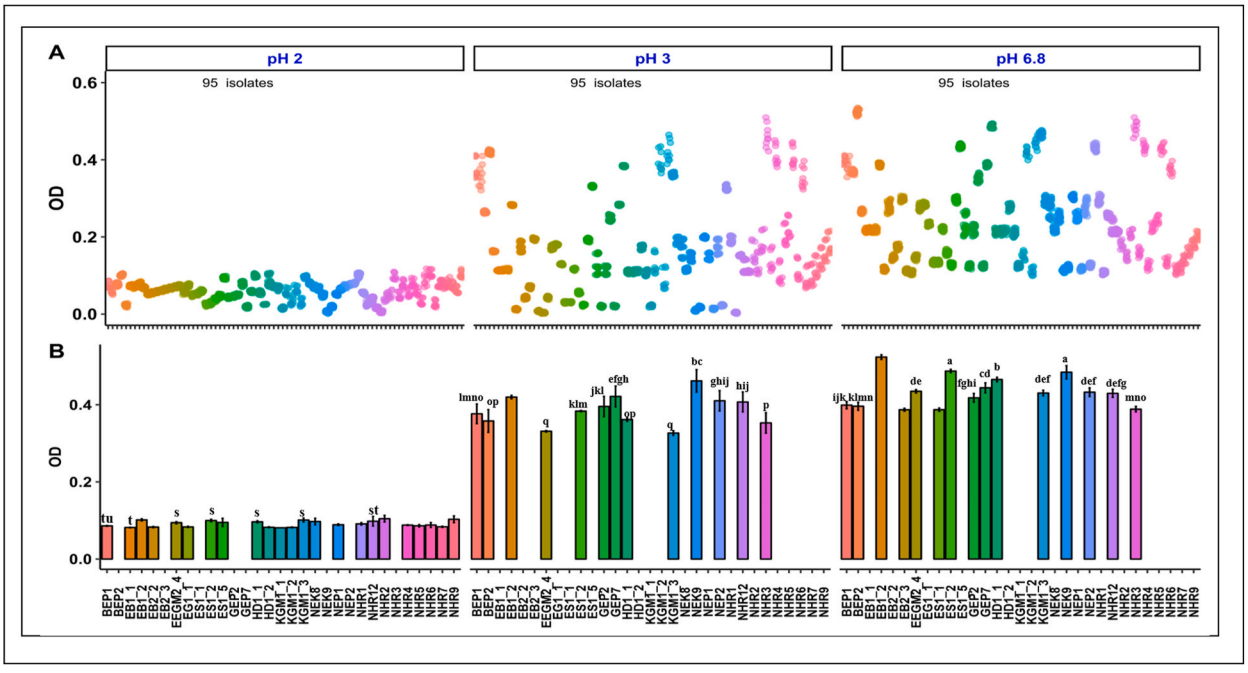

Fig. 2. Yeast isolated showing tolerance to acid at pH 2, and pH 3 and pH 6.8 (control). Data represent the mean ± SD, standard deviation of three independent replication as indicated by the vertical line. The vertical bars with different alphabets are statistically significant at $p < 0.05$.

2.5.4. Functional characterization

OmicsBox conducted efficient high-throughput functional annotations by employing EggNOG-Mapper and PfamScan. Gene prediction within assembled sequences was executed using Prodigal 2.6.3 [41] as an integral step in the OmicsBox pipeline for functional characterization. Subsequently, the predicted genes were annotated through EggNOG Mapper [42], and PfamScan https://ftp.ebi.ac.uk/pub/databases/Pfam/Tools/offering detailed functional information.

2.5.5. Statistical analysis

Analysis of variance (ANOVA) with Tukey' HSD post hoc test was performed in RStudio to find the significant differences between the means and for pairwise comparisons. The Shannon diversity indices, Fisher alpha, Chao1 were calculated with the help of EstimateS and PAST software for the metagenomic data [43,44]. Beta-diversity was calculated using *Bray-Curtis* and *Jaccard* dissimilarity indices in PAST software [44] and represented in Principle Coordinate Analysis (PCoA) drawn in RStudio. The rarefaction curve was plotted using iNEXT (iNterpolation and EXTrapolation), an R-based package [45]. Pearson correlation between various samples and their functional profile was presented through Heat Map using ggplot2 package in R. Random Forest Model was performed to check the variable importance in R.

3. Results

3.1. Acid and bile tolerance

Among the 95 isolates selected on the basis of colony morphology, 13 isolates showed promising acid survival capacity, among which BEP1, BEP2, EB1_2, EEGM2_4, ES1_2, GEP2, GEP7, HD1_1, KGM1_3, NEK9, NEP2, NHR12, and NHR3 could attain more than 0.3 OD at 620 nm within 3 h at pH 3 (Fig. 2). Highest CFU/ml was observed in EB1_2 (7.86 ± 0.07), GEP7 (7.69 ± 0.06), and NEK9 (7.27 ± 0.03) (Supplementary Table 2). The isolates showed an increasing survival rate with increasing concentrations of cholic acid and taurocholic acid while the survival rate was lower in the case of oxbile. Overall, different isolates showed different survival potential in the presence of bile salts. GEP7 and HD1_1 showed the highest bile tolerance at 0.3% cholic acid, KGM1_3, and NEP2 showed the highest growth at 0.5% taurocholic acid, and NEK9, and NHR12 at 1% oxbile as illustrated in Fig. 3.

3.2. Co-aggregation

Coaggregation analysis was conducted using four distinct indicator strains across various time points. All tested probiotic strains demonstrated differences in the ability to aggregate with the tested pathogenic bacteria. Overall, coaggregation behaviour was not statistically different ($p < 0.05$) across the pathogenic strains. EEGM2_4 exhibited the greatest coaggregation capacity, followed by

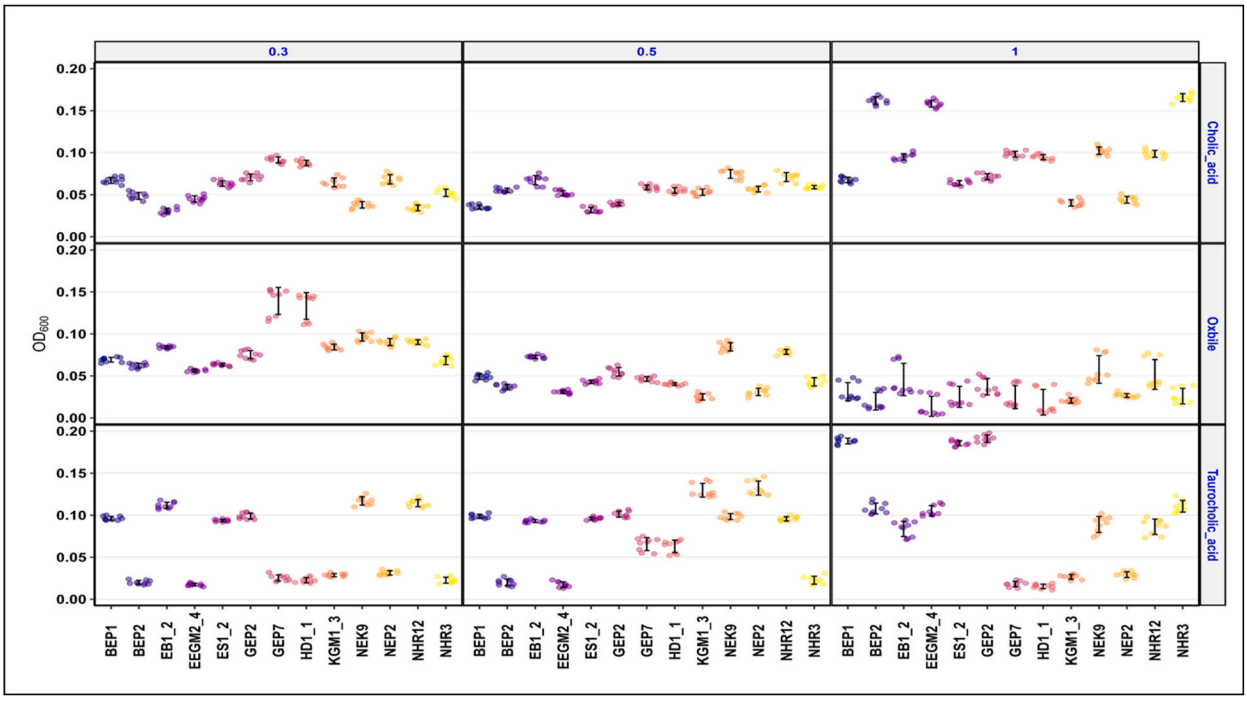

Fig. 3. Yeast isolates exhibiting tolerance to cholic acid, oxbile and taurocholic acid at 0.3 %, 0.5 %, and 1 %. Data represent the mean ± SD, standard deviation of three independent replicates.

GEP7, NEP2 and ES1_2 (Fig. 4 and Supplementary Table 3).

3.3. Autoaggregation and hydrophobicity

The auto-aggregation behaviour of the yeast isolates increased significantly ($p < 0.05$) during 3 h reaching as high 84.063 ± 0.911 (in case of HD1_1) (Fig. 5 and Supplementary Table 4). The adhesion of yeast to hydrocarbons measures their ability to adhere to nonpolar organic compounds. A higher degree of surface non-polarity corresponds to increased adhesion to hydrocarbons and greater hydrophobicity. The percent adhesion of strains to different hydrocarbons has been presented graphically in (Fig. 6). Isolate BEP1, BEP2 and NHR3 had the highest hydrophobicity in chloroform, while ES1_2, GEP2, HD1_1, NEP2, and NHR3 had the highest hydrophobicity in hexadecane. NHR3, NEP2, ES1_2 and GEP2 had the highest hydrophobicity in xylene.

3.4. Tolerance to lysozyme and antifungal activity

Among 13 isolates of yeast, 3 isolates showed the highest tolerance (>90 %) to lysozyme (Fig. 7). Isolate GEP2 showed the highest tolerance to lysozyme of 93.3 ± 2.74%, followed by EB1_2, and NHR12 with the tolerance of 93.2 ± 3.12%, and 93.2 ± 3.24% respectively. The selected yeast strains were tested against various commercially available antifungals and their sensitivity and resistivity are illustrated in Fig. 8 and Supplementary Table 5. All the yeast strains were resistant to itraconazole, griseofulvin, and clotrimazole (Supplementary Table 5). Yeast isolates were sensitive to terbinafine and fluconazole among which isolate EB1_2, and ES1_2 showed the highest sensitivity to terbinafine (18 mm), followed by NEK9 to fluconazole (17 mm).

3.5. Deconjugation of bile salt (BSH activity)

The probiotic cultures were screened for BSH activity by qualitative direct plate assay. The yeast isolates EB1_2, HD1_1, NEK9 were able to deconjugate taurocholate while all the yeast isolates were able to deconjugate glycocholate. The plate assay showed an opaque halo zone of more than 10 mm by all the isolates (Supplementary Fig. 1 and Supplementary Table 6).

3.6. Antimicrobial activity

The yeast strains were tested for their antimicrobial activity against some pathogenic bacteria by agar layover method and the results of this assay are shown in Table 1 and Supplementary Fig. 2. BEP1 showed the highest activity against *Bacillus cereus* MTCC 430

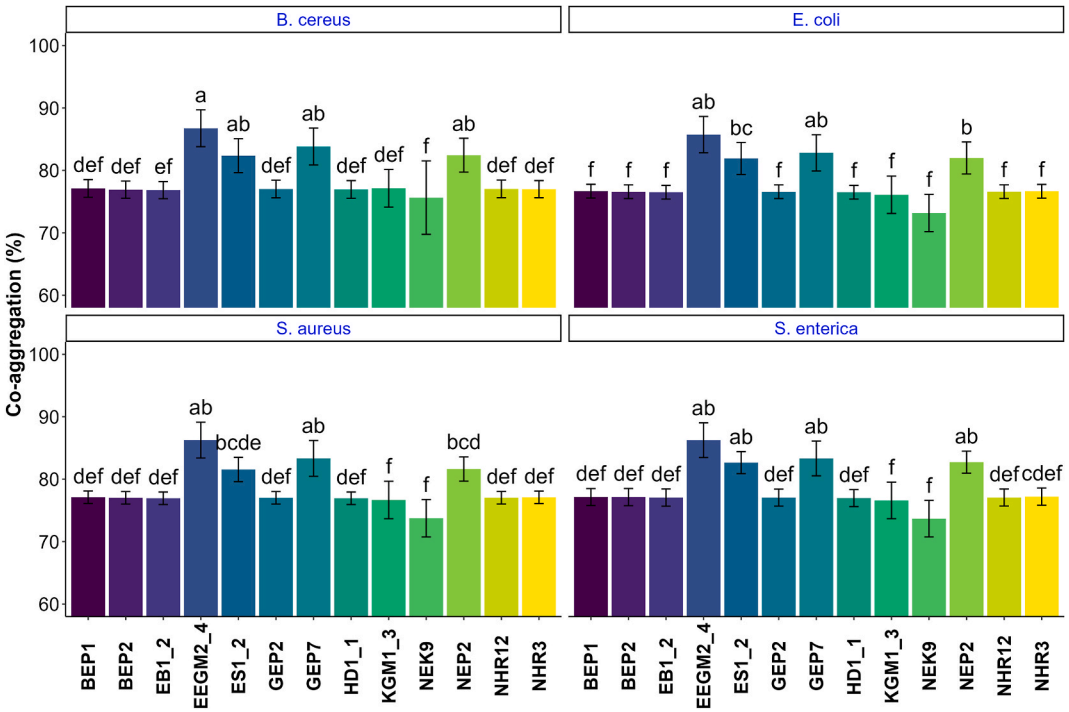

Fig. 4. Co-aggregation property of selected yeast isolates with *B. cereus, E. coli, S. aureus,* and *S. enterica.* Data represent the mean ± SD, Standard deviation of three independent replicates as indicated by the vertical line. The vertical bars with different alphabets are statistically significant at $p < 0.05$.

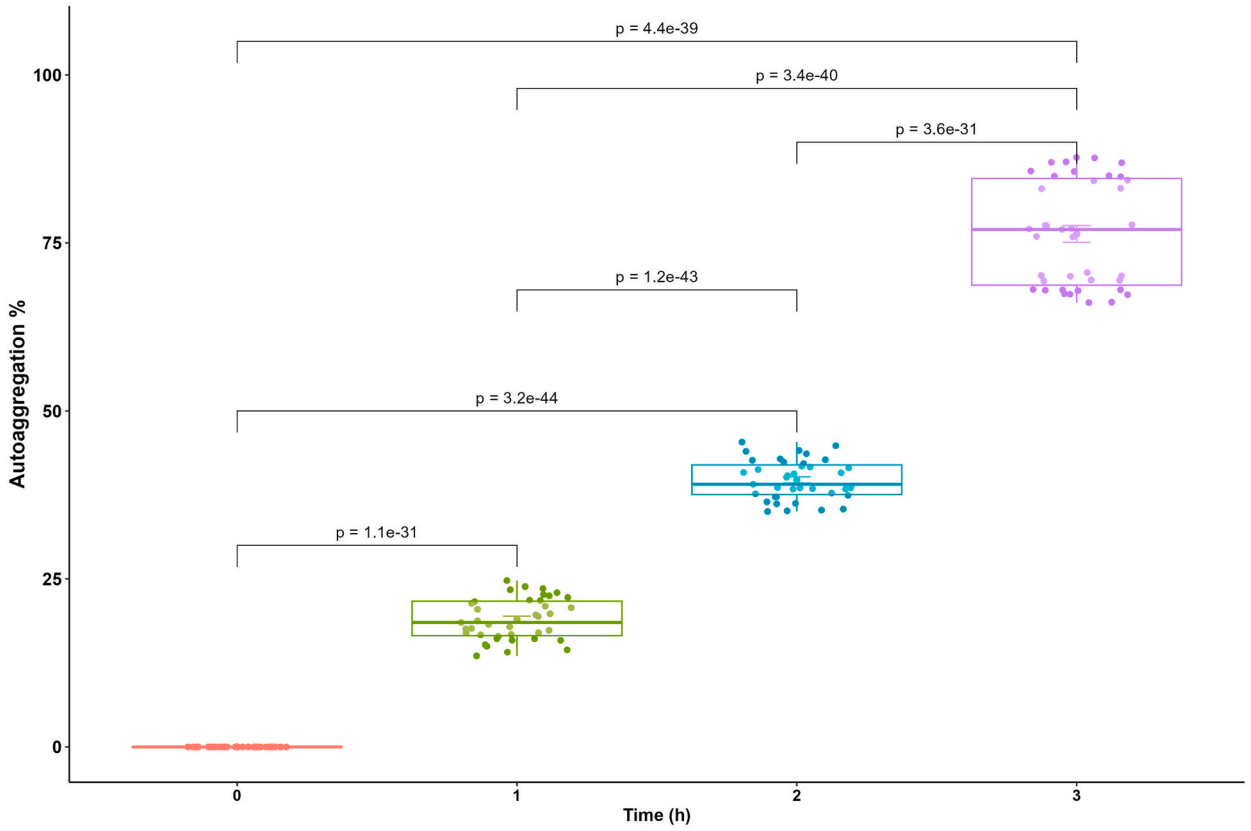

Fig. 5. Autoaggregation percentage of yeast isolates. Increase in autoaggregation percentage of yeast isolates are statistically significant at $p < 0.05$.

(mm) with the zone of inhibition of 22 mm, followed by NHR3 of 20 mm with *Salmonella enterica* MTCC 3223, BEP1 of 22 mm with *Escherichia coli* MTCC 1583, and GEP2, BEP1 of 18 mm with *Staphylococcus aureus* MTCC 740.

3.7. Bioinformatic analysis

Thirteen yeast isolates screened for probiotic characteristic were identified and belonged to four major genus *Pichia, Candida, Meyerozyma*, and *Saccharomyces*. BEP1, KGM1_3 and NHR3 were identified as *Meyerozyma guilliermondii,* GEP7 as *Meyerozyma caribbica,* ES1_2, EB1_2, EEGM2_4, and GEP2 were identified as *Candida orthopsilopsis*, NEK9, and HD1_1 was identified as *Candida parasilopsis,* NHR12 was identified as *Pichia kudriavzevii,* and BEP2 was identified as *Pichia fermentans,* and NEP2 was identified as *Saccharomyces cerevisiae* (Fig. 9).

3.8. Screening of genes for probiotic traits

Following the initial screening for probiotics, additional analysis was conducted to detect probiotic genes using specific target genes, traits associated with probiotic activity, and designated primers. Genes for acid tolerance, bile tolerance, antimicrobial activity, and adhesion were detected, isolates BEP1, BEP2, EEGM2_4, ES1_2, GEP2, GEP7, HD1_1, NEK9, NHR12, and NEP2 were detected to have acid tolerance gene *TPS1* (Fig. 10a and Supplementary Fig. 3). All the 13 selected isolates were detected to have adhesion gene *FLO5R* (Fig. 10b and Supplementary Fig. 3). EB1_2, KGM1_2, BEP1, BEP2, EEGM2_4, ES1_2, GEP2, GEP7, HD1_1, and NHR12 showed the presence of *Apid* gene which encodes for antimicrobial activity (Fig. 11a and Supplementary Fig. 3). BEP1, BEP2, ES1_2, GEP2, NHR3, HD1_1, and NHR12 showed the presence of *YOR1* gene which encodes for bile tolerance (Fig. 11b and Supplementary Fig. 3).

3.9. Culture independent analysis

3.9.1. Metagenomic data analysis

Amplicon sequencing of Arunachal Pradesh's FBS revealed varying read counts: Basar averaged 7131 reads, with BEK and BHR highest (9798 and 9563 reads); Ganga Market averaged 1560 reads, highest in GHR (2405); Naharlagun averaged 5817 reads, with NEK highest (14622), all exhibiting high quality.

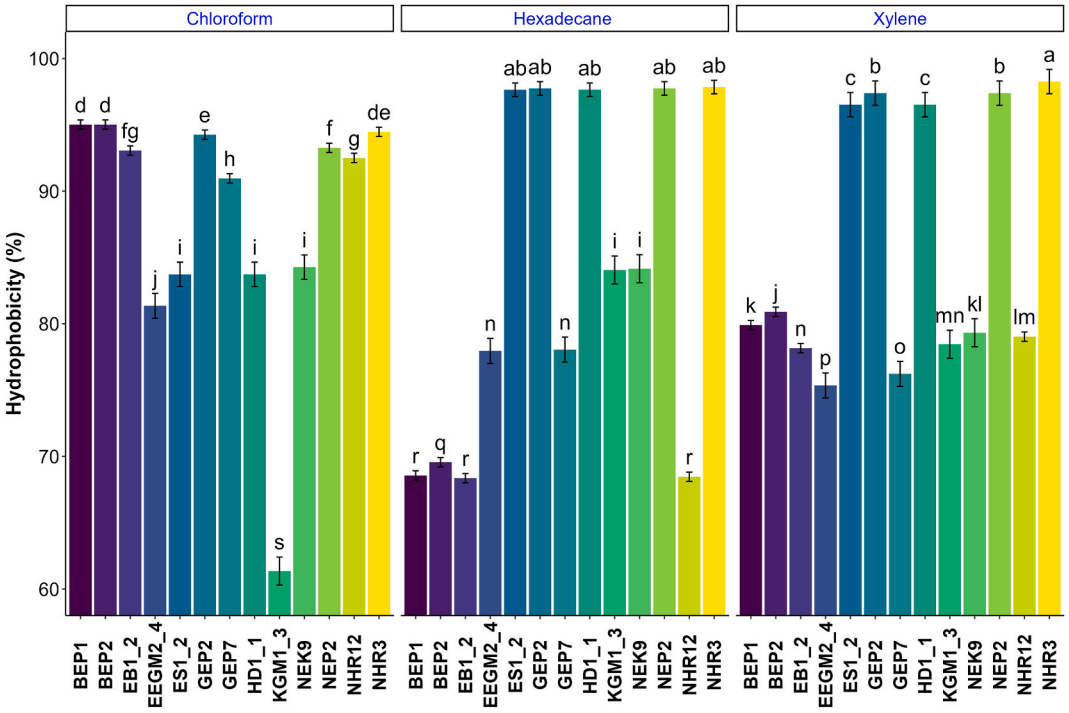

Fig. 6. The hydrophobicity percentage shown by the selected yeast isolates. Data represent the mean ± SD, standard deviation of three independent replicates as indicated by the vertical line. The vertical bars with different alphabets are statistically significant at $p < 0.05$.

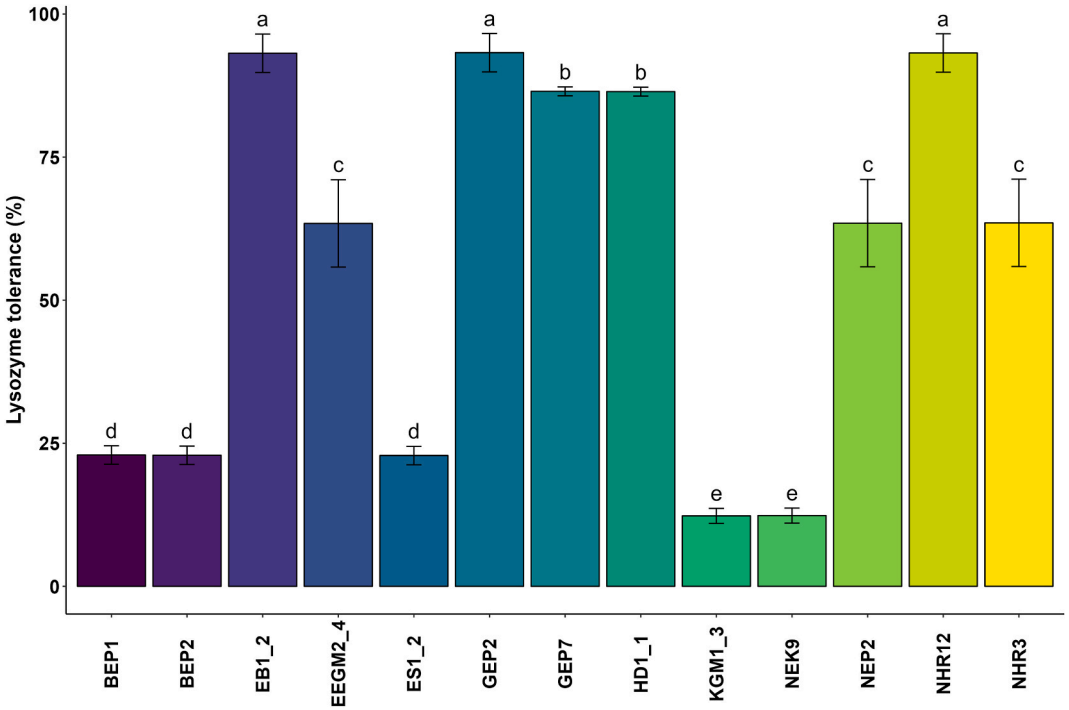

Fig. 7. Bar plot shows the survival percentage of yeast isolates in lysozyme. Data represent the mean ± SD, Standard deviation of three independent replicates as indicated by the vertical line. The vertical bars with different alphabets are statistically significant at $p < 0.05$.

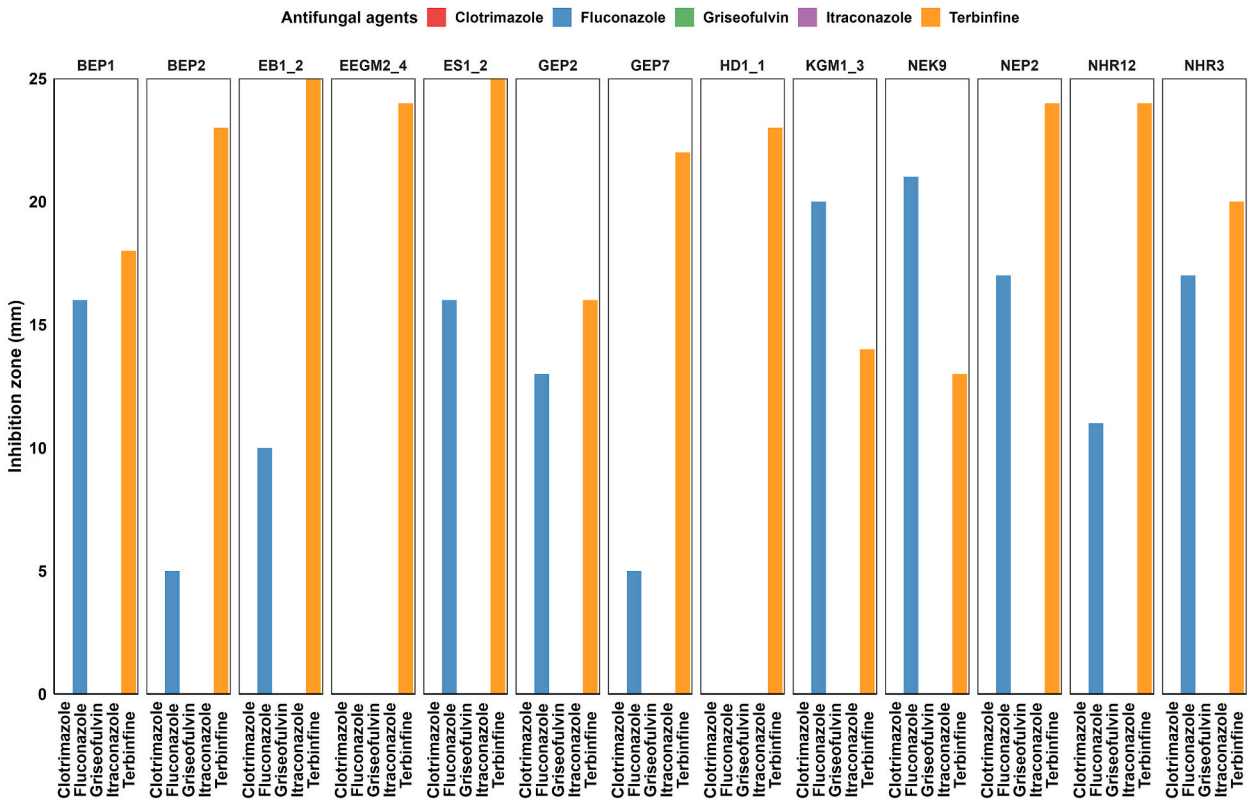

Fig. 8. The selected isolates showing sensitivity against selected antifungal agents.

Table 1
Antimicrobial activity of yeast isolates from fermented bamboo shoot.

Isolate code	*Bacillus cereus* MTCC 430 (mm)	*Salmonella enterica* MTCC 3223 (mm)	*Escherichia coli* MTCC 1583 (mm)	*Staphylococcus aureus* MTCC 740 (mm)
EB1_2	12	10	14	12
EEGM2_4	16	12	16	12
ES1_2	14	14	12	10
HD1_1	12	14	10	10
KGM1_3	12	12	10	14
NHR12	15	13	17	15
BEP2	19	15	19	15
NEP2	17	17	15	13
GEP7	15	17	13	13
NEK9	15	15	13	17
GEP2	18	16	20	18
BEP1	22	18	22	18
NHR3	20	20	18	16

3.9.2. Diversity indices and rarefaction curves

Diversity indices calculated using PAST software revealed Ganga Market bamboo shoot samples with the highest alpha diversity (Fisher alpha: 1.89) followed by Naharlagun (1.62). Similar trends were seen in Shannon diversity, with Ganga Market exhibiting higher diversity (1.84) compared to Naharlagun and Basar (1.49), indicating more even species distribution in Ganga Market samples.

Beta diversity analysis showed higher values (0.31) between EK and EP bamboo shoots compared to EK vs HR (0.29) and EP vs HR (0.23) samples, consistent with Whittaker's concept. PCA based on Bray-Curtis beta diversity confirmed significant differences, indicating a negative correlation between HR and EP/EK samples. Fig. 12a. Similarly, the PCA based on Jaccard's beta diversity analysis demonstrated a positive correlation between EK and EP, while both were negatively correlated with HR Fig. 12b. Consequently, both statistical approaches suggest that there is minimal difference in beta diversity among EK and EP bamboo shoot samples, while there is a substantial difference in beta diversity between HR and EK/EP samples.

Examining rarefaction curves, with plot species numbers against the number of samples, revealed an initial rapid rise, indicating the discovery of common species. However, Basar and Naharlagun region samples displayed higher peaks, indicating the accumulation

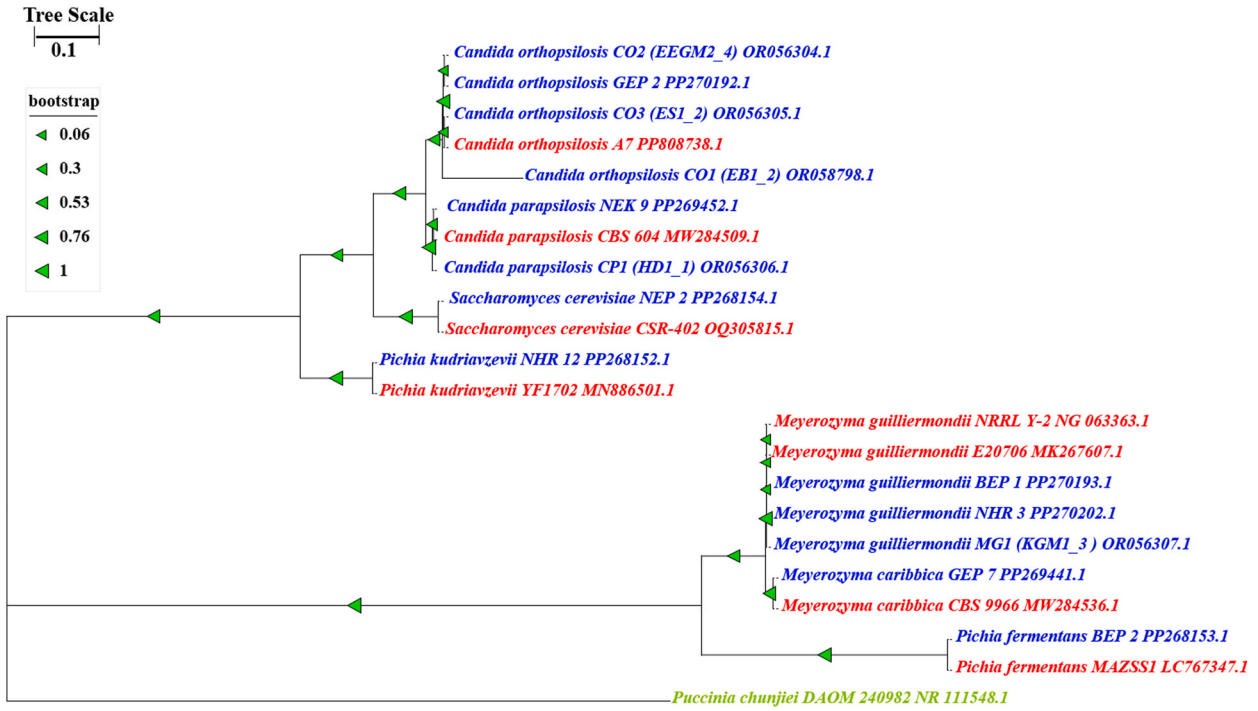

Fig. 9. Phylogenetic tree of yeast isolates constructed using MEGA11 and iTOL software where names in blue colour font represent yeast isolates obtained from FBS of Arunachal Pradesh, whereas the names in the red colour font represent the Refseq extracted from NCBI. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

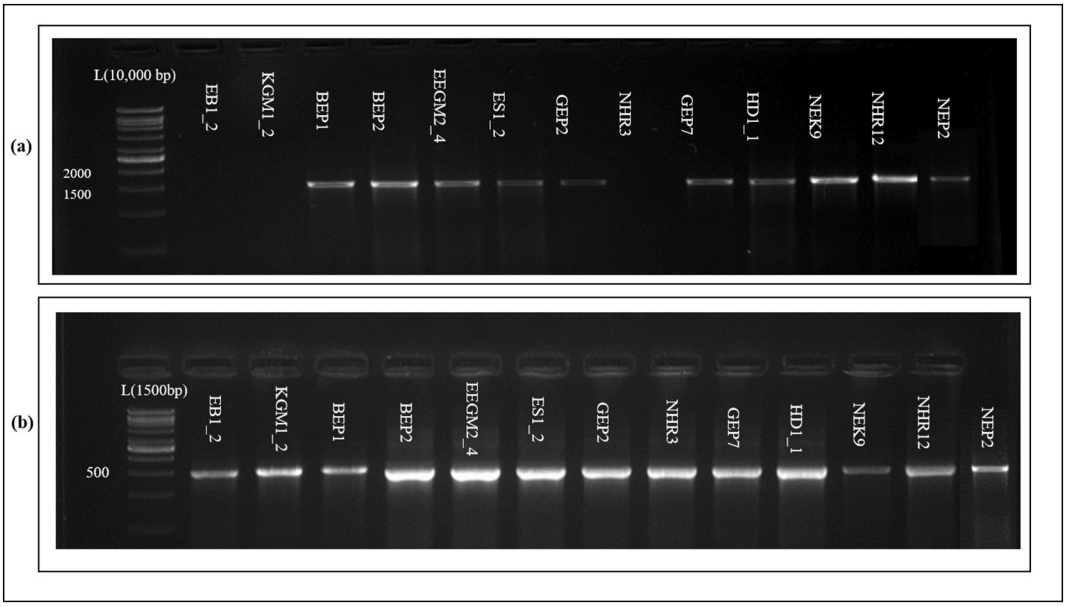

Fig. 10. a. Probiotic genes identified using specific primers *TPS1* for acid tolerance. b.Probiotic genes identified using specific primers *FLOR5* for adhesion. L is the ladder well and rest of the wells were for the yeast isolates.

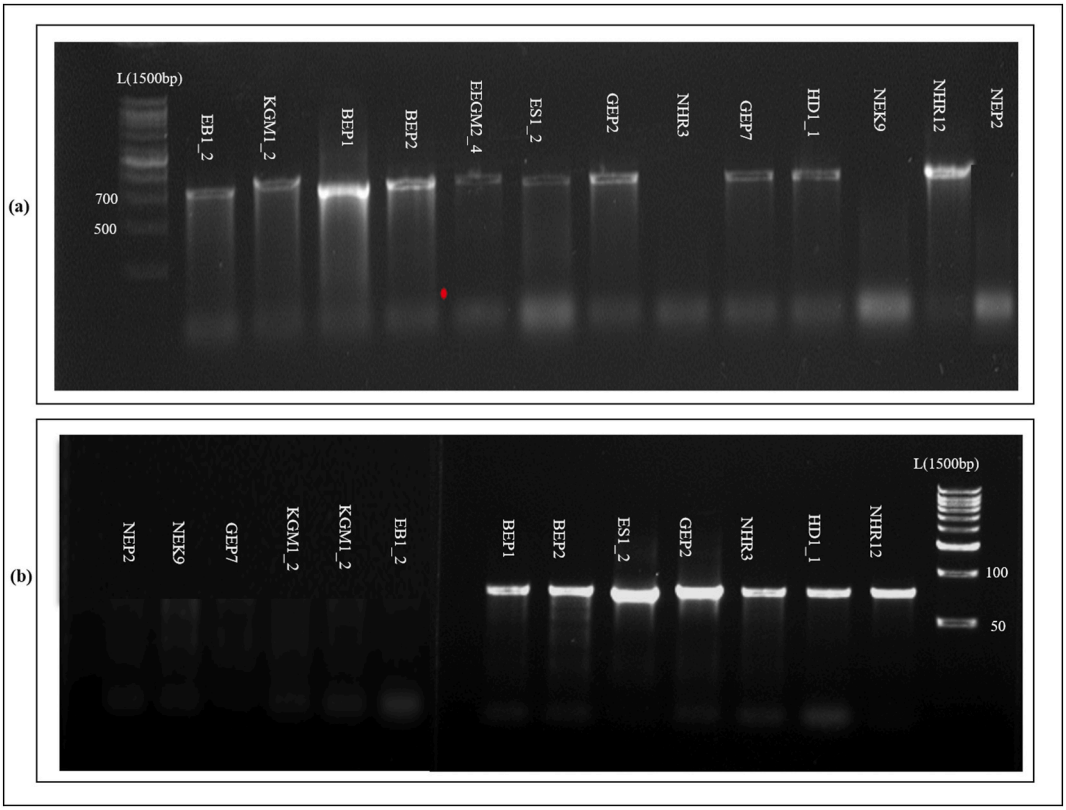

Fig. 11. a. Probiotic genes identified using specific primers *Apid* for antimicrobial activity. Probiotic genes identified using specific primers *YOR1* for bile tolerance. L is the ladder well and rest of the wells were for the yeast isolates.

of rarer species. This aligns with the diversity indices results mentioned earlier, reinforcing the diversity patterns observed in these regions Fig. 12c.

3.9.3. Fungal diversity analysis

The examination of phylum-level diversity revealed prominent abundance of major phyla, including Ascomycota, Basidiomycota, and Pseudomonadota. However, distinct variations in the abundance of these phyla were observed across different sampling regions and bamboo shoot samples. In the Basar region, Ascomycota dominated with abundances of 74%, 5.6%, and 60.5% in BEK, BEP, and BHR shoot samples, respectively. Conversely, Ganga Market samples exhibited Ascomycota as the major phylum, with GEK at 5%, GEP at 4.5%, and GHR at 7.4%. In Naharlagun region shoot samples, Ascomycota prevailed, particularly in NEK (96%), NHR (5.21%), and to a lesser extent in NEP (4.8%). These findings indicate that Ganga Market samples have lower diversity abundance compared to the other two regions. Similarly, the EP shoot samples displayed a lower abundance of fungal phyla in comparison to the EK and HR shoot samples Fig. 13a.

At the genus level, the fungal diversity analysis highlighted the prevalence of genera such as *Pestalotiopsis*, *Penicillium*, *Aspergillus*, *Trichosporon*, and *Fusarium*. The percentage abundance of these genera varied significantly across bamboo shoots in distinct regions. In the Basar region, *Pestalotiopsis* was dominant (BEK-31.3 %, BEP-2.1 %, BHR-28.9 %), along with *Penicillium* (BEK-25.5%, BEP-0%, BHR-10 %). Notably, BEP exhibited lower fungal abundance. Conversely, Ganga Market shoot samples displayed a lower abundance of different genera compared to the other two regions. Here, the prevalent genera were *Penicillium* (GEK-2%, GEP-0.7%, GHR-0.34%) and *Pestalotiopsis* (GEK-1.4 %, GEP-0.4%, GHR-1.5%). In Naharlagun region shoot samples, the abundant genera included *Pestalotiopsis* (NEK-53.1%, NEP-0.67%, NHR-1.1%), *Penicillium* (NEK-16.8%, NEP-1.6%, NHR-0.92%), and *Aspergillus* (NEK-24.4 %, NEP-1.8%, NHR-2.12%). Yeast isolates were present in very minimal abundance for instance. Among the yeast, *Candida* had the highest abundance (BHR-83.4%, and GEP-16.5%), followed by *Pichia* (BEK-24.24%, BEP-27.27%, and NEK-48.48%). Similar to the phylum-level fungal diversity, Ganga Market exhibited lower abundance. Furthermore, EP bamboo shoots demonstrated a lower abundance of genus-level fungal diversity compared to EK and HR bamboo shoot samples Fig. 13b.

3.9.4. Correlation of fungal diversity with physico-chemical parameters

The impact of various elements, including pH, acidity, and moisture content, on fungal diversity in bamboo shoot samples was examined. A Random Forest Model was implemented in R to assess variable importance. The results indicated that calcium and moisture content play more significant roles in shaping fungal diversity, followed by Fe, Cu, Zn, and acidity. The variable with the least

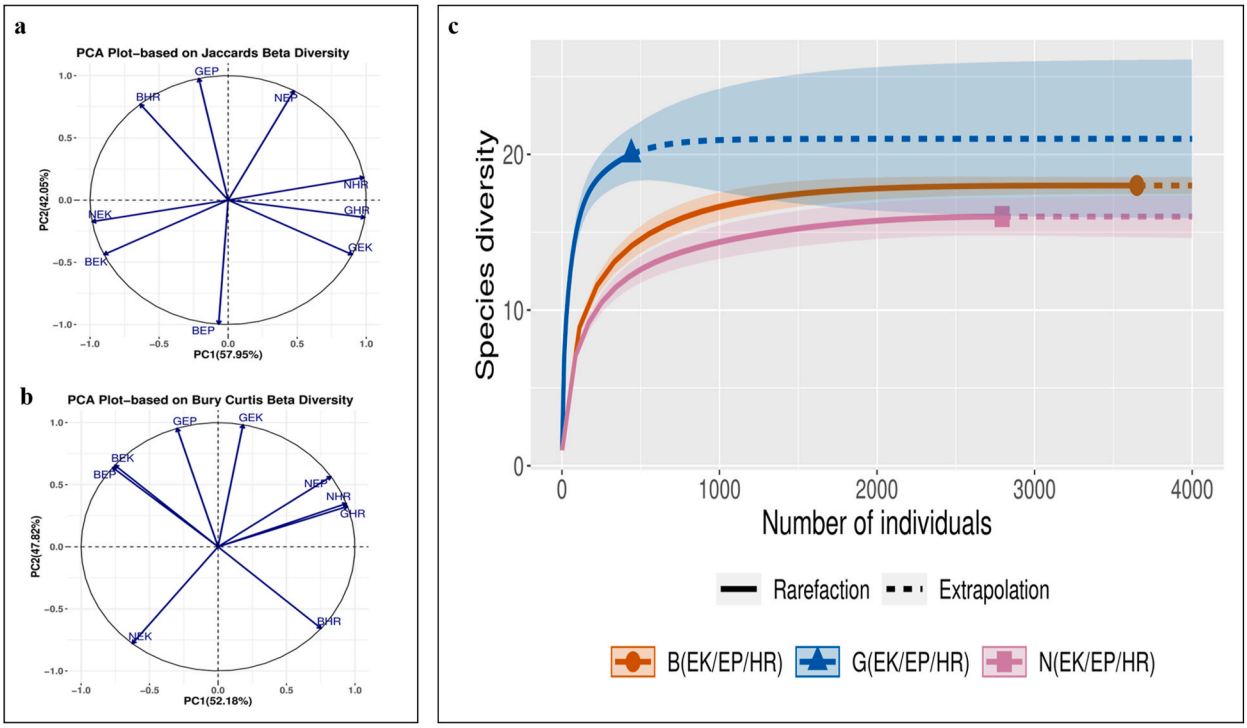

Fig. 12. a. PCA plot of Beta diversity obtained using Bray-Curtis algorithm. b. PCA plot of Beta diversity obtained using Jaccard algorithm. c. Rarefaction curves showing common species.

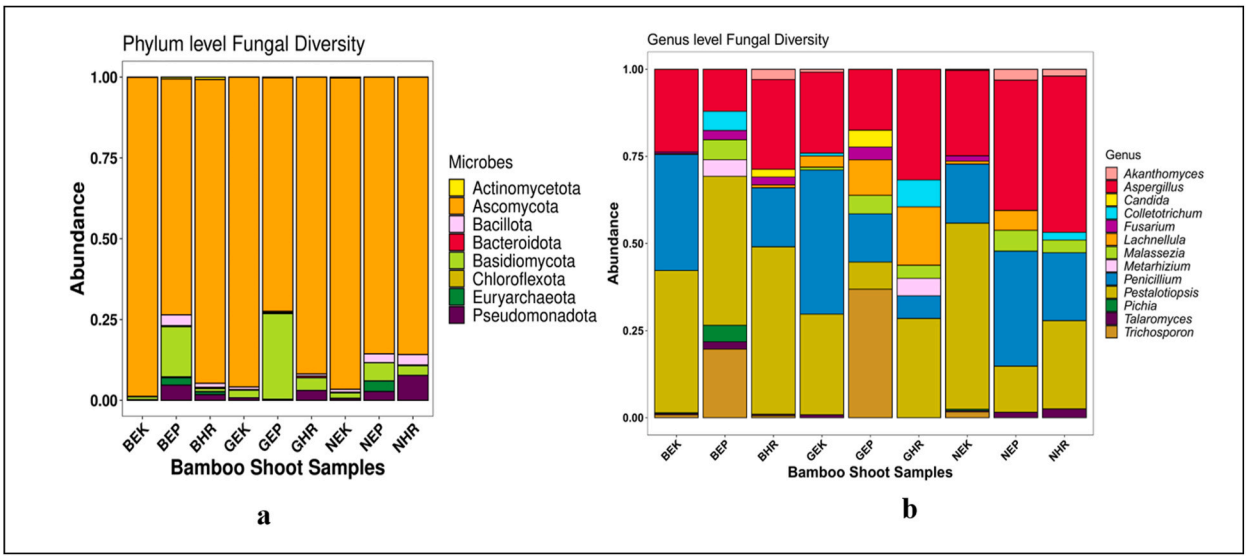

Fig. 13. Abundance profiling at (a) phylum and (b) genus level.

contribution was identified as magnesium Fig. 14.

3.9.5. Functional characterization

Functional analysis of bamboo shoot samples using EggNOG-Mapper and PfamScan highlighted genes related to replication, translation, lipid and carbohydrate metabolism, energy production, and cell motility. Pearson correlation showed positive associations among NEP, NEK, NHR, BEK, BHR, BEP, and GHR samples, contrasting with negative correlations with GEP and GEK shoots Fig. 15. These findings align with the previously discussed results, indicating that GEP and GEK exhibit lower fungal diversity and do not

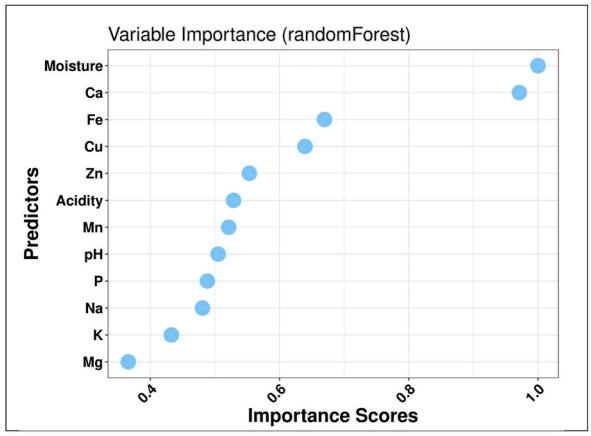

Fig. 14. Random forest plot showing Calcium and moisture as the key component; Fe, Cu, Zn, acidity follow; magnesium least.

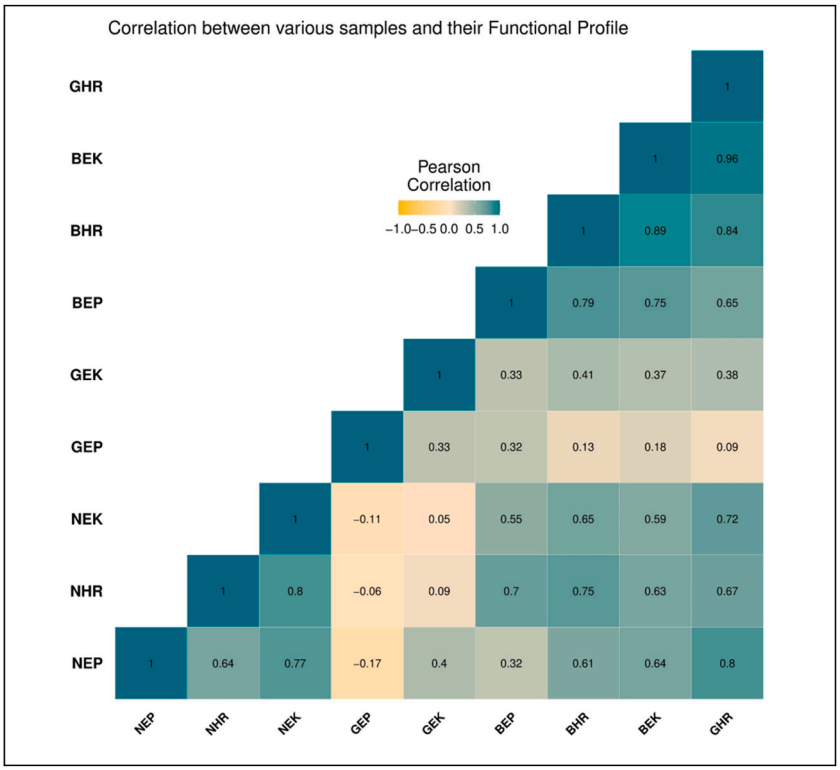

Fig. 15. Correlation heat map of functional profiling and samples.

accumulate rarer species. However, the study revealed a lower gene count predicting these functions, underscoring the insufficient characterization of the fungal functional profile Supplementary Table 3.

4. Discussion

This is the first report on the isolation, identification, and probiotic characterization of yeast isolates along with the amplicon sequencing from the FBS of Arunachal Pradesh. The earlier studies on the ethnic fermented bamboo shoots whether culture-based or culture-independent were mostly focused on bacteria [2,46–48]. Recent metagenomic shotgun sequencing has shown the presence of yeasts and filamentous moulds although in lower abundance [19]. Other non-alcoholic fermented products of plant origin where yeasts have been reported are kimchi [5,7], sauerkraut [6], fermented olive [4], fermented cucumber [8]. As in other fermented vegetables, fermented bamboo shoots also harbour a sizeable population of fungal communities including yeast which we were able to

isolate and identify such as *Candida orthopsilopsis, Candida parasilopsis*, *Pichia fermentans*, *Meyerozyma caribbica*, *Pichia kudriavzevii, Saccharomyces cerevisiae*, and *Meyerozyma guillermondii.*

Microorganisms associated with the natural fermentative process of bamboo shoots, especially yeasts, have wide biotechnological potential with applications in the food sector to improve the quality, sensory and functional properties [49,50]. Out of 95 isolates tested, 13 strains met all probiotic criteria, demonstrating safety, resilience in the digestive system, colonization abilities, antimicrobial properties, and pathogen inhibition. These strains fulfilled essential requirements, including identification and functional characterisation. 6 out of 13 isolates selected from the 95 isolates belonged to the genus *Candida*, which includes well-known yeasts found all over the world, and certain strains can be harmful to humans [51]. Despite the fact that pathogenicity is common in these yeasts, several species of *Candida* have been studied as potential probiotics [52]. *Candida orthopsilosis* was earlier isolated from the Camu-Camu fruit of the North Brazilian Amazon and was reported to have fermentative quality [53]. Diverse yeast species such as of *Candida parapsilosis, Candida orthopsilosis, Clavispora lusitaniae*, and *Rhodotorula mucilaginosa* has been reported from Brazilian Ameriindian fermented beverages through the culture-dependent and independent methods [54]. *Candida parapsilosis* was found in coffee fruit beverages [55], while *Meyerozyma guillermondii* exhibited potent enzymatic activity in wine [56]. Various *Candida* species from indigenous Brazilian foods and cocoa, along with *Meyerozyma caribbica* from fermented olives, exhibited probiotic potential [57,58]. *Pichia kudriavzevii* from African cereal-based foods also showed promising probiotic properties [59].

Saccharomyces cereviase and *Pichia kudriavzevii* isolated from 4 different fermented food of Thailand, showed antimicrobial against *B. cereus* and *E.coli.* similarilty, the above isolate showed survival at pH 3.0, and 0.3% bile, with hydrophobicity of 65–70% xylene. Most of the yeast isolate we isolated also showed similar characteriestics [60]. Twenty yeast strains isolated from traditional Indian fermented foods exhibited strong probiotic potential, with seven strains showing 100% survival at gut-like conditions (pH 2.0–2.5, bile salt 1%). These strains demonstrated antimicrobial activity against enteric pathogens, produced beneficial enzymes, assimilated cholesterol, and were generally safe for use. Identified strains included *Saccharomyces cerevisiae, Candida tropicalis, Aureobasidium* sp., and *Pichia manschurica.* [61]. *Saccharomyces cerevisiae* (14 strains), and *Pichia kudriavzevii* (2 strains) were isolated from *Rabilé*, a Traditionally Fermented Beer Produced in Burkina Faso, this yeast strains exhibited strong resistance under simulated gastrointestinal conditions. Auto-aggregation ranged from 70.20 ± 10.53% to 91.82 ± 1.96%, *Candida parasilopsis* HD1_1 siolated from FBS (*Hirring*) of Arunchal Pradesh showed highest autoaggregation of 84.063 ± 0.911, while co-aggregation with *E. coli* varied between 24.92 ± 3.96% and 80.68 ± 9.53%, and with *S. enterica* serovar *Typhimurium* from 40.89 ± 8.18% to 74.06 ± 7.94%. Similarly, *Candida orthopsilopsis* EEGM2_4 showed a higest coaggregation of 86.75 ± 2.96 with *Bacillus cereus* MTCC 430, 85.73 ± 2.91 with *Escherichia coli* MTCC 1583, 86.26 ± 2.87 with *Staphylococcus aureus* MTCC 740, and 86.25 ± 2.78 with *Salmonella enterica* MTCC 3223. Additionally, the hydrophobicity of the strains toward n-hexane ranged from 43.17 ± 5.07% to 70.73 ± 2.42%. Isolates *Meyerozyma guilliermondii* BEP1, *Pichia fermentans* BEP2 and *Meyerozyma guilliermondii* NHR3 had the highest hydrophobicity in chloroform, while *Candida orthopsilopsis* ES1_2, *Candida orthopsilopsis* GEP2, *Candida parasilopsis* HD1_1, *Saccharomyces cerevisiae* NEP2, and *Meyerozyma guilliermondii* NHR3 had the highest hydrophobicity in hexadecane. *Meyerozyma guilliermondii* NHR3, *Saccharomyces cerevisiae* NEP2, *Candida orthopsilopsis* ES1_2 and *Candida orthopsilopsis* GEP2 had the highest hydrophobicity in xylene [62]. In a research conducted by Lama and Tamang (2022) [35] total of 3438 yeast strains were isolated from 40 samples of *dahi* (1779 isolates) and 40 samples of *chhurpi* (1659 isolates) and screened for probiotic potential based on their survival in low pH, bile salt resistance, and hydrophobicity. From these, 20 yeasts were selected for further in vitro and genetic screening. *Saccharomyces cerevisiae* DJT-2 showed the highest hydrophobicity at 97.54% and 98.33%, respectively, while *S. cerevisiae* DRC-42 and *S. cerevisiae* CGI-29 exhibited high auto-aggregation rates of 93.88 % and 91.69 %. All strains demonstrated co-aggregation with pathogenic bacteria, and *Kluyveromyces marxianus* DPA-41 and *Pichia kudriavzevii* CNT-3 showed strong deconjugation activities. Probiotic genes were also screened for acid and bile tolerance, adhesion, and antimicrobial activity were detected in select strains, similar work has also been seen in the yeast isolates isolated from fermented bamboo shoot of Arunachal Pradesh.

There are differences in both the abundance and diversity revealed by the metagenomic analysis of fungi in various types of fermented bamboo shoots across different locations within Arunachal Pradesh. This means that Ganga Market samples have higher diversity levels compared to those from Basar and Naharlagun areas when it comes to alpha diversity indices like Fisher α and Shannon diversity, hence suggesting the possibility of more uniform distribution patterns in terms of fungal species among bamboo shoot samples collected from Ganga Market. The beta diversity study backs up these findings, indicating significant differences in fungal composition between locales. The dominance of different fungal phyla, such as Ascomycota, Basidiomycota, and Pseudomonadota, varies by location and sample, with Ascomycota dominating in the Naharlagun and Basar areas. *Pestalotiopsis*, *Penicillium*, *Aspergillus, Trichosporon,* and *Fusarium* are examples of genus-specific species with varying abundance patterns, showing regional colonization preferences. Notably, Ganga Market samples demonstrate reduced fungal diversity abundance at both phylum and genus levels compared to other places. The yeast isolates from *Candida, Pichia, Meyerozyma*, and *Debaryomyces* had a low abundance (<1 %). Despite their low prevalence, these isolates may play important roles in the fermentation process of fermented bamboo shoots. These findings highlight the complex interplay between environmental conditions and fungal community dynamics in fermented bamboo shoot ecosystems, and interaction with other dominant microorganisms especially with lactic acid bacteria, paving the way for future study on microbial diversity and its implications for food fermentation. These findings were consistent with fungal amplicon sequencing of Guangxi fermented bamboo shoots, which revealed that ascomycetes dominated, followed by basidiomycetes [63].

In FBS of Arunachal Pradesh, *Pestalotiopsis* and *Penicillium* were more abundant, whereas in *suansun*, a Chinese FBS, *Pichia* was reported as the dominant genus. *Candida* and *Debaryomyces* are two genera found thriving more in *Guangdong* than elsewhere among other areas where *Pichia* predominates in sour bamboo shoots; the reverse being the case for sour bamboo shoots in *Guangx*i compared with *suancai* [63,64]. The relationship between physical and chemical parameters and fungal diversity in bamboo shoots opens up calcium and moisture content's great importance through which they influence fungal communities. Functional characterization

reveals what certain genes do in bamboo shoot samples, with some notable positive relationships among areas, although the identification of negative ones among low fungal diversity samples calls for a more comprehensive approach to understand functional profiles of fungi.

The results of this study reveal exciting opportunities for the use of yeast as a starter culture in the fermentation of bamboo shoots. Yeasts can thus be used as useful biological agents in the production of probiotic-rich foods. The identification of these 13 strains having a strong probiotic quality opens up exciting possibilities toward the inclusion of useful microorganisms in non-dairy products to fill up the usual failure in such products. The integration of non-dairy and non-lactic probiotics into an enlarged portfolio of food substrates with improved health benefits of the consumer is shown to be possible according to this study.

5. Conclusion

This is a unique study because it is a comprehensive investigation into probiotic potential and fungal ecology within bamboo shoot samples. Promising candidates that showed strong probiotic traits were selected, which included substantial acid and bile tolerance, autoaggregation, co-aggregation ability, and antagonistic activity against pathogens. Furthermore, culture-independent metagenomic analysis revealed the fungal diversity, showing distinct abundances at the phylum and genus levels. More importantly, the correlation of fungal diversity with environmental factors, such as calcium and moisture content, revealed the complexity of the factors that influence the makeup of microbial communities. The research aims to classify the fungal community and provide critical insight into putative probiotic yeast strains in the fermented bamboo shoots of the region. Future research can be directed towards in vitro and *in vivo* studies on the safety and functional features of these probiotic candidates in the cell lines followd by animal models. Further, these yeast isolates can be explored for their use as starter cultuire for FBS production in order to produce hygenic and functional products.

CRediT authorship contribution statement

Rohit Das: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Buddhiman Tamang:** Writing – review & editing, Methodology, Investigation, Formal analysis, Conceptualization. **Ishfaq Nabi Najar:** Writing – original draft, Formal analysis. **Marngam Bam:** Methodology, Investigation. **Prabal Khesong Rai:** Methodology, Investigation.

Data availability statement

The data of all the metagenomic amplicon sequences is publicly available in NCBI with BioProject bearing PRJNA1079901. The 28s sequence of the isolated yeast isolates are also publicly available in NCBI with accession number *Candida orthopsilosis* CO1 (EB1_2) OR058798.1, *Meyerozyma guilliermondii* MG1 (KGM1_3) OR056307.1, *Candida orthopsilosis* CO3 (ES1_2) OR056305.1, *Candida parapsilosis* CP1 (HD1_1) OR056306.1, *Candida orthopsilosis* CO2 (EEGM2_4) OR056304.1, *Meyerozyma guilliermondii* NHR 3 PP270202.1, *Meyerozyma guilliermondii* BEP 1 PP270193.1, *Candida orthopsilosis* GEP 2 PP270192.1, *Candida parapsilosis* NEK 9 PP269452.1, *Meyerozyma caribbica* GEP 7 PP269441.1, *Saccharomyces cerevisiae* NEP 2 PP268154.1, *Pichia fermentans* BEP 2 PP268153.1, and *Pichia kudriavzevii* NHR 12 PP268152.1.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors used Grammarly in order to improve the correctness of the English. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

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.e39500.

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