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Research Article

Potential Probiotic Yeasts of the *Pichia* Genus Isolated from '*Dadih*', a Traditional Fermented Food of West Sumatra, Indonesia

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ABSTRACT

Fermented buffalo milk, known as *dadih*, serves as a reservoir of potential probiotic yeasts. Over the past two decades, probiotic yeasts have gained increasing attention in both basic and clinical sciences due to their health benefits. This study aimed to isolate and characterize probiotic yeasts from *dadih*. Yeasts were isolated using yeast Extract, peptone, and dextrose (YPD) medium, and molecularly identified through 18S-rRNA sequencing. Probiotic potential was assessed by evaluating resistance to acidic pH, bile salts, proteolytic, lipolytic, and hemolytic activities. Secondary metabolites produced during fermentation were tested for antimicrobial properties. GBT30 and GBT37 isolates were selected based on their superior performance in probiotic property assays for further analysis. Molecular identification revealed these isolates as *Pichia occidentalis* (GBT30) and *Pichia kudriavzevii* (GBT37). Both strains demonstrated *in vitro* survivability under simulated gastrointestinal conditions and exhibited antimicrobial activity. Whole-genome sequencing of *P. kudriavzevii* GBT37 identified a genome size of 10,906,850 base pairs, distributed across four chromosomes with a GC content of 38.26%. Notably, secondary metabolite biosynthesis genes were located on contig 7. In addition, 26 probiotic-related genes, including *GSY1, HSC82, HSP104, TPS1, ARN1, FLO1, ALA1, SIR2*, and others, were identified in *P. kudriavzevii* GBT37, indicating its potential as a probiotic yeast. The traditional fermentation process of *dadih* offers probiotic yeasts with promising health benefits, supporting its potential as a functional food.

1. Introduction

The food and agriculture organization (FAO) of the United Nations defines probiotics as "live microorganisms that, when administered in adequate amounts, confer a health benefit to the host". The consumption of fermented foods, which has been recognized for its health benefits for centuries, predates the discovery of functional microorganisms. The scientific exploration of microorganisms with positive

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health effects began over a century ago by Metchnikoff, who postulated that the gut microbiome could be altered with beneficial bacteria found in yogurt to improve health and delay aging (Mackowiak 2013). Over the past 20 years, probiotics have received increasing attention in both basic and clinical sciences, resulting in over 6,000 publications in the biomedical context. More than 60% of these studies have been published in the last five years, many in highly reputable scientific journals (Didari *et al.* 2014). The surge of interest reflects the development of functional foods containing probiotic strains that offer health benefits, such as reducing the risk of inflammatory bowel disease, controlling serum cholesterol levels, mitigating diarrhea, improving gut microbiome, and modulating the immune system (Hu *et al.* 2018).

West Sumatra, located in the western part of Central Sumatra, Indonesia, borders the provinces of North Sumatra, Riau, Jambi, and Bengkulu to the west, as well as the Indian Ocean (Hashfi 2023). West Sumatra is situated at 361.86 meters (1187.2 feet) above sea level, and its climate is classified as tropical rainforest. The warm temperature, high humidity, and extended rainy season make meat and vegetables highly perishable. In rural areas, preserving and storing perishable food to extend shelf life and ensure safety for consumption is crucial. Fermentation is a technique widely employed in West Sumatra to achieve this. Beneficial organoleptic changes during fermentation result from the interaction of yeasts and lactic acid bacteria with the substrate, successively impacting the flavor, texture, and aroma of food (Narzary *et al.* 2021). Traditional fermented foods in Indonesia are predominantly produced through simple, spontaneous fermentations that do not require sophisticated or expensive equipment. Natural microorganisms are sourced from ingredients, tools, and surroundings, which, under appropriate conditions, foster the growth of specific microorganisms responsible for food fermentations (Mannaa *et al.* 2021). There is a growing interest in fermented foods, accompanied by an increasing awareness of food safety and the need for standardization (Galimberti *et al.* 2021).

Yeasts represent the second most important group of microorganisms in fermented products consumed by humans, following lactic acid bacteria. However, yeasts have received less attention in research compared to lactic acid bacteria, with most studies focusing on their role in alcohol production or the brewing industry rather than fermented foods. There are limited reports discussing the contribution of yeasts in fermented foods. These reports mainly address species within genera *Candida, Saccharomyces, Lachancea, Pichia, Zygosaccharomyces, Debaryomyces*, and *Hanseniaspora* (Kuda *et al.* 2009; Tamang & Lama 2023). Yeasts are gaining more attention for their flavor and aroma-forming capabilities and their potential as probiotics. Several studies have highlighted yeasts such as *Debaryomyces hansenii, Torulaspora delbrueckii, Kluyveromyces lactis, Yarrowia lipolytica* and *Kluyveromyces marxianus*, which can withstand gastric juices, survive in the human digestive tract, and resist gastrointestinal pathogens (Psani & Kotzekidou 2006). Their health benefits drive the growing demand for chemical-free and natural foods. Furthermore, recent studies suggested the potential for discovering novel strains of probiotic yeasts (Boonanuntanasarn *et al.* 2019), which, despite their minor proportion in the intestinal microbiota, play essential roles within the GI tract (Gatesoupe 2007; Boonanuntanasarn *et al.* 2019). Among potential sources of probiotic yeasts, *dadih*, a fermented buffalo milk indigenous to West Sumatra, Indonesia, presents a unique opportunity for bioprospecting. *Dadih*, traditionally fermented in bamboo culms, is a yogurt-like product with a thicker consistency, smooth texture, and pleasant flavor, providing nutritional benefits to the indigenous people. The spontaneous fermentation of *dadih* involves a variety of microorganisms, including yeasts, lactic acid bacteria, and acetic acid bacteria (Rizqiati *et al.* 2015). Despite its long history of consumption, the specific yeast species responsible for the probiotic properties of *dadih* remain largely unexplored.

In this study, we investigated the probiotic potential of yeast strains isolated from *dadih*, a traditional fermented food from West Sumatra, Indonesia. Of the 55 isolates obtained, two yeast strains, GBT37 and GBT30, exhibited irregular colony morphology, distinct from the other isolates, making them interesting candidates for further biochemical and genetic screening. By integrating traditional microbiological techniques with molecular methods, we aimed to identify novel yeast strains with potential probiotic properties and enzymatic activities. The significance of this research extends beyond the scientific domain, with implications for public health, food security, and economic development in the region. Understanding the yeast diversity in *dadih* and harnessing its probiotic potential could lead to the development of new functional foods, dietary supplements, and biotechnological applications. Moreover, this study contributes to the preservation of indigenous knowledge while promoting sustainable food production and consumption practices.

2. Materials and Methods

2.1. Materials

A total of 600 ml of *dadih* was collected from three places: Lintau Tanah Datar (LTD), Gadut Bukittinggi (GBT), and Kota Bukittinggi (KBT), in West Sumatra Province, Indonesia. Samples were transported to the Laboratory of Genomics, National Research and Innovation Agency (BRIN), Cibinong, Indonesia, and stored at 4 degree of Celcius (°C). Pathogenic bacterial strains, *Staphylococcus aureus* ATCC, *Bacillus cereus*, Enteropathogenic *E. coli* K1.1 (EPEC), and *Listeria* spp. were obtained from the Laboratory of Microbiology, IPB University, Bogor, Indonesia.

2.2. *Dadih* Yeast Isolation

Approximately 0.5 milliliters (ml) of *Dadih* samples from each location were enriched using liquid Yeast extract, Glucose, and Chloramphenicol (YGC) media, and incubated at 30 and 37°C with agitation at 250 revolutions per minute (rpm) for 24 hours (h). Dilutions of 10^5, 10^6, and 10^7 were prepared using 0.85% NaCl, and 0.1 ml of each dilution was spread on YGC agar. The plates were incubated at 30 and 37°C for 24-48 h. Single colonies were transferred to Yeast extract, Peptone, and Dextrose (YPD) agar media using the streaking method and incubated at 30°C (isolate GBT30) and 37°C (isolate GBT37) for 24-48 h. The isolates were then cultured in liquid YPD media and shaken for 24 h.

2.3. Yeast Identification Using 18S rRNA PCR

Two yeast isolates (GBT30 and GBT37) were further identified using PCR targeting the 18S rRNA gene. The identification process employed amplification primers FR3F (5'-ATTGGAGGGCAAGTCTGGTG-3') and FR3R (5'-CCGATCCCTAGTCGGCATAG-3') (Roostita *et al.* 2011; Magalhães *et al.* 2022). The isolates, based on their probiotic potential, underwent DNA isolation and amplification. The resulting PCR products were sequenced, and the sequences were then submitted to the GenBank sequence database. Accession numbers PP897640 and PP897641 were obtained for GBT30 and GBT37, respectively. The obtained consensus sequence was compared with other sequences of closely related species retrieved from GenBank/NCBI and then aligned using BioEdit version 7.2. The evolutionary analyses were conducted in MEGA11 version 10.2.6. The taxonomic connection was inferred using the maximum likelihood method and the Tamura-Nei model.

2.4. Scanning Electron Microscopy (SEM)

Selected colonies were cultured in 5 ml of YPD, incubated at 30 and 37°C. Colonies were observed using an optical microscope and SEM (Hitachi SU-3500, Netherlands). After dehydration with glutaraldehyde for 3 h at 4 °C, the samples were rinsed for 15 minutes (min) in a series of ethanol concentrations (50, 70, 90, and 100%). After overnight drying, the samples were placed inside a specimen aluminum tub with double sticky tape and then coated with a sputter coater for 2 min at a voltage of 10 kV.

2.5. Growth Curve

YPD liquid medium (2% peptone, 1% yeast extract, 2% dextrose) was used routinely for GBT30 and GBT37 cultures. Other culture media utilized in this study included buffered glycerol complex (BMGY) medium (1% yeast extract, 2% peptone in 100 mM potassium phosphate buffer (pH 6.0), 1.34% yeast nitrogen base (YNB), and 1.64×10^{-3} mM of biotin containing 2% glycerol), which was used for cell growth, and buffered methanol complex (BMMY) medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate buffer (pH 6.0), 1.34% yeast nitrogen base (YNB), 1.64×10^{-3} mM of biotin, and 0.5% methanol, which was used for induction. During all experiments, the yeasts were cultivated in 250-ml flasks (with a working volume of 50 ml) at 30 and 37°C with an agitation rate of 250 rpm. Optical density was measured at 600 nm (OD_{600}) at intervals ranging from 0 to 96 h. The yeast sample was collected during the stationary phase for further probiotic tests.

2.6. Bile Salt Tolerance

Different bile salt concentrations (0.1%, 0.3%, and 0.5%) were prepared by dissolving bile salts in YPD media to determine the survival of selected yeast colonies under varying bile concentrations. Survival in bile salts was assessed following a modified method based on Helmy *et al.* (2019). Kristoffersen *et al.* (2007) reported that the concentration of bile salt in the small intestine starts at 0.2%; hence, in this test, 0.1% was used as the control. Two yeast isolates (GBT37 and GBT30) were initially cultured in YPD media containing different bile concentrations and then incubated at 37 °C and 250 rpm for 24 h. After the incubation period, serial dilutions of the yeast culture (10^{-7}, 10^{-8}, and 10^{-9}) were prepared using a sterile 0.85% NaCl solution. Subsequently, 0.1 ml of each dilution

was spread on YPD agar plates using the spread plate method. The YPD agar plates were incubated at 30 and 37°C for 48 h, and colony counts were recorded at 24-h intervals. Colonies were counted to assess yeast survival under each bile salt concentration, and CFU/ml was calculated according to Equation below:

$$\frac{\text{CFU}}{\text{ml}} = \frac{\text{No. of colonies} \times \text{Total dilution factor}}{\text{Volume of culture plated in ml}}$$

2.7. pH Tolerance

To assess the pH tolerance of the two selected yeast isolates (GBT30 and GBT37), the test was conducted using YPD media adjusted to various pH values (pH 2, 3, 4, 5, 6, and 7). The yeast isolate was inoculated into each prepared YPD medium at the specified pH levels. The inoculated cultures were then incubated at 30 and 37°C with agitation at 250 rpm for 24 h to allow for growth and adaptation to the respective pH environments. Following the incubation period, serial dilutions of the yeast cultures were prepared using a sterile 0.85% NaCl solution. Serial dilutions of 10^{-7}, 10^{-8}, and 10^{-9} were made to facilitate colony counting and ensure the accuracy of the results. Subsequently, 0.1 ml of each dilution was spread onto YPD agar plates using the spread plate method to obtain isolated colonies. The agar plates were then incubated at 30 and 37°C for 48 h to allow colony formation. Colony counting was performed at 24-hour intervals to monitor growth under different pH conditions. The number of colonies on each plate was recorded to assess the ability of the yeast strains to survive and multiply at varying pH levels. Data analysis involved calculating the colony-forming units per milliliter (CFU/ml) for each yeast culture at different pH levels. The survival and growth of yeast colonies were analyzed to evaluate pH tolerance and identify optimal pH conditions for the yeast isolate, and CFU/ml was calculated according to Equation below:

$$\frac{\text{CFU}}{\text{ml}} = \frac{\text{No. of colonies} \times \text{Total dilution factor}}{\text{Volume of culture plated in ml}}$$

2.8. Proteolytic Activity Test

The test was conducted using the method described by Muche *et al.* (2023) with minor modifications. To determine the proteolytic activity of the strains, selected GBT37 and GBT30 strains were streaked on skim milk agar media. The inoculated agar plates were then incubated at 30 and 37°C for 72 h. Colonies surrounded by a clear (halo) were identified as strains exhibiting proteolytic activity.

2.9. Lipolytic Activity Test

The test was performed following a method described by Muche *et al.* (2023) with minor modifications. Briefly, GBT37 and GBT30 isolates were streaked on tween 80 media (10 ml/L tween 80, 10 g/L peptone, 5 g/L sodium chloride, 0.1 g/L calcium chloride, and 20 g/L agar) on a separate plate and incubated at 30 and 37°C for 24 h. Lipolytic activities by strains were indicated by dispersed growth forms through precipitation of calcium salts formed from the fatty acids released during the hydrolysis of Tween 80. Yeast colonies displaying this precipitation around streak-inoculated media were considered lipolytic isolates.

2.10. Hemolytic Activity Test

A hemolytic activity assay was carried out to determine whether each yeast isolate could cause damage to red blood cells, which is an important criterion for choosing a probiotic strain. The strains were assessed for hemolytic activity using blood agar (5% v/v sheep blood, 0.5% peptone, 0.5% yeast extract, 0.5% sodium chloride, and 1.5% agar) described by Muche *et al.* (2023). Twenty microliters of the selected isolate suspensions were spotted on sterile blood agar. The plates were incubated at 37°C for 48 h and then observed for the formation of a lysis zone around the colonies (indicating a positive reaction or β-hemolysis). The nonhemolytic reaction will be recorded by observation of green-halo zones around the colonies (α-hemolysis).

2.11. Antagonistic (Antimicrobial) Test of Yeast Isolates

This test was conducted to determine the ability of yeast isolated from *dadih* to inhibit the growth of pathogenic bacteria. The testing process is carried out according to a modified method by Muche *et al.* (2023). Yeast colonies of GBT30 and GBT37 were cultured in 5 ml of different liquid YPD media and then incubated at 30 and 37°C for 24 hours with a speed of 250 rpm using a shaker incubator. 1 ml of the yeast culture was then centrifuged at 4°C for 10 minutes at 4,000 rpm to obtain a pellet. The cell suspension was prepared using 1× phosphate-buffered saline (PBS). Pathogenic bacteria *Staphylococcus aureus* ATCC, *Bacillus cereus*, Enteropathogenic *E. coli* K1.1 (EPEC), and *Listeria* spp. are cultured in 5 ml of liquid Luria-Bertani (LB) media and incubated at 37°C with a speed of 150 rpm for 18 hours using a shaker incubator. A standardized amount

of pathogenic culture, 0.1 ml, using 0.5 McFarland, is inoculated onto nutrient agar media and poured into Petri dishes until dry. Then, test discs soaked in yeast isolate suspensions are placed on labelled Petri dishes and incubated at 37°C for 24 hours. The same procedure was repeated with a positive control (ampicillin) and a negative control (distilled water). Observation is made by examining the clear zones formed around the test discs.

2.12. Whole Genome Sequencing, Genome Assembly, and Bioinformatic Analysis

In brief, a ligation sequencing kit (SQK-NBD114-24 Oxford Nanopore Technologies, Oxford, UK) was used to prepare a library using 250 ng of DNA as input, with a few minor modifications. To prevent excessive size selection, the produced library was filtered using 1.0X AMPure XP magnetic beads and then washed once more using a 1:1 ratio of short fragment buffer to long fragment buffer, as previously described by Zainulabid *et al.* (2022). The genomic DNA was then sequenced using PromethION 24 (Oxford Nanopore Technologies, Oxford UK), and base calling was carried out with modifications using Dorado v7.2.13. Raw reads were first visualized using NanoPlot v1.42.0 (https://doi.org/10.1093/bioinformatics/btad311). The reads were then filtered using Filtlong v0.2.1 to obtain 90% of the best reads with a minimum length of 1000 bp (Wick RR. Filtlong. 2017. https://github.com/rrwick/Filtlong). The filtered reads were subjected to de novo assembly with Flye v2.9.3, the resulting contigs were polished using Medaka v1.11.3 (medaka: Sequence correction provided by ONT Research. https://github.com/nanoporetech/medaka). Then, the polished contigs were assembled into a scaffold using ragtag v2.1.0 (https://doi.org/10.1186/s13059-022-02823-7). Assembly quality assessment was checked using QUAST v5.0.2 integrated into the Patho Systems Resource Integration Center (PATRIC) platform. The raw data analysis was performed with default parameters unless otherwise stated (Sylvere *et al.* 2023). Moreover, the circular genome was visualized using Genious Prime, and genome annotation was performed using the Rapid Annotation using Subsystem Technology (RAST) server (Sylvere *et al.* 2023). Antibiotic resistance genes were predicted from the Comprehensive Antibiotic Resistance Database (CARD) and ResFinder 4.1. Virulence factors were identified by the Center for Genomic Epidemiology (CGE) (Brettin *et al.* 2015). To predict a cluster gene that correlates to the probiotic properties (secondary metabolites) of the potential probiotic yeast, AntiSMASH was used. In addition, Antibiotic Resistant Target Seeker Version 2 (ARTS) was used to detect antibiotic resistance-encoding genes.

2.13. Probiotic-Related Genes Analysis

Genome assembly of *P. kudriavzevii* GBT37 was compared to two reference assemblies: *Saccharomyces cerevisiae* S288C (Goffeau *et al.* 1996) and *Saccharomyces boulardii* unique28 (Khatri *et al.* 2017). A list of probiotic-related proteins from *S. cerevisiae* S288C was retrieved from the Yeast Genome Database (Cherry *et al.* 2012). BLASTP v2.6.0 was employed to search for homologous proteins in the predicted proteomes of *P. kudriavzevii* GBT37 and *S. boulardii* unique28. The BLASTP search was conducted using the parameters "-task blastp -outfmt '6 qseqid sseqid pident qcovs' -evalue 10e-5."Protein alignments were performed using Clustal Omega v1.2.4 with default parameters (Sievers *et al.* 2011). ProgressiveMauve was used to align the genome assemblies of S288C, GBT37, and unique28 under default conditions (Darling *et al.* 2010). Orthologous groups shared and unique to the proteomes of S288C, GBT37, and unique28 were identified using OrthoVenn2 (Xu *et al.* 2019). Proteins unique to the GBT37 orthologous group were extracted and further analyzed by BLASTP to identify the closest homologous strains.

3. Results

3.1. Yeast Identification Using 18S rRNA PCR

Yeast identification at the species level was conducted by molecular methods such as PCR targeting the 18S rRNA gene (Table 1). GBT30 was confirmed to be closely related to *P. occidentalis*, and GBT37 was related to *P. kudriavzevii* when compared to the GenBank on the National Center for Biotechnology Information (NCBI). Phylogenetic analysis was used to confirm the close relationships between yeast strains based on their 18S rRNA gene sequences (Figure 1). Based on the phylogenetic tree, GBT30 was further confirmed to be closely related to *P. occidentalis*, and GBT37 was related to *P. kudriavzevii*, as they were

Table 1. Similarity of isolates compared to sequences on the NCBI website

Isolate code	Species name	Percent identify (%)	Sequence ID
GBT37	*Pichia kudriavzevii*	99.54	MH545928.1
GBT30	*Pichia occidentalis*	99.54	KX150657.1

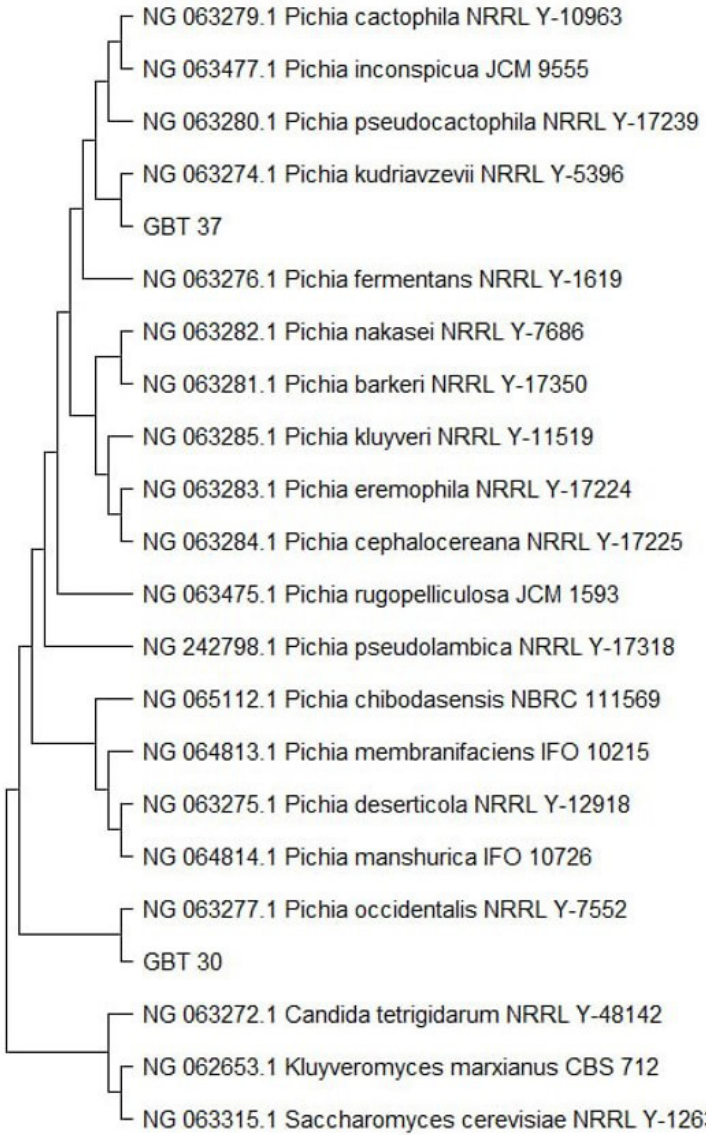


Figure 1. The phylogenetic tree was constructed based on 18S rRNA gene sequences showing a taxonomic connection. *Pichia kudriavzevii* (GBT37), and *Pichia occidentalis* (GBT30) strains with the closest hits retrieved from GenBank/NCBI. The evolutionary history was inferred by using the maximum likelihood method and the Tamura-Nei model

located in the same branch as *P. occidentalis* strains PG6 and *P. kudriavzevii* N-X Hac1, respectively.

3.2. Scanning Electron Microscopy (SEM)

The GBT37 cells showed an oval shape (Figure 2), with an average size of 3.5 µm. At 11,000× magnification, the GBT37 surface showed rough patches, and the cell wall appeared thick with no visible defect or irregularities on the cell wall. No indication of biofilm, extracellular fibrils, or filamentous structures were observed in GBT37. The characteristics of the GBT30 cells were similar to those of the GBT37 cells, except that the GBT30 cells were oval-to-elongated, had a smooth cell surface, and a size of 4 µm.

3.3. Growth Curves

The growth curve (Figure 3) analysis for the yeast isolates GBT30 and GBT37 revealed a rapid initial growth for both isolates, with their OD_{600} reaching approximately 8.0 after 24 h. This indicates a swift transition into the stationary phase, where the OD_{600} of both isolates remained around 8.0 for the next 24 to 48 h. During the 48 to 71 h period, both isolates maintained a steady OD_{600}, with GBT30 showing a slightly higher OD_{600} compared to GBT37. After 71 hours, the OD_{600} of GBT30 began to slowly decrease, reaching approximately 8.0 at 96 hours. In contrast, the OD_{600} of GBT37 decreased more significantly, dropping to approximately 6.0 at 96 h.

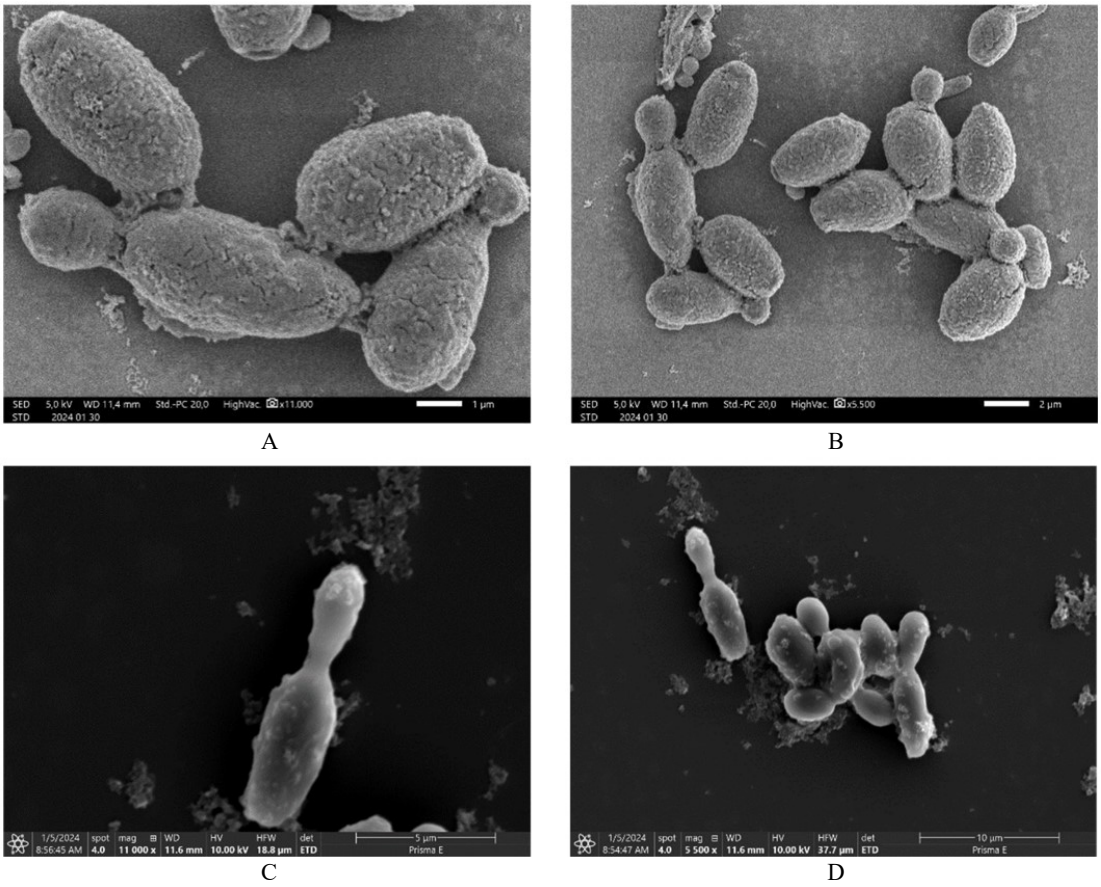


Figure 2. SEM analysis of (A) GBT37 at 11000X (B) GBT37 at 5500X (C) GBT30 at 11000X (D) GBT30 at 5500X

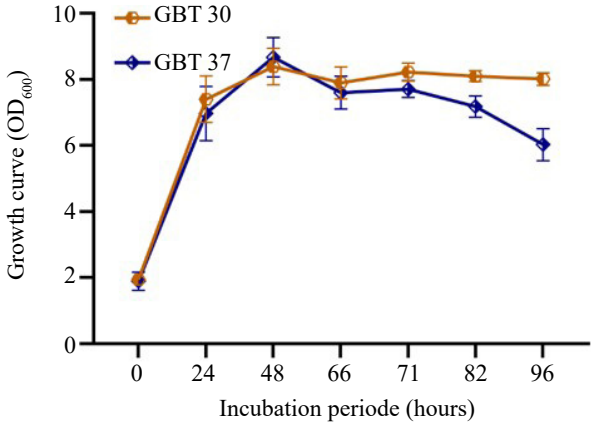


Figure 3. Growth curves of GBT37 and GBT30 cells in inductive medium containing BMMY. Error bars represent standard errors

3.4. Probiotic Tests

3.4.1. pH Tolerance Test

The stability of *Pichia* strains at different pH mediums allows them to survive different pH changes in the host body. The growth of two yeast isolates at acidic pH values was evaluated at various pH values: 2, 3, 4, 5, and 6, and the control pH was 7 (Figure 4). All 2 isolates exhibited growth at pH 2, indicating their survival ability in simulated gastric juice *in vitro*. The tolerance of GBT30 at pH 3 was greater than that at pH 2 under similar incubation conditions, while the opposite trend was observed for GBT37, which indicates that there might be a relationship between the strains and pH. The growth of both isolates at all pH values was almost the same for an increase in incubation time from 24 h to 48 h except for at pH 5 and pH 6 in GBT30. Generally, there was an increase in colony count from pH 2 to pH 6 in both colonies with minor anomalies in pH 4 and pH 6 in GBT30 and pH 3 in GBT37.

3.4.2. Bile Salt Tolerance Test

As shown in Figure 5, the 2 yeast isolates (GBT30 and GBT37) showed high tolerance to bile salts at concentrations of 0.3% and 0.5% when compared to the control of 0.1%. The colony counts of GBT30 were consistent across all bile salt concentrations, while GBT37 showed similar results but with a slight decrease in cell viability at higher bile salt concentrations. The

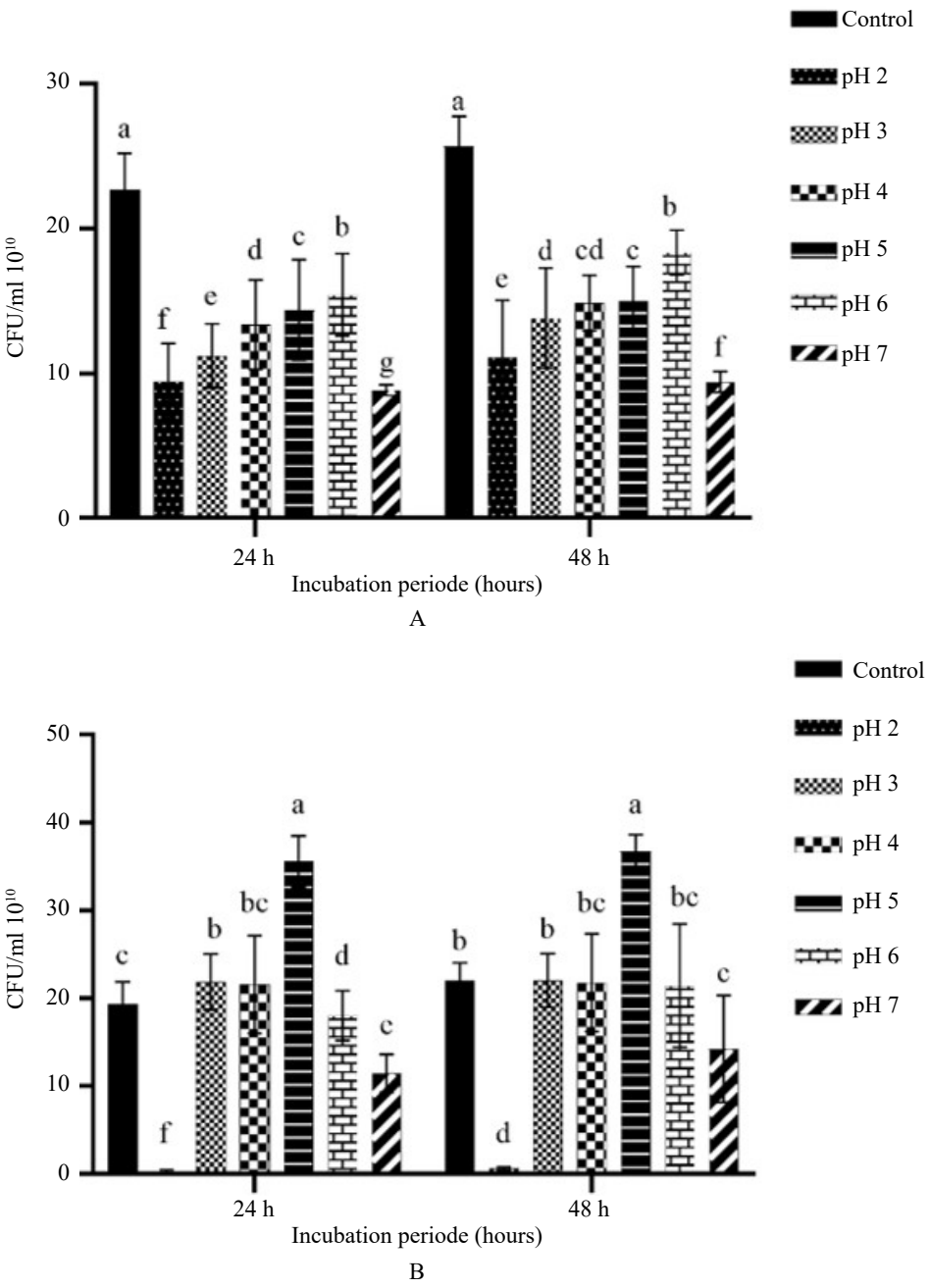
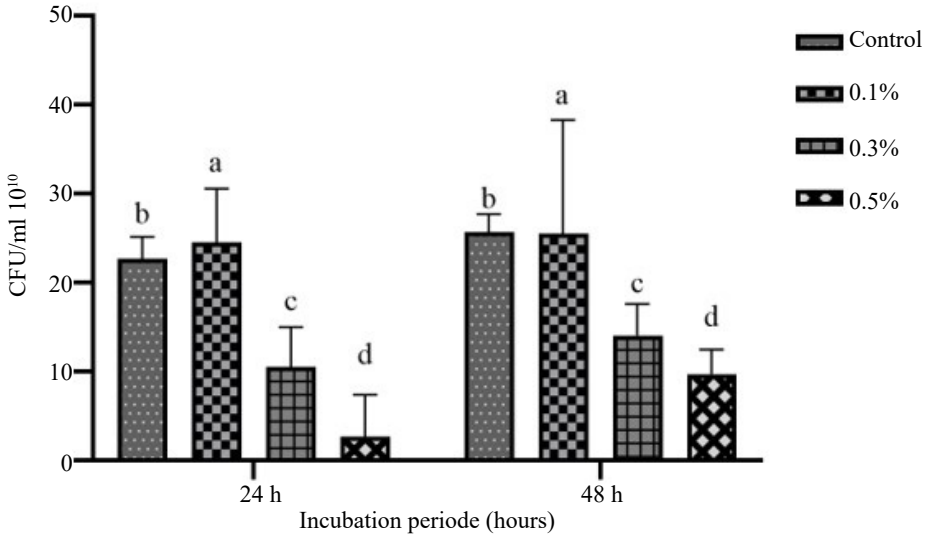


Figure 4. pH tolerance test of (A) GBT30 and (B) GBT37. Data are expressed as mean ± SEM, n = 3

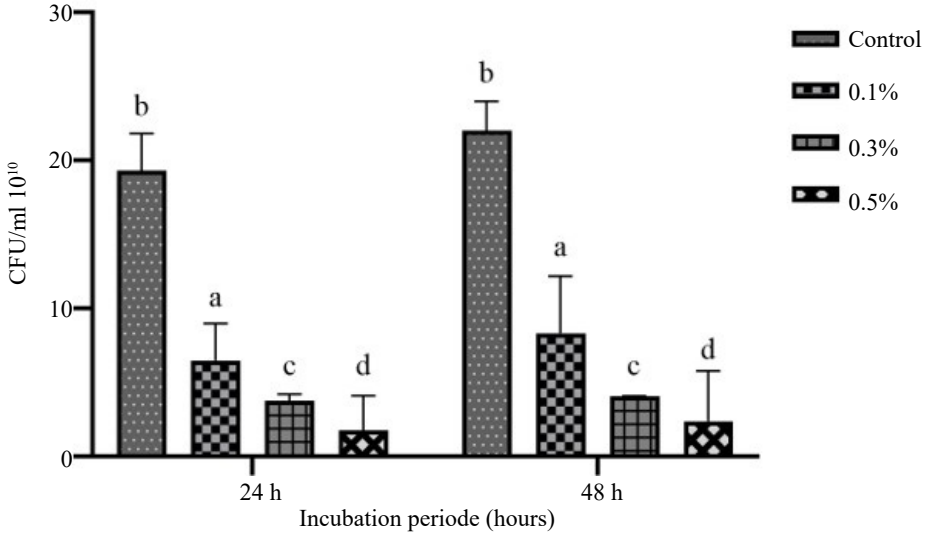
incubation time of the 2 yeast isolates did not affect the cell viability of either isolate, as the isolates showed constant cell viability at all incubation times. Cell proliferation remains almost the same in both isolates at 0.3% and 0.5% bile salt concentrations.

3.5. Proteolytic, Lipolytic, and Hemolysis Testing of Yeast Isolates

Probiotics produce a variety of beneficial enzymes that facilitate food digestion and prospectively boost human health. This step aimed to evaluate the functional properties and safety of *dadih* as a potential probiotic candidate for yeast isolates by performing proteolysis, lipolysis, and hemolysis (Table 2). Based on the results of the proteolytic tests, two yeast isolates from *dadih* demonstrated significant proteolytic activity. This was evidenced by the formation of clear zones around each isolate on the test medium, which indicates the breakdown of proteins. The test results indicate that



A



B

Figure 5. Bile salt tolerance of (A) GBT30 and (B) GBT37. The data are presented as the average ± SEM, n = 3

Table 2. Proteolytic, lipolytic, and hemolysis testing of yeast isolates from fermented food

Isolate	Proteolytic	Lipolytic	Hemolysis
Pichia kudriavzevii (GBT37)	Positive	Positive	Negative
Pichia occidentalis (GBT30)	Positive	Negative	Negative

GBT37 possesses lipolytic activity or the ability to produce lipase enzymes, while GBT30 has the opposite effect, as evidenced by the absence of clear zones or color changes in the test media. Hemolysis testing revealed that neither of the 2 yeast isolates from *dadih* exhibited hemolytic activity, indicating that they do not produce hemolysins, which are substances capable of lysing red blood cells. The antibacterial activity of the yeast isolates GTB30 and GBT37 was assessed against several pathogens, with results measured in millimeters of inhibition (Table 3). Against *S. aureus* ATCC, GTB30 exhibited an inhibition zone of 3.40±0.32 mm, while GBT37 showed 4.20±0.33 mm, compared to 8.55±0.33 mm with the ampicillin control. For Bacillus cereus, GTB30 and GBT37 showed inhibition zones of 2.50±0.17 mm and 4.55±0.20 mm, respectively, with the ampicillin control displaying 7.55±0.18 mm. In the case of EPEC K1.1, GTB30 showed 4.00±0.25 mm, GBT37 showed 3.68±0.27 mm, and the ampicillin control showed 5.20±0.26 mm. Lastly, against *Listeria* spp., GTB30 and GBT37 produced inhibition zones of 7.67±0.17 mm and 5.50±0.19 mm, respectively,

Table 3. Antibacterial activity of yeast isolated fermented food

Pathogen	Antibacterial activity (mm)		Control	
	Pichia occidentalis (GBT 30)	*Pichia kudriavzevii* (GBT 37)	Ampicilin	Aquadest
S. aureus ATCC	3.40±0.32 mm^{b}	4.20±0.33 mm^{c}	8.55±0.33 mm^{d}	0.00^{a}
B. cereus	2.50±0.17 mm^{b}	4.55±0.20 mm^{c}	7.55±0.18 mm^{d}	0.00^{a}
EPEC K.1.1	4.00±0.25 mm^{b}	3.68±0.27 mm^{bc}	5.20±0.26 mm^{c}	0.00^{a}
Listeria sp.	7.67±0.17 mm^{c}	5.50±0.19 mm^{b}	7.30±0.20 mm^{c}	0.00^{a}

Data are presented as the mean ± SEM. The finding was based on the average values of three independent assays. All values were significant $p<0.001$ against negative control

compared to 7.30±0.20 mm with the ampicillin control. No antibacterial activity was observed with aquadest as the negative control.

3.6. Whole-Genome Analysis

3.6.1. Annotation

Based on the results of the probiotic test, GBT37 (*P. kudriavzevii*) showed good results; hence, it was selected for whole-genome sequencing. An Oxford Nanopore Technologies sequencer was used to produce reads and quality assessment was done using QUAST with a total sequence length of 10 906 850 bp and an average guanine + cytosine content (GC%) of 38.26%. The sequencing data are available at NCBI BioProject ID SAMN33688472. The assembled genome had a minimum contig length to cover 50% (N_{50}) and 90% (N_{90}) of 1461763 bp and 977519 bp respectively, and several contigs to cover 50% (L_{50}) and 90% (L_{90}) of the genome of 3 and 6, respectively. The assembled gene had an auN length of 1833489 bp and 49 contigs and the size of the contigs is shown in Table 4. A total of 5 chromosomes were identified (Table 5), when compared to P. kudriavzevii strain: CBS573 Genome sequencing and assembly (GenBank, GCF_003054445.1). On the other hand, a total of 9103 CDSs were identified using ARTS (Antibiotic Resistant Target Seeker Version 2), and 119 genes were identified as core genes (Figure 6).

Table 4. QUAST analysis of Contigs showing their size and quantity across the reads

Metric	Value
Contigs (≥0 bp)	49
Contigs (≥1000 bp)	29
Contigs (≥5000 bp)	17
Contigs (≥10000 bp)	12
Contigs (≥25000 bp)	9
Contigs (≥50000 bp)	9
Contigs length (≥0 bp)	10906850
Contigs length (≥1000 bp)	10893805
Contigs length (≥5000 bp)	10893929
Contigs length (≥10000 bp)	10828861
Contigs length (≥25000 bp)	10783922
Contigs length (≥50000 bp)	10783922

Table 5. Genome assembly of GBT37 at a chromosomal level

Chromosome	GenBank	RefSeq	Size (bp)	GC content (%)
Chr 1	CP028773.1	NC_042506.1	2.852272	38.5
Chr 2	CP028774.1	NC_042507.1	2.745854	38.5
Chr 3	CP028775.1	NC_042508.1	2.541747	38.0
Chr 4	CP028776.1	NC_042509.1	1.383544	39.0
Chr 5	CP028777.1	NC_042510.1	1.289138	38.5

3.6.2. Genome Annotation Using the RAST Server

The Rapid Annotation using Subsystem Technology (RAST) server was used to predict the protein-coding sequences (CDSs) and their gene functions in the assembled genome. The genome annotation revealed 9103 CDSs and 210 RNA-encoding genes. The subsystem covered 456 genes (6%) in total, and their distribution is shown in Figure 7. On the other hand, Figure 8 shows the genome in a circular form with a GC content of 38.26% when blasted against genes of *P. kudraivzevii*. The green ring denotes the presence of the anticipated functional genes. The prediction of protein-coding sequences (CDSs) revealed the presence of several genes involved in stress response, such as cardiolipin synthase, fhs_2 (formyltransferase enzyme), and tet2_2 (tetracycline resistance protein).

3.6.3. Prediction of Antibiotic Resistance Genes and Pathogenicity

In the present study, GBT37 whole-genome sequence data was used for the prediction of antibiotic resistance, in which the assembled genome of GBT37 was uploaded and then analyzed via the CARD and ResFinder online software. Neither CARD nor ResFinder 4.5.0 detected phenotypic antimicrobial resistance-associated genes encoded in the genome of GBT37. ResFinder 4.5.0 was used to identify antibiotic resistance genes against 90

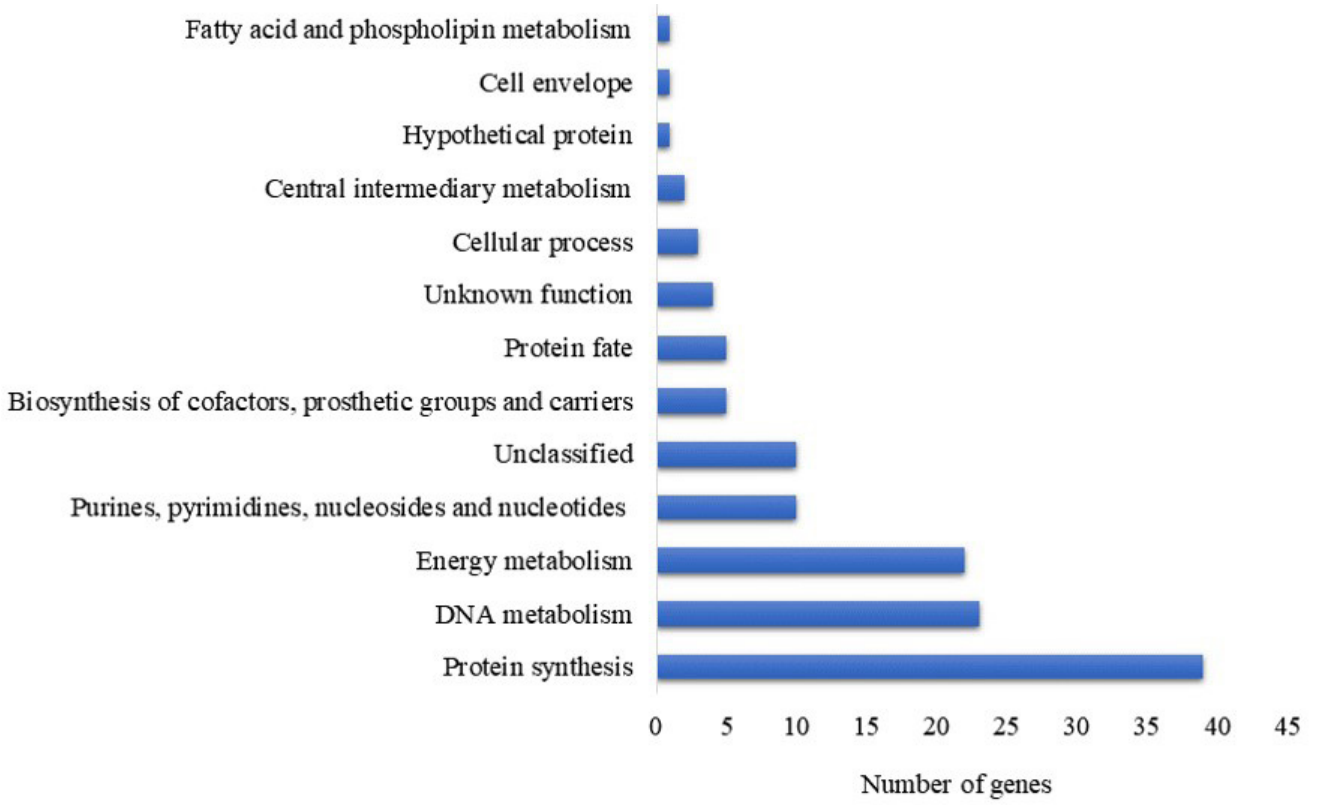


Figure 6. Core genes and their subcategories were identified using Antibiotic Resistant Target Seeker (ARTS). Protein synthesis genes were the most abundant whilst fatty acid and phospholipin metabolism genes were the least in quantity

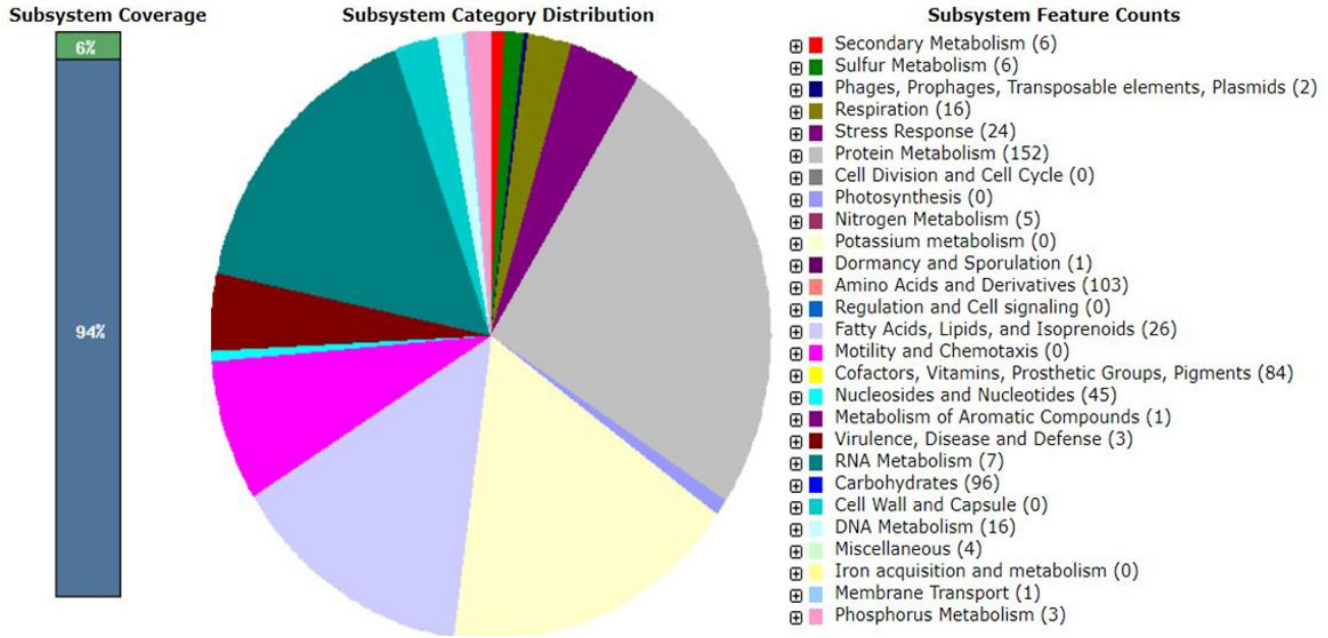


Figure 7. Functional gene prediction of subsystems using the RAST function. 18 categories were identified as having coding genes and protein metabolism counts dominated the list with 152 CDSs, followed by carbohydrates counts with 96 CDSs

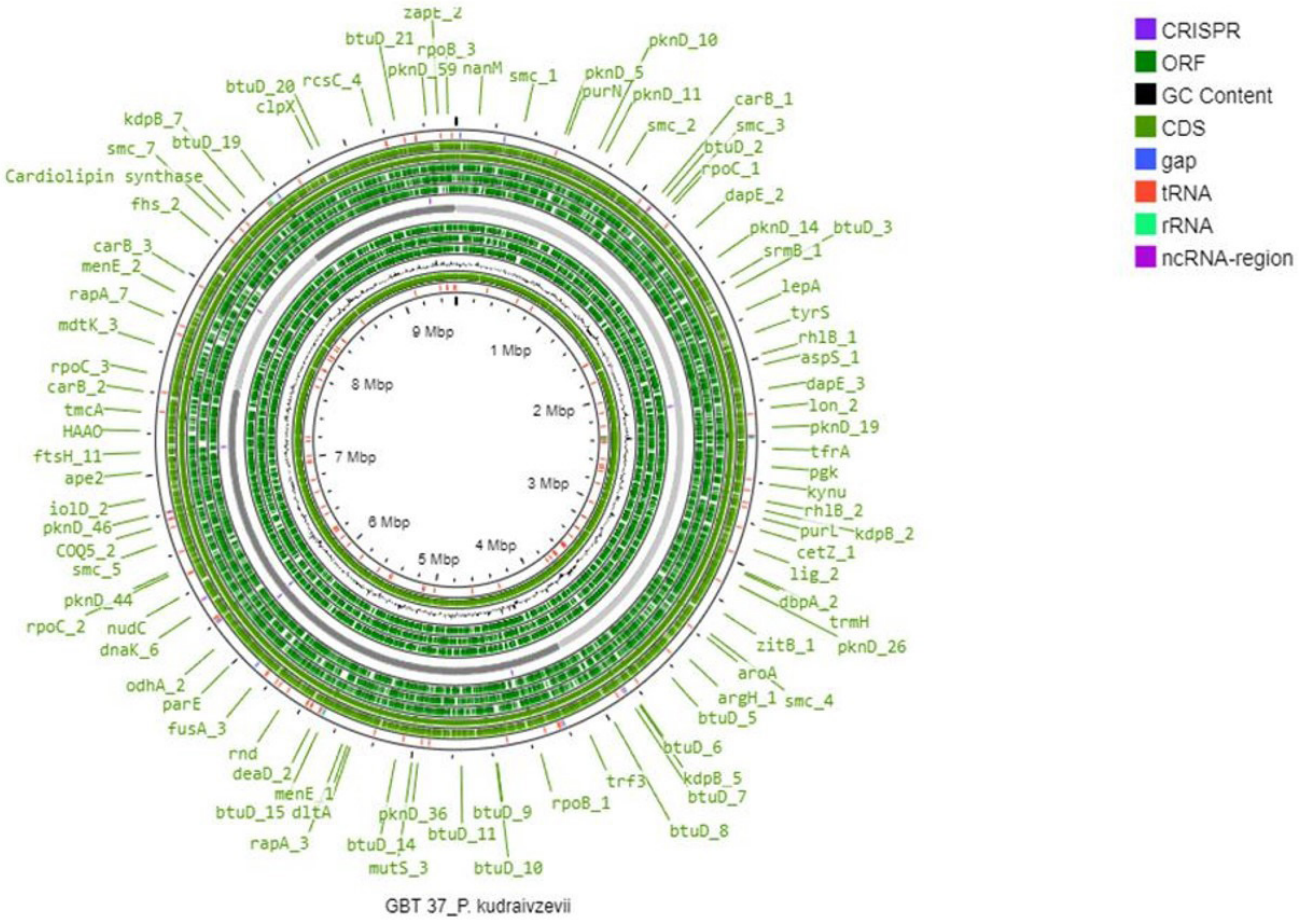


Figure 8. Circular genome visualization and annotation using geneious Prime when compared to the genes of *P. kudraivzevii*. The green ring shows the protein-coding sequences (CDSs) identified

antibiotics, including aminoglycoside, aminoglycoside, quinolone, betalactam, and tetracycline. In terms of pathogenicity, GBT37 was determined to be nonpathogenic using the VirulenceFinder-2.0 server. According to the VirulenceFinder tool analysis, GBT37 did not exhibit any hits or genes coding for *Listeria, Escherichia coli, Enterococcus faecium, Enterococcus lactis, Enterococcus*, exoenzyme-encoding genes for *Staphylococcus aureus*, toxin genes for *S. aureus*, hostimm genes for *S. aureus* or *Shiga toxin* genes.

3.6.4. Secondary Metabolites

Secondary metabolites were determined using AntiSMASH. The results revealed that secondary metabolites were only found in contig 7 (Figure 9). Contig 7 produced nonribosomal peptides (NRPSs) like type of secondary metabolites. Five secondary metabolites genes were identified when compared to GenBank from NCBI (Table 6).

3.7. *Pichia kudriavzevii* GBT37 and *Saccharomyces* Species Share Genes Implicated in Probiotic Phenotypes

A compiled list of 27 genes were involved in stress tolerance, adherence, and biosynthesis of *Pichia kudriavzevii* GBT37 as compared to the genes from *Saccharomyces cerevisiae* S288C, and *Saccharomyces boulardii* unique28 (Figure 10). Of these, 26 genes were identified as related to the probiotic characteristics of *P. kudriavzevii* GBT37, with one gene remaining unclassified. Specifically, 13 genes were linked to heat and pH tolerance. The heat tolerance genes included *GSY1, HSC82, HSP104, HSP26, HSP82,* and *SSA4,* while the general stress regulators *TPS1* and *NTH1* were involved in overall stress response. Additionally, the long-term heat stress response was associated with SSQ1. Genes related to acidic pH tolerance included the metal metabolism genes *ARN1, AFT1*, and *HAA1*, while *FET4* was identified as contributing to alkaline

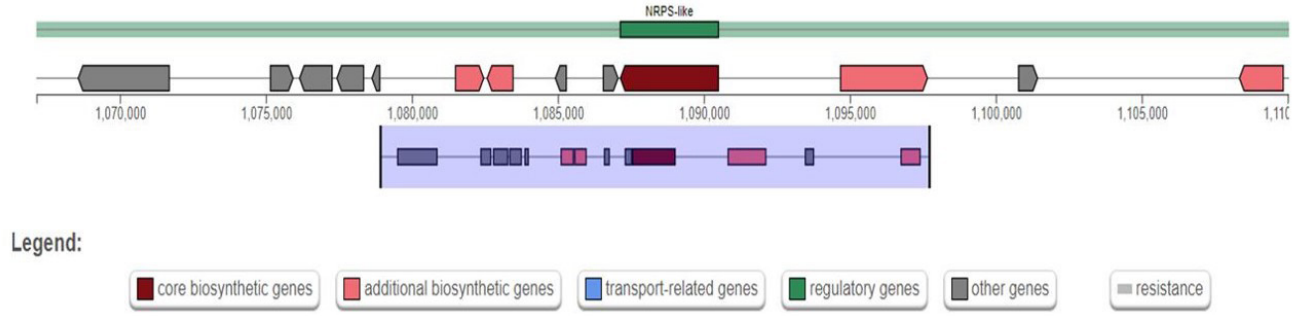


Figure 9. Schematic representation of identified secondary metabolite gene clusters in contig 7 and their components. Core biosynthetic genes and additional biosynthetic genes were produced at several locations along the contig

Table 6. Distribution of secondary metabolites genes in contig 7 according to BLAST searches

BLAST description	BLAST strain	% Identity	Accession number
L-aminoadipate-semialdehyde dehydrogenase	*Pichia kudriavzevii*	99.00	ONH77123.1
Putative secondary metabolism biosynthetic enzyme	*Candida californica*	81.00	KAG0680792.1
Trifunctional aldehyde reductase/xylose reductase	*Pichia kluyveri*	99.00	GMM46937.1
Putative hydrolase	*Pichia kluyveri*	100.00	GMM46936.1
Carboxypeptidase S	*Pichia kudriavzevii*	76.00	ONH71882.1

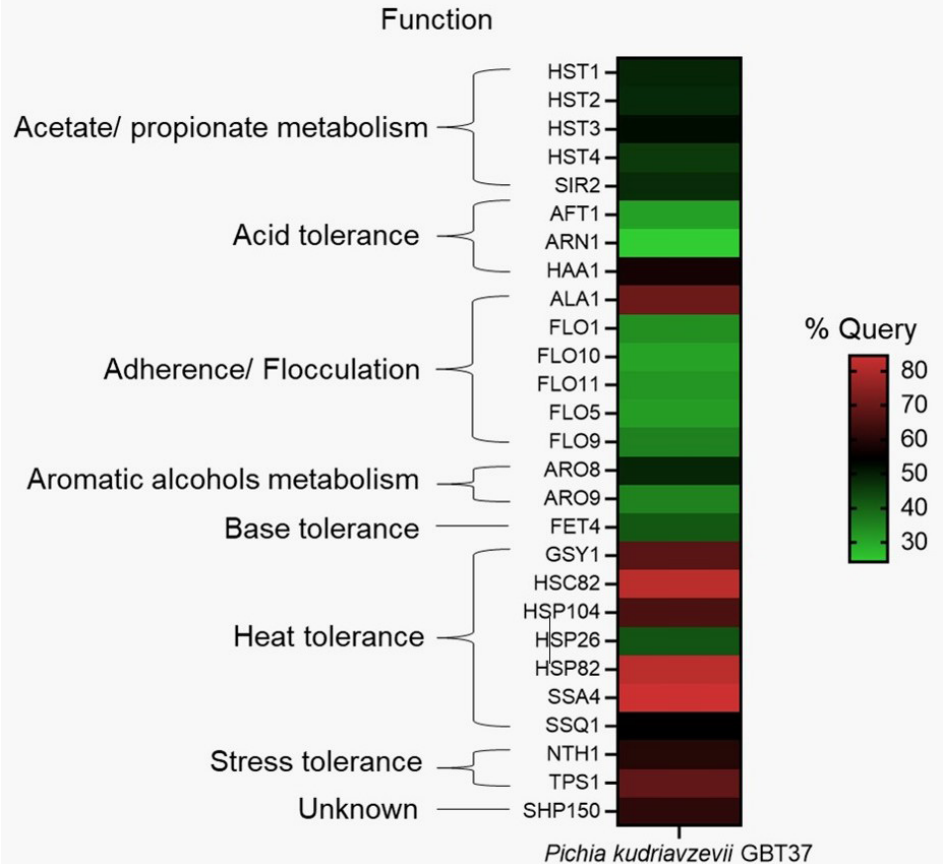


Figure 10. Percent identity of proteins associated with probiotic phenotypes. The BLASTp hits for *Pichia kudriavzevii* GBT37 and *Saccharomyces boulardii* unique28 strains are displayed. Hits with less than 80% query coverage are indicated by black-to-green color

pH tolerance (Figure 10). Six genes associated with adherence were also identified, primarily from the *FLO* family, including *FLO1, FLO10, FLO11, FLO5*, and *FLO9*, along with *ALA1*. Furthermore, seven genes involved in metabolic pathways relevant to probiotic activity were identified, consisting of *SIR2, HST1, HST2, HST3, HST4, ARO8*, and *ARO9*.

4. Discussion

Recently, probiotics have gained much popularity because of their multifaceted traits in producing natural antimicrobial agents for application in medicines, and food preservatives (Tegegne & Kebede 2022). To ensure the safety and efficacy of probiotics, it is essential to evaluate key properties, such as survival in the human digestive tract and enzymatic activities. Among probiotic yeasts, strains of *P. kudriavzevii* were studied for their positive effects on the intestinal microbiota, including mucus adhesion, immune enhancement, cholesterol reduction, and varied fermentation capabilities (Wulan *et al.* 2021). Using traditional cultivation techniques, 55 yeast colonies were isolated from *dadih* samples collected from three locations in West Sumatra namely Lintau Tanah Danah (LTD), Gadut Bukit Tinggi (GBT), and Kota Bukit Tinggi (KBT), revealing a high degree of morphological diversity.

The GBT37 (Colony-3) isolate showed an irregular colony shape, different from the other isolates, which had a circular morphology. These irregularities may indicate the presence of unique physiological or genetic traits warranting further investigation, contradicting the findings of Zha *et al.* (2023), that most *Pichia* cells are generally milky-white with a smooth surface and a bulging appearance. Colony height varied significantly, with isolates from LTD showing the greatest increase in fat, while others showing increased height. Key nutrients, including sugars, amino acids, and nitrogen compounds, are required for yeast cells to grow at distinct rates and with different developmental programs (Broach 2012). These variations can be a result of differences in nutrient uptake and metabolism which also reflect differences in growth rates (Zakhartsev & Reuss 2018) or genetic diversity among isolates (Al Halim *et al.* 2024). The uniformity of all edges and smooth surface properties across colonies is in line with some previous findings which stated that *Pichia* colonies are typically smooth with milky-white surfaces and moldings (Wulan *et al.* 2021). The potential for species variation or environmental factors to influence colony morphology has been suggested (Ezaka *et al.* 2021).

Detailed morphological insights of GBT37 and GBT30 were further visualized through SEM imaging, which revealed distinctive structural forms that may have an impact on the overall functionality and probiotic potential of the isolates. The GBT37 cells have an average size of 3.5 µm and are primarily oval Jasme *et al.* (2022) reported the shape of *P. kudriavzevii* as ovoid, and Krainer *et al.* (2012) reported its size to be in the range of 3 µm to 5 µm. This size falls within the normal range of yeast cells, indicating a population of healthy cells with active metabolism and reproductive capacity. Rough spots may be seen on the GBT37 cell surface at 11,000× magnification. This roughness may be a sign of active budding sites or cell wall modification, which would indicate rapid cell division and growth. Yeast cells are characterized by a thick cell wall, as observed in GBT37 cells, which is advantageous for withstanding challenging circumstances such as the acidic environment of the stomach (Levin 2005). It is possible that GBT37 may not produce complex extracellular structures (Honorato *et al.* 2022) under the conditions described because there is no considerable biofilm formation. The shape of GBT30 cells ranged from oval to elongated, suggesting some morphological diversity. This variation in form could be attributed to various phases of the cell cycle or to adaptive reactions to external factors (Van Rossum *et al.* 2020).

Survival in acidic environments is crucial for probiotics to exert their full potential which enable them for application in the food industry and transit through the GI tract in an optimum load. The commonly reported mechanism by which yeast cells survive at low pH is by modifying their cell walls. A mechanism that triggers the cell wall integrity pathway is observed in yeast cells that have been adapted to low pH and in the stomach, where strong inorganic acids such as HCl are present (Lucena *et al.* 2020). Fletcher *et al.* (2017) demonstrated that acid tolerance may be established by modifying the sterol composition and decreasing iron intake. This was demonstrated in *S. cerevisiae*, whose acidic tolerance reached pH 2.8. A low acidic medium pH of 2 to 6 was employed in this investigation as a preliminary indicator for any probiotic characteristics that our isolates might possess. Probiotic yeasts are generally able to withstand strong inorganic acid by adjusting their cell walls, activating cell wall integrity, and activating general stress response pathways (Kapteyn *et al.* 2001). Both isolates (GBT37 and GBT30) exhibited growth at pH 2, suggesting a significant capacity for survival in extremely acidic environments. This is an important discovery

since gastric acid can cause the pH of the stomach to decrease to as low as 1.5 to 3.5 (Menezes *et al.* 2020). The ability of these isolates to survive at such low pH levels indicates their resistance to stomach acidity, which is a crucial trait for effective probiotics. Over 24 to 48 h of incubation, both isolates displayed consistent growth rates across all pH levels tested, except for pH 5 and pH 6 in GBT30. This growth rate stability suggests that these isolates are viable for prolonged periods, which is critical for their long-lasting probiotic action. At pH 5 and pH 6, which are closer to the intestinal pH neutral zone, GBT30 growth did not decrease but instead continued at a steady rate. This adaptability across GI conditions highlights the potential of GBT30 to thrive in less acidic environments. This was similar to the pH tolerance of *P. kudraivzevii* reported by Vergara Alvarez *et al.* 2023). Hsu & Chou (2020) reported that *D. bruxellensis, P. kudraivzevii* and *S. cerevisiae*, which were isolated from kombucha, fermented vinegar and milk kefir originally from Taiwan, also showed good growth at pH 3, which was consistent with our results. The tolerance to low pH conditions was not surprising, as this level of acidity is commonly found in *dadih* (Usmiati *et al.* 2013), where yeast cells cohabited with the lactic and/or acetic acid produced by bacteria. In light of this evidence, relative to the control, it is possible to reliably estimate that at least 95% of the isolated cells will be able to survive passage through stomach acids and reach the duodenum.

Bile salts, which are amphipathic substances, play an essential role in emulsifying fats within the digestive system. Their tolerance is essential for bacterial survival in the gut, as bile salts can disrupt cell membranes and directly impact bacterial viability, particularly in the small intestine (Makzum *et al.* 2023). The optimum bile concentration for the human gut environment ranges from 0.2% to 0.6%. The small intestines contain pancreatic juice and bile released from the liver to continue digestion (Alkalbani *et al.* 2022a). Previously, Urdaneta & Casadesús (2017) reported that bile salts possess detergent properties, which can be harmful to the human gastrointestinal tract, including exerting antimicrobial effects on yeast. Therefore, resistance to bile salts is a crucial criterion for microorganisms to qualify as probiotics. Lipid droplets have been identified as enhancing resistance to bile salts by forming a protective layer (Alkalbani *et al.* 2022b). Ivashov *et al.* (2013) reported several lipid droplets in *Pichia pastoris* and related species, which may be attributed to bile salt resistance in GBT37 and GBT30. High resistance to bile salts was demonstrated by GBT30 and GBT37 at concentrations as high as 0.5%. This demonstrates their resilience and survival capacity in the small intestine, where bile salts are present in significant amounts. Throughout the study, GBT30 maintained a constant colony count across all tested bile salt concentrations. This consistency suggested that GBT30 possesses robust defense mechanisms against bile salts, effectively neutralizing their harmful effects, and ensuring steady viability and growth. This feature is especially beneficial for probiotics since it guarantees the ability of microbes to flourish in the gut despite fluctuations in bile salt levels (Sahadeva *et al.* 2011; Ruiz *et al.* 2013). The cell viability of GBT37 decreased slightly at greater bile salt concentrations, although it still showed a high tolerance to bile salts. This minor decline suggests that while GBT37 exhibits resilience, its tolerance mechanisms may be less effective than GBT30 (Sahadeva *et al.* 2011). Importantly, neither isolate showed a decline in viability with prolonged incubation periods, indicating long-term stability and further highlighting the potential of GBT30 and GBT37 as human probiotics.

Proteolytic and lipolytic activities (Table 2) were assayed to determine the ability of yeast isolates in hydrolyzing proteins and lipids, respectively, which are important for proper digestion and nutrient absorption by the host. Both isolates *P. kudraivzevii* (GBT37) and *P. occidentalis* GBT30 showed remarkable proteolytic activity, as evidenced by the formation of clear zones, which indicates the production of proteases by the isolates capable of degrading the casein (Muche *et al.* 2023). Proteolytic activity in probiotics is crucial, as protein hydrolysis is necessary to fulfill the amino acid and nitrogen requirements of microorganisms during the fermentation process (Lata *et al.* 2022). This proteolytic activity is desirable for probiotic candidates, as it suggests that these strains can aid in protein digestion and improve nutrient availability when used as probiotics. The secretion of proteases reflects the prophylactic and therapeutic potential of the probiotic strain which may protect the host from intoxication by enteric bacterial pathogens (Gut *et al.* 2019). Furthermore, both isolates showed positive lipolytic activity, showing that isolates produced lipase and can therefore hydrolyze lipids (Muche *et al.* 2023). Zheng *et al.* (2018) showed that flavor compounds are produced during cheese ripening mainly through lipolysis and fatty acid metabolism through the lipase of *P. kudraivzevii.* Screening probiotic strains primarily hinges on their nonpathogenic nature. Hemolytic activity, which can be assessed by observing the clear zone on blood agar plates, indicates the potential harm of yeast strains by lysing red blood cells. Hemolysin is produced

by several pathogenic microbes, including *Klebsiella pneumoniae* and *S. typhimurium*. The protein exoenzyme or nonprotein toxin known as hemolysin is responsible for the breakdown of hemoglobin. The microorganisms that produce hemolysin can access iron bound to hemoglobin in red blood cells (Wulan *et al.* 2021). Alpha-hemolysin causes partial damage to red blood cells, beta-hemolysin completely damages/lyses red blood cells, and gamma-hemolysis does not lyse red blood cells (negative). GBT37 and GBT30 displayed gamma hemolytic activity on blood agar plates. Gamma hemolysis indicates that these strains do not lyse red blood cells, which is a characteristic of nonpathogenic microorganisms. This finding aligns with the study performed by Menezes *et al.* (2020), who reported that yeast probiotics isolated from fermented foods do not show hemolytic activity.

GTB30 and GBT37 were checked for their antimicrobial activities against clinically-isolated pathogenic bacteria. The results showed moderate antimicrobial activity when compared to the control (ampicillin). This is in agreement with studies done by Muche *et al.* (2023) which showed moderate antibacterial activity of *P. kudriavzevii* against *S. aureus* and *E. coli*. Both isolates showed the highest inhibition activity against *Listeria* as compared to other pathogens. This is the same with studies done by Hatoum *et al.* (2012) which showed strains of *Pichia* can reduce *Listeria monocytogenes* counts by 7 log cycles. In this study, the yeast isolates GBT30 and GBT37 were evaluated for their probiotic potential, demonstrating promising in vitro characteristics. These findings suggest that both strains are viable candidates for use in the dairy industry, particularly in the development of fermented food products with potential health benefits.

Genomic analysis of novel yeast probiotic strains is highly recommended because it greatly aids in recognizing the biotechnological capabilities of new yeast strains and offers valuable information regarding probiotic functional diversity, metabolic pathways, and health-promoting mechanisms. Therefore, this study performed genome-based analyses to evaluate the safety and probiotic properties of GBT37 (*P. kudriavzevii*) isolated from *dadih*. Long-read sequencing data are available from Oxford Nanopore Technologies are useful for chromosomal structure completion, repetitive region resolution, and complex genome assembly (Amarasinghe *et al.* 2020). The genome length of GBT37 was determined as 10,906,850 bp, and the GC content as 38.26%, which is similar to other yeasts submitted in the NCBI database of *P. kudriavzevii*, which has a size in the range of 5-13 Mb, for example, *P. kudriavzevii* SD108 (12.9 Mb, 38.50%, GenBank Id: GCA_000764455.1). Genomic integrity and stability are indicated by a stable and balanced nucleotide composition (Musa *et al.* 2023), which is suggested by a consistent size of contigs across different positions in the genome (Table 6) and an average GC content (%) of 38.5% across the five identified chromosomes (Table 7). In comparison to the other chromosomes, Chr 4 has a marginally greater GC content of 39%, which could be a sign of regional differences in gene density, replication origins, or particular functional elements (Fan *et al.* 2023). Core genes refer to a set of genes that are found in the core genome of a species or a group of related species, are essential for basic cellular functions, and are conserved across different strains or isolates of the same species (Steinberg *et al.* 2022). Efficient protein synthesis is crucial for growth, reproduction, and metabolic activity, ensuring that probiotic yeast can thrive and perform its beneficial functions in the host environment (Markowiak & Ślizewska 2017). Figure 8 shows that protein synthesis dominated the core genes, which also included ribosomal proteins. Woolford & Baserga (2013) reported that the production of ribosome proteins is closely related to the growth and proliferation of cells. According to the subsystem coverage (Figure 9), 6% of the annotated genes, are linked to known biological subsystems. This represents the portion of the genome whose function can be categorized into specific biological roles. Notably, the presence of stress response genes is an important factor needed for probiotics as it allows the adaptability and survival of probiotic yeast under different conditions (Feng & Wang 2020). Circular visualization of the genome of *P. kudriavzevii* (GBT37) showed important genes such as cardiolipin synthase, pknD, rpoB, ftsH, and smc, as well as important protein-coding sequences (CDS) and GC content (Figure 10). These genes are essential for maintaining the robustness and adaptability of yeast as probiotics because they are involved in transcription, cellular signaling, mitochondrial function, protein quality control, and chromosome preservation. Comprehending these genomic characteristics offers a significant understanding of the metabolic capacities and possible health advantages of *P. kudriavzevii*, hence supporting its use in probiotics and biotechnology (Fan *et al.* 2023).

Analyses with CARD, ResFinder 4.5.0, and VirulenceFinder-2.0 showed that GBT37 does not have any antibiotic-resistance genes or pathogenicity factors. These findings confirm that the yeasts generally recognized as safe (GRAS) designation is safe. Thus, GBT37 exhibits potential as a probiotic candidate for use in biotechnology and medicine. AntiSMASH provides an

in-depth analysis of an organism's genome to pinpoint biosynthesis-related gene clusters (BGCs) in charge of producing secondary metabolites (Blin *et al.* 2019). AntiSMASH exhibited distinct biosynthetic capabilities in contig 7 and it produced NRPSs like type of secondary metabolites, which are a class of secondary metabolites that are synthesized outside of the ribosomal machinery and have a variety of properties, such as cytostatic, immunosuppressants or anticancer agents, antibiotics, and pigments. NRPSs in fungi are known to assemble peptide backbones of different secondary metabolites with biological and medicinal activities. The biosynthetic genes associated with the NRPS cluster, such as putative hydrolases, are involved in the synthesis of complex secondary metabolites, such as those involved in defense (Martínez-Núñez & López 2016).

Several probiotic phenotypes are associated with specific genotypes, including tolerance, adherence, and metabolite biosynthesis. In the gastrointestinal tract, probiotic microbes must tolerate both acidic and alkaline pH conditions, as well as temperatures of 37°C. Adherence to intestinal epithelial cells is crucial, as the natural flow of materials through the gastrointestinal tract can rapidly eliminate non-adherent microbes. Additionally, the biosynthesis of certain metabolites, such as aromatic alcohols and short-chain fatty acids, is linked to functional probiotic attributes. Acetate and propionate have been shown to reduce colon inflammation, modulate the secretory activity of the gut by interacting with the enteric nervous system, and enhance gut immunity (Koh *et al.* 2016). Aromatic alcohols such as tryptophol and phenylethanol have been demonstrated to inhibit the filamentation of *Candida albicans* (Kunyeit *et al.* 2021). Previous studies have confirmed that *P. kudriavzevii* GBT37 exhibits tolerance to pH and heat, as well as adherence to intestinal cells (Lohith and Anu-Appaiah 2018). These findings reinforce the potential of *P. kudriavzevii* GBT37 as a robust probiotic strain capable of thriving in the gastrointestinal environment and contributing to host health.

The microenvironment of the human gastrointestinal tract presents conditions that inhibit microbial growth. However, several microorganisms, including *Pichia kudriavzevii*, can survive the gut's adverse conditions by regulating genes that modulate their response to stresses such as extreme temperature, osmolarity, and the availability of essential micronutrients, including trace elements. Specifically, the GBT37 isolate has been reported to possess 26 genes associated with probiotic characteristics. We identified 13 genes involved in heat and pH tolerance (*GSY1, HSC82, HSP104, HSP26, HSP82, SSA4, TPS1, NTH1, SSQ1, ARN1, AFT1, HAA1,* and *FET4*). Large-scale studies have demonstrated the involvement of these genes in regulating general stress responses, including metal homeostasis (Yun *et al.* 2000). These findings suggest that these genes play a critical role in modulating the adaptation of food-derived yeasts to the challenging conditions of the human gastrointestinal tract.

Six genes involved in adherence were identified: *FLO1, FLO10, FLO11, FLO5*, and *FLO9*, which encode cell-wall-associated surface proteins and regulate the cell surface properties of *Saccharomyces cerevisiae* at both genetic and epigenetic levels (Halme *et al.* 2004; Hope and Dunham 2014). ALA1 was included due to its role in binding extracellular matrices (Gaur and Klotz 1997). The FLO family is of particular interest for its significant impact on probiotic characteristics. *FLO1, FLO5, FLO9*, and *FLO10* mediate cell-to-cell adhesion and adhesion to abiotic surfaces in *S. cerevisiae* (Teunissen and Steensma 1995), while *FLO1, FLO5*, and *FLO9* are critical for biofilm formation (Yang *et al.* 2018). *FLO11* plays a role in adhesion to agar and abiotic surfaces, sliding motility, filament formation, invasive growth, and substrate adhesion (Guo *et al.* 2000; Halme *et al.* 2004; Bayly *et al.* 2005). Higher expression of *FLO1, FLO5*, and *FLO11* has also been linked to enhanced thermotolerance and viability in *S. cerevisiae* (Vergara-Alvarez *et al.* 2019) (Figure 10).

Seven genes were identified to be associated with metabolic pathways essential for probiotic yeasts. Specifically, *SIR2, HST1, HST2, HST3*, and *HST4* were associated with the metabolism of acetate and propionate (Starai *et al.* 2003). Additionally, *ARO8* and *ARO9* were recognized for their roles in the biosynthesis of aromatic alcohols, including tryptophol and phenylethanol (Chen and Fink 2006) (Figure 10). The presence of these 26 genes was confirmed in *Pichia kudriavzevii* GBT37, *Saccharomyces cerevisiae* S288C, and *Saccharomyces boulardii* unique28 through BLASTP analysis. The percent identity of the top protein hits to the query sequences is illustrated in Figure 10. Interestingly, both GBT37 and *S. boulardii* unique28 exhibited query coverages exceeding 80% for *HSC82, HSP82*, and *SSA4* (Figure 10), indicating high levels of similarity in these stress response genes across the strains.

In conclusion, Probiotic yeasts involved in the fermentation process were successfully identified from *dadih* samples. The study identified 2 of the isolates GBT30 and GBT37 as *P. occidentalis* and *P. kudriavzevii*

respectively. Both isolates showed the ability to survive in acidic environments from pH 2 up to pH 6. Furthermore, GBT30 and GBT37 showed the ability to survive in a bile salt environment with high cell viability from a bile salt concentration of 0.3% and 0.5%. Another interesting feature of the isolates GBT30 and GBT37 all showed positive results for the proteolytic test showing that they can hydrolyze proteins, while for lipolytic test only GBT37 showed a positive result which means it has an ability to hydrolyze lipids. Both isolates showed a negative result for hemolytic activity, showing that they are non-pathogenic. This research holds significant implications for understanding the beneficial role of probiotic yeast in human health. Moreover, these findings can be leveraged in the commercial production of *dadih* and other fermented foods. The yeast isolates GTB30 and GBT37 demonstrated notable antibacterial activity against various pathogens. GTB37 generally showed stronger inhibition than GTB30, particularly against *Bacillus cereus* and *Staphylococcus aureus*. Both isolates exhibited significant activity against *Listeria* spp., with GTB30 being particularly effective. We also successfully reported that the results of genome analysis of *P. kudriavzevii* GBT37 produced 26 genes related to probiotic properties using the yeast genus *Saccharomyces* as its reference gene. Genetic engineering studies focused on enhancing probiotic traits, such as pH resistance, represent a promising direction for future research. Consequently, molecular investigations are crucial for advancing our understanding and application of probiotic yeasts.

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