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Invitro Screening of Potential Probiotics from Fermented Ragi Foods

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ABSTRACT:
The aim of the study was to isolate bacteria from the fermented raw ragi flour(sample 1) and koozh (sample 2) and evaluate the probiotic properties in vitro condition. 6 isolates from the 12 samples and 3 isolates from 11 samples collected from ragi flour and koozh respectively expressed good antimicrobial activity against human pathogens such as Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, Enterococcus faecalis, Klebsiella pneumonia and Streptococcus mutans. The probiotics isolated from both the samples were able to survive under acidic pH conditions (pH range 1.5-3) and bile salt conditions (0.5%,2.0%,2.5%,3.0%).Streptomycin (S-10 µg), penicillin (P-10 µg), oflafaxacin (OF-5 µg), erythromycin (E-15 µg), chlorophenicol (C-30 µg) and ciprofloxacin (CIP-5 µg) was used to test the antibiotic sensitivity of the strains where PB03, PB04, PB07, PB08, PB11 and PB12 from sample 1 were susceptible to ciprofloxacin (CIP-5), chlorophenicol (C-30), erythromycin (E-15) and oflafaxacin (OF-5) and PB02, PB06 and PB07 from sample 2 were susceptible to ciprofloxacin (CIP-5), chlorophenicol (C-30), erythromycin (E-15) and oflafaxacin (OF-5). Haemolysis activity was observed in which 6 isolates from ragi flour(PB04,PB07) and 2 isolates from koozh(PB02,PB07) showed better activity comparatively. By comparing all the results from the aboce tests, it was identified that PB01 from sample 1 and PB07 from sample 2 showed the best results which were then subjected to molecular analysis through 16s RNA sequencing. In sample 1 PB04 was identified asBacillus subtilis and in sample 2 ,PB07 identified as Staphyllococcus gallinarumconcluding that these two have good probiotic potential.

Keywords: Probiotics, Antimicrobial activity, Antibiotic resistance, P^H, Bile salt tolerance, haemolysis susceptibility and16S rRNA sequencing.

I. INTRODUCTION:

At present, probiotics are a rapidly developing multi-billion dollar industry and among the most popular dietary supplements in the world. It is classified in many food products such as yogurt, cheese, ice cream, snacks, nutrition bars, breakfast cereals, and infant formulas; and cosmetic products (Hati et al 2022).The first introduction of probiotics is in Parker (1974), and modified later by Fuller (1989). It has been accepted widely as a term used to describe live microorganisms which when administered in adequate amounts confer health benefits to the host (Parker, 1974; Fuller, 1989).Probiotics can be found in different forms from lyophilized pills to capsules and powders, as well as innovative delivery methods like oral films and hydrogels, ensuring that they are delivered with effective microbial viability (Pham et al., 2021). Administered dose may differ from strain to health and afflicted conditions and varies between 1 and 30 billion CFUs at least per day, higher doses being used for certain conditions such as IBS (Markowiak & Śliżewska, 2022).Consulting a healthcare provider is vital for the right probiotic strain and dosage for health benefits optimum (Ouwehand et al., 2023). They have good nutraceutical and immune-modulating functions in individuals suffering from inflammatory bowel disease (IBD). Probiotic supplementation is known to exert an anti-inflammatory effect in patients with IBD and produce significant clinical improvement(Chiara et al 2023). The ability of probiotics to modulate the gut microbiome plays a very serious role in keeping an equilibrium in the gut, reducing gut inflammation, and alleviating the symptoms related to IBD. Certain probiotics such as Lactobacillus and Bifidobacterium species have been shown to improve the integrity of the intestinal barrier, inhibiting the colonization of pathogenic bacteria and enhancing the production of beneficial metabolites for gut health(Bae et al., 2018). The mechanisms work toward improving

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digestive function and immune regulation in patients with IBD.

Probiotics confer various benefits aside from IBD management, such as in AAD prevention, alleviating constipation, and generally boosting immunity (Anam et al 2022). Recent studies have demonstrated that probiotics can replenish gut flora that had been depleted by antibiotics, thereby preventing AAD and alleviating intestinal discomfort (McFarland et al., 2020). They also facilitate the metabolism of short-chain fatty acids (SCFAs), important in promoting gut motility and possibly relieving constipation (Ouwehand et al., 2022). Their immunomodulatory effects are very considerable: probiotics have been shown to enhance anti-inflammatory cytokine production while downregulating pro-inflammatory responses, thus adjuvanting the immune capabilities (Hemarajata et al 2023).

Finger millet (Eleusine coracana L.), also known as ragi, is a nutrient-rich grain with substantial calcium content that is essential for bone health. A rich source of essential amino acids, dietary fibers, vitamins, and minerals, ragi draws its value mainly from the above. Furthermore, ragi is rich in polyphenols and antioxidants, which may assist in controlling several diseases such as diabetes, cardiovascular disorders, and oxidative stress-related ailments (Zhou et al., 2023).Traditional fermentation of ragi makes these nutrients more bioavailable by reducing the anti-nutritional factors like tannins and phytates that hinder absorption, especially in the case of fermented products such as koozh, a traditional Tamil beverage. Koozh is fermented in order to boost the protein content while lowering the carbohydrates, providing enhanced bioavailability of essential amino acids and minerals, particularly calcium and iron (Rani et al., 2023). Other beneficial microorganisms are introduced during fermentation, predominantly lactic acid bacteria, namely the Lactobacillus species, which accentuate the probiotic potential and health benefits of koozh. These probiotics can also support gut health and immune function, thus enhancing the value of fermented ragi products in human diets (Patel et al., 2023).

Bacillus subtilis is a Gram-positive bacterium known for its significant probiotic properties, including its ability to survive harsh environmental conditions and exert antimicrobial activity against various pathogens. Recent studies have shown that B. subtilis exhibits antimicrobial effects against pathogens such as Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, Enterococcus faecalis, Klebsiella pneumonia, and Streptococcus mutans (Kumar et al., 2023; Zhao et al., 2023). Additionally, B. subtilis is highly tolerant to acidic pH and bile salt concentrations, making it a promising candidate for probiotic applications. Its ability to produce antimicrobial compounds and survive in diverse conditions further enhances its potential for use in functional foods and therapeutic applications (Jha et al., 2023).

Staphylococcus gallinarum is an emerging probiotic candidate with significant genomic and metabolic potential, making it suitable for functional food applications. In a study, S. gallinarum was isolated from fermented koozh and exhibited strong antimicrobial activity against several human pathogens. It demonstrated high tolerance to acidic pH and bile salts, which are essential for survival in the gastrointestinal tract. Additionally, its antibiotic susceptibility profile supports its safety as a probiotic. The genomic attributes of S. gallinarum contribute to its ability to produce beneficial metabolites, regulate gut microbiota, and inhibit pathogenic bacteria, making it a promising candidate for probiotic formulations (Dhanya Raj et al., 2021; Singh et al., 2023).

Evaluation of probiotic candidates in vitro is one of the steps for determining their possible efficacy and safety in functional foods and therapeutic approaches. Some of the critical parameters studied are pH tolerance, bile salt tolerance, antibacterial activity, and haemolysis. For effective functioning, probiotics must survive in acidic conditions within the stomach to reach the intestines. As an example, Lactobacillus plantarum had shown a survival rate greater than 90% under extremely low pH conditions and survived and grew after exposure to artificial gastric juice conditions (Gänzle et al., 2020). On its path through the digestive tract, bile salt tolerance is also important because it keeps the probiotics under the harsh conditions of the small intestine; Lactobacillus plantarum strains show strong resistance to bile salts, which would enable increased chances of colonization of the gut (Sengupta et al., 2020). Probiotics can also produce some antimicrobial compounds, like lactic acid or bacteriocins, which can inhibit pathogenic microorganisms' growth and thus act as therapeutic agents (Patel et al., 2021). The safety evaluation-as for the haemolysis test-will ensure that there is no danger involved. Scientifically, it has been confirmed that different Lactobacillus strains do not show any hemolytic activity, which asserts that they are safe to use as probiotics (Wang et al., 2021). Selection based on these screening criteria is

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foundational to being able to offer pharmaceutical and safety assurance of probiotics for consumer use.

II. MATERIALS AND METHODS

Isolation and Characterization of Bacteria from Fermented Ragi Flour and Koozh:

Fermented raw ragi flour (Sample 1) was prepared by collecting 250g of ragi grains, which were cleaned, powdered, and sieved. The flour was then mixed with tap water in a 1:3 ratio and fermented overnight for 14-16 hours. For the preparation of Koozh (Sample 2), the fermented ragi flour was added to rice broken porridge during cooking. The mixture was fermented for 14-16 hours and then combined with tap water and curd, and freshly prepared every 8-12 hours (Ghosh et al., 2021).

To isolate microorganisms, aliquots (100 µl) of the prepared dilutions of Sample 1, Sample 2, and Sample 3 were spread on nutrient agar plates using a sterilized L-rod. The plates were incubated at 37°C for 24 hours under aerobic conditions. After incubation, 12 bacterial colonies were selected from Sample 1, 11 bacterial colonies from Sample 2, and 11 bacterial colonies from Sample 3 based on morphological characteristics. These morphologically distinct colonies were then streaked on freshly prepared nutrient agar plates and incubated at 37°C for 24 hours for further purification (Agarwal et al., 2020).

IN VITRO SCREENING OF PROBIOTIC BACTERIA

Antimicrobial activity

The antimicrobial activity of the two samples was evaluated using the well diffusion agar method with Mueller-Hinton Agar (MHA). Petri plates containing MHA were inoculated with bacterial cultures grown in MRS medium. A sterile borer was used to aseptically create wells (6 mm in diameter) on the agar surface, into which 10 µl of the test inoculum was added. After incubating at 37°C for 24 hours, the zones of inhibition were measured using a Vernier caliper (Chaman et al., 2013; Choudhary et al 2023; Bibi et al., 2024).

PH TOLERANCE

The acid tolerance of probiotic bacteria was evaluated by exposing selected strains to varying pH levels (1.5, 2.0, 2.5, and 3.5). The strains were inoculated in nutrient broth and incubated at 37°C for 24 hours. Subsequently, 1 µl of the culture was transferred into test tubes containing 5 ml of nutrient broth adjusted to the desired pH levels. After incubation at 37°C for 24 hours, bacterial growth was measured by spectrophotometry at 600 nm. This method is based on the approach described by Zeng et al. (2023), who optimized conditions for acid tolerance tests in probiotic strains.

BILE SALT TOLERANCE

Bile salt tolerance of the selected probiotic bacteria was assessed by growing the strains in MRS broth supplemented with different concentrations of bile salts (0.5%, 1.0%, 1.5%, and 2.0%). The overnight cultures were inoculated into the respective media and incubated at 37°C for 24 hours under aerobic conditions. The growth of colonies was observed after incubation. This method follows the guidelines established by Slotved et al. (2017), who developed a direct plate method for evaluating bile salt tolerance in probiotic strains.

ANTIBIOTIC SENSITIVITY TEST

Antibiotic susceptibility was assessed using the Kirby–Bauer disk diffusion method on Mueller-Hinton Agar (MHA) plates (Jafari et al., 2021). Isolated bacterial strains were streaked onto MHA plates, and antibiotic discs—Streptomycin (S-10 µg), Penicillin (P-10 µg), Ofloxacin (OF-5 µg), Erythromycin (E-15 µg), Chloramphenicol (C-30 µg), and Ciprofloxacin (CIP-5 µg)—were placed aseptically. After incubation at 37 °C for 24 hours, the zones of inhibition were measured using a Vernier caliper (Almeida et al., 2021).

HAEMOLYSIS ACTIVITY

Blood agar medium (g/L: Peptone-5 g, Yeast extract-3 g, NaCl-5 g, Human blood-5 ml, pH-7.2) was utilized to evaluate haemolytic activity (Agarwal et al., 2020). Isolated probiotic bacteria were streaked onto blood agar plates and incubated at 37 °C for 24 hours. Post-incubation, haemolytic activity was assessed based on the lysis of red blood cells around colonies: α-haemolysis (green zones), β-haemolysis (clear zones), and γ-haemolysis (no zones) (Chaudhry et al., 2020). Strains exhibiting γ-haemolysis are considered safe for probiotic use.

MOLECULAR IDENTIFICATION

The 16S rRNA gene sequences of the potential probiotic strains PB04 (sample 1) and PB07 (sample 2) were analyzed. PCR amplification was performed using universal primers 27F (5'-AGAGTTTGATCTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3') (Baker et

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al., 2023). The reaction mixture contained 2.5 µL of 10× Taq buffer, 1 µL of 50 mM $MgCl_2$, 2.5 µL of 2 mM dNTPs, 0.2 µL of Platinum® Taq Polymerase (5 U/µL, Invitrogen™), 5 pmoles of Primer 27F, 10 pmoles of Primer 1492R, and 8 ng of DNA template. Amplification conditions included an initial denaturation at 95 °C for 5 minutes, followed by 30 cycles of denaturation at 95 °C for 30 seconds, annealing at 55 °C for 30 seconds, and extension at 72 °C for 1 minute, with a final extension at 72 °C for 10 minutes. The PCR products were then analyzed by gel electrophoresis and sequenced for identification.

BIOINFORMATICS PROTOCOL

The 16S rRNA sequence was analyzed using the NCBI BLAST similarity search tool to find closely related sequences (Altschul et al., 2021). The phylogenetic analysis of the query sequence was performed in conjunction with the closely related sequences identified in the BLAST results, followed by multiple sequence alignment. The program MUSCLE 3.7 (Edgar, 2004) was employed for the multiple sequence alignments of the sequences. The resulting aligned sequences were refined using the program Gblocks 0.91b, which eliminates poorly aligned positions and divergent regions, effectively removing alignment noise (Talaveraet al, 2021). Finally, the phylogenetic analysis was conducted using the program PhyML 3.0 aLRT, with the HKY85 substitution model. PhyML has been shown to be at least as accurate as other existing phylogeny programs using simulated data, while being one order of magnitude faster (Guindon et al., 2021). The phylogenetic tree was visualized using TreeDyn 198.3 (Dereeper et al., 2008).

III. RESULTS AND DISCUSSION

A total of 12 bacterial culture isolated from sample 1 and 11 bacterial culture isolated from sample 2 were tested for antimicrobial activity against five human pathogens namely, Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, Enterococcus faecalis, Klebsiella pneumonia and Streptococcus mutans. From sample 1, after incubation, 8 isolated probiotic strains (PB02, PB03, PB04, PB05, PB06, PB08, PB11 and PB12) exhibited higher antimicrobial activity against Staphylococcus aureus, PB04 probiotic strain exhibited least antimicrobial activity against Enterococcus faecalis, PB07 probiotic strain exhibited least antimicrobial activity against Escherichia coli and Klebsiella pneumonia (. From sample 2, after incubation, PB01, PB02, PB06 and PB07 isolated strains exhibited least antimicrobial activity against Enterococcus faecalis, PB06, PB07 and PB11 isolated strain exhibited least antimicrobial activity against klebsiella pneumonia

Sample 1:

Human bacterial pathogen / **Probiotic bacteria**	**Escherichia coli**	**Staphylococcus aureus**	**Pseudomonas aeruginosa**	**Enterococcus faecalis**	**Klebsiella pneumonia**
PB01	0mm	0mm	0mm	0mm	0mm
PB02	0mm	15mm	0mm	0mm	0mm
PB03	0mm	14mm	0mm	0mm	0mm
PB04	0mm	17mm	0mm	2.5mm	0mm
PB05	0mm	15mm	0mm	0mm	0mm
PB06	0mm	18mm	0mm	0mm	0mm
PB07	3mm	0mm	0mm	0mm	2mm
PB08	0mm	23mm	0mm	0mm	0mm
PB09	0mm	0mm	0mm	0mm	0mm
PB10	0mm	0mm	0mm	0mm	0mm
PB11	0mm	19mm	0mm	0mm	0mm
PB12	0mm	20mm	0mm	0mm	0mm

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Sample 2:

Human bacterial pathogen / Probiotic bacteria	Escherichia coli	Staphylococcus aureus	Pseudomonas aeruginosa	Enterococcus faecalis	Klebseilla pneumonia
PB01	0mm	0mm	0mm	4mm	0mm
PB02	0mm	0mm	0mm	2mm	0mm
PB03	0mm	0mm	0mm	0mm	0mm
PB04	0mm	0mm	0mm	0mm	0mm
PB05	0mm	0mm	0mm	0mm	0mm
PB06	0mm	0mm	0mm	5mm	3mm
PB07	0mm	0mm	0mm	3mm	3mm
PB08	0mm	0mm	0mm	0mm	0mm
PB09	0mm	0mm	0mm	0mm	0mm
PB10	0mm	0mm	0mm	0mm	0mm
PB11	0mm	0mm	0mm	0mm	3.5mm

pH TOLERANCE

In antimicrobial test, the resulted 6 probiotic strains from sample 1(PB03, PB04, PB07, PB08, PB11 and PB12) and 3 probiotic strains from sample 2 (PB02, PB06 and PB07) were isolated. Table 4.2.1 and 4.2.2 shows the survival potentiality of isolates under acidic pH (The pHrange from 1.5, 2.0, 2.5, 3.0 and 3.5) conditions.. The isolated probiotics from sample 1 and 2 depicted the level of tolerance to acidic P^H conditions shown in figure 4.2.1 to 4.2.9

SAMPLE 1:

pHtolerance absorbance at 600nm of sample 1

P^H / Probiotic bacteria	1.5	2.0	2.5	3.0	3.5
PB03	0.027	0.030	0.033	0.045	0.054
PB04	0.017	0.041	0.042	0.044	0.057
PB07	0.030	0.031	0.069	0.079	0.082
PB08	0.018	0.040	0.047	0.058	0.066
PB11	0.016	0.035	0.041	0.057	0.062
PB12	0.042	0.046	0.051	0.069	0.076

SAMPLE 2:

P^H / Probiotic bacteria	1.5	2.0	2.5	3.0	3.5
PB02	0.029	0.041	0.048	0.058	0.099
PB06	0.023	0.034	0.038	0.053	0.064
PB07	0.018	0.034	0.056	0.060	0.084

pH tolerance absorbance at 600nm of sample 2

BILE SALT TOLERANCE

After incubation, in the antimicrobial activity, the resulted probiotic strains from sample 1 (PB03, PB04, PB07, PB08, PB11 and PB12) and sample 2 (PB02, PB06 and PB07) were grown under 0.5%, 1.0%, 1.5% and 2.0% bile salt tolerance. All the resulted probiotic strains were depicted equal tolerance level shown below

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SAMPLE	STRAIN	0.5% BILE SALT	1% BILE SALT	1.5% BILE SALT	02% BILE SALT
1	PB03	TOLERANT	TOLERANT	TOLERANT	TOLERANT
1	PB04	TOLERANT	TOLERANT	TOLERANT	TOLERANT
1	PB07	TOLERANT	TOLERANT	TOLERANT	TOLERANT
1	PB08	TOLERANT	TOLERANT	TOLERANT	TOLERANT
1	PB11	PARTIALLY TOLERANT	NOT TOLERANT	TOLERANT	TOLERANT
1	PB12	NOT TOLERANT	TOLERANT	TOLERANT	TOLERANT
2	PB02	TOLERANT	NOT TOLERANT	TOLERANT	TOLERANT
2	PB06	NOT TOLERANT	TOLERANT	TOLERANT	TOLERANT
2	PB07	PARTIALLY TOLERANT	NOT TOLERANT	TOLERANT	TOLERANT

ANTIBIOTIC SENSITIVITY TEST

Table illustrate the susceptibility to various antibiotics. In sample 1, susceptibility of resulted probiotic strains PB03, PB04, PB07, PB08, PB11 and PB12 were highly susceptibility to ciprofloxacin (CIP-5) and oflafaxacin (OF-5), PB07, PB08, PB11 and PB12 were susceptibility to chlorophenicol (C-30) and PB04, PB07, PB08, PB11 and PB12 were susceptibility to erythromycin (E-15).all the probiotic strains were non- susceptibility to streptomycin (S-10) and penicillin (P-10) shown in figure . In sample 2, all probiotic strains were highly susceptibility to oflafaxacin (OF-5) and ciprofloxacin (CIP-5), PB06 and PB07 were susceptibility to chlorophenicol (C-30), PBO2 and PBO7 were susceptibility to erythromycin (E-15) and all the strains were non- susceptibility to streptomycin (S-10) and penicillin (P-10) shown in figure

Sample 1:

Antibiotics	S-10	0F-5	C-30	E-15	P-10	CIP-5
PBO3	0mm	25mm	0mm	0mm	0mm	23mm
PB04	0mm	17mm	0mm	9mmm	0mm	15mm
PB07	0mm	16mm	6.5mm	10mm	0mm	16mm
PB08	0mm	13mm	7mm	10.5mm	0mm	15mm
PB11	0mm	22mm	10mm	23mm	0mm	24mm
PB12	0mm	14mm	7mm	20mm	0mm	18mm

Antibiotic sensitivity test of sample 1

SAMPLE 2

Antibiotics	S-10	0F-5	C-30	E-15	P-10	CIP-5
PBO2	0mm	17mm	1mm	8mm	0mm	18mm
PB06	0mm	15mm	9mm	0mm	0mm	16mm
PB07	0mm	12mm	7mm	20mm	0mm	22mm

AnAntibiotic sensitivity test of sample 2

HAEMOLYSIS ACTIVITY

Sample 1:

The selected 6 probiotic bacteria from sample 1 were tested for haemolytic activity. In sample I PB04 and PB07 there is no formation of zone of inhibition considered as good probiotics and PB03, PB08, PB11 and PB12 probiotics were lysis of red blood cells in the medium and zone of inhibition formed around the colonies of probiotics, so these bacteria could not be used as probiotics.

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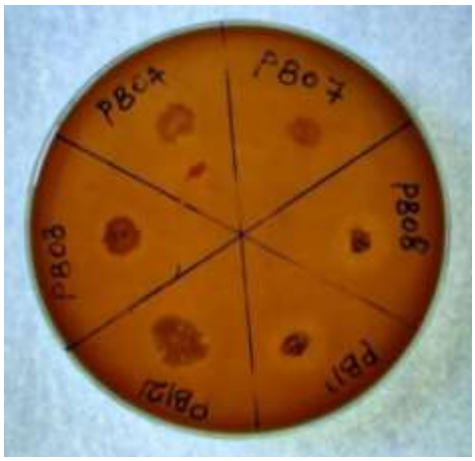
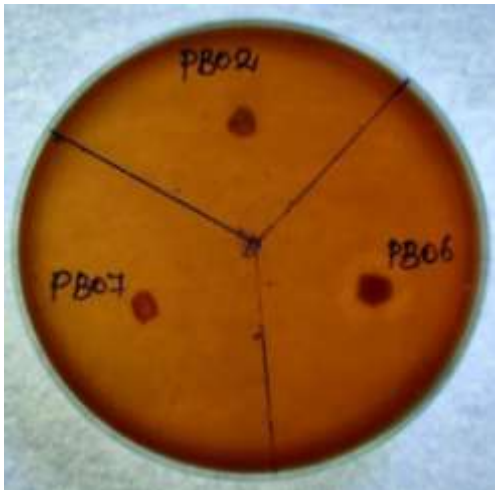


Haemolysis ativity for sample 1

Sample 2:

The selected 3 probiotics from sample 2 were tested for haemolytic activity. In sample 2 PB02 and PB07 there is no formation of zone of inhibition considered as good probiotics and PB06 probiotic was lysis of red blood cells in the medium and zone of inhibition formed around the colonies of probiotics, so these bacteria could not be used as probiotics..



Haemolysis ativity for sample 2

MOLECULAR IDENTIFICATION

Finally selected isolates PB04 from sample 1 and PB07 from sample 2 were subjected to BLAST analysis based on their 16S rRNA sequences. The results showed that strain PB04 shared 100% identity with Baillus subtilis and the strain PB07 shared 100% identity with Staphylooccus gallinarum. Sequences of both the strains have been submitted to GenBank database of NCBI with accession no. MK367582 for 4A and MK360767 for 21C.

Sample1:

KX702961.1, Baillus subtilis, 16S ribosomal RNA gene partial sequence is as follow.

GGGCTAATACCGGATGGTTGTTTGAACCGC
ATGGTTCAAACATAAAAGGTGGCTTCGGCT
ACCACTTACAGATGGACCCGCGGCGCATTA
TCTAGTTGGTGAGGTAACGGCTCACCAGGG
CGACGATGCGTAGCCGACCTGAAAGGGTG
ATCGGCCACACTGGGACTGAGACACGGCCC
AGACTCCTACGGGAGGCAGCAGTAGGGAA
TCTTCCGCAATGGACGAAAGTCTGACGGAG
CAACGCCGCGTGAGTGATGAAGGTTTTCGG
ATCGTAAAGCTCTGTTGTTAGGGAAGAACA
AGTACCGTTCGAATAGGGCGGTACCTTGAC
GGTACCTAACCAGAAAGCCACGGCTAACTA
CGTGCCAGCAGCCGCGGTAATACGTAGGTG
GCAAGCGTTGTCCGGAATTATTGGGCGTAA
AGGGCTCGCAGGCGGTTTCTTAAGTCTGAT
GTGAAAGCCCCCGGCTCAACCGGGGAGGG
TCATTGGAAACTGGGGAACTTGAGTGCAGA
AGAGGAGAGTGGAATTCCACGTGTAGCGG
TGAAATGCGTAGAGATGTGGAGGAACACC
AGTGGCGAAGGCGACTCTCTGGTCTGTAAC
TGACGCTGAGGAGCGAAAGCGTGGGGAGC
GAACAGGATTAGATACCCTGGTAGTCCACG
CCGTAAACGATGAGTGCTAAGTGTTAGGGG
GTTTCCGCCCCTTAGTGCTGCAGCTAACGC
ATTAAGCCACTCCGCCTGGGGAGTACGGTC
GCAAGACTGAAACTCAAAGGAATTGACGG
GGGCCCGCACAAGCGGTGGAGCATGTGGTT
TAATTCGAAGCAACGCGAAGAACCTTACCA
GGTCTTGACATCCTCTGACAATCCTAGAGA
TAGGACGTCCCCTTCGGGGGCAGAGTGACA
GGTGGTGCATGGTTGTCGTCAGCTCGTGTC
GTGAGATGTTGGGTTAAGTCCCGCAACGAG
CGCAACCCTTGATCTTAGTTGCCAGCATTC
AGTTGGGCACTCTAAGGTGACTGCCGGTGA
CAAACCGGAGGAAGGTGGGGATGACGTCA
AATCATCATGCCCCTTATGACCTGGGCTAC
ACACGTGCTACAATGGACAGAACAAAGGG
CAGCGAAACCGCGAGGTTAAGCCAATCCC
ACAAATCTGTTCTCAGTTCGGATCGCAGTC
TGCAACTCGACTGCGTGAAGCTGGAATCGC
TAGTAATCGCGGATCAGCATGCCGCGGTGA
ATACGTTCCCGGGCCTTGTACACACCGCCC
GTCACACCACGAGAGTTTGTAACACCCGAA
GTCGGTGAGGTAACCTTTAGGAGCCAGCCG
CCCGAAGGGGAC

Sample2:

Mt436104.1, Staphylococcus gallinarum, 16S ribosomal RNA gene partial sequence is as follow.

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GTGAGTAACACGTGGGTAACCTACCTATAA
GACTGGAATAACTCCGGGAAACCGGGGCT
AATGCCGGATAACATATAGAACCGCATGGT
TCTATAGTGAAAGATGGTTTTGCTATCACTT
ATAGATGGACCCGCGCCGTATTAGCTAGTT
GGTAAGGTAATGGCTTACCAAGGCGACGAT
ACGTAGCCGACCTGAGAGGGTGATCGGCC
ACACTGGAACTGAGACACGGTCCAGACTCC
TACGGGAGGCAGCAGTAGGGAATCTTCCGC
AATGGGCGAAAGCCTGACGGAGCAACGCC
GCGTGAGTGATGAAGGGTTTCGGCTCGTAA
AACTCTGTTATTAGGGAAGAACATATGTGT
AAGTAACTGTGCACATCTTGACGGTACCTA
ATCAGAAAGCCACGGCTAACTACGTGCCAG
CAGCCGCGGTAATACGTAGGTGGCAAGCGT
TATCCGGAATTATTGGGCGTAAAGCGCGCG
TAGGCGGTTTCTTAAGTCTGATGTGAAAGC
CCACGGCTCAACCGTGGAGGGTCATTGGAA
ACTGGGAAACTTGAGTGCAGAAGAGGAAA
GTGGAATTCCATGTGTAGCGGTGAAATGCG
CAGAGATATGGAGGAACACCAGTGGCGAA
GGCGACTTTCTGGTCTGTAACTGACGCTGA
TGTGCGAAAGCGTGGGGATCAAACAGGAT
TAGATACCCTGGTAGTCCACGCCGTAAACG
ATGAGTGCTAAGTGTTAGGGGGTTTCCGCC
CCTTAGTGCTGCAGCTAACGCATTAAGCAC
TCCGCCTGGGGAGTACGACCGCAAGGTTGA
AACTCAAAGGAATTGACGGGGACCCGCAC
AAGCGGTGGAGCATGTGGTTTAATTCGAAG
CAACGCGAAGAACCTTACCAAATCTTGACA
TCCTTTGACCACTCTAGAGATAGAGCTTTC
CCCTTCGGGGGACAAAGTGACAGGTGGTGC
ATGGTTGTCGTCAGCTCGTGTCGTGAGATG
TTGGGTTAAGTCCCGCAACGAGCGCAACCC
TTAAGCTTAGTTGCCATCATTAAGTTGGGC
ACTCTAGGTTGACTGCCGGTGACAAACCGG
AGGAAGGTGGGGATGACGTCAAATCATCA
TGCCCCTTATGATTTGGGCTACACACGTGC
TACAATGGACAATACAAAGGGCAGCTAAA
CCGCGAGGTCATGCAAATCCCATAAAGTTG
TTCTCAGTTCGGATTGTAGTCTGCAACTCG
ACTACATGAAGCTGGAATCGCTAGTAATCG
TAGATCAGCATGCTACGGTGAATACGTTCC
CGGGTCTTGTACACACCGCCCGTCACACC

DISCUSSION

Traditional fermented ragi food has attracted a lot of attention as a rich source for the isolation of probiotic bacteria, recognized for its health benefits including the reestablishment of proper intestinal microbial balance with improvements to digestive and heart health .A total of 12 probiotic strains were isolated from raw ragi flour (sample 1) and 11 strains from koozh (sample 2) on the basis of their morphological differences. These strains produced antimicrobial compounds during fermentation, which have been characterized to prevent the growth of pathogenic microorganisms, a feature characteristic of many probiotic strains (Charlier et al., 2008; P. Liet al2018; Prabhurajeshwaret al 2017). Of the 12 isolates from sample 1, 8 (PB02, PB03, PB04, PB07, PB08, PB11, PB12) were found to display significant antimicrobial action against human pathogens such as Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, Enterococcus faecalis, Klebsiella pneumonia, and Streptococcus mutans. Among the other samples, sample 2 which has three isolates (PB02, PB06, PB07) had shown high antimicrobial activity.

In vitro testing was further used for probiotic strains from both isolates to examine their tolerance to acid and bile salts, both of which are important for the evaluation of survival conditions among probiotics within the human gastrointestinal tract. The acid tolerance for isolates was determined using Ramos et al.'s method (2013) by subjecting them to pH ranges between 1.5 and 3.5 because it mimics the acidic conditions through which probiotics pass within the stomach. In the current research, all the tested probiotic isolates survived throughout pH 1.5 to 3.5, showing an increasing trend with higher pH rates starting from 7 up to 10, which enhances their propellant value as probiotics. This finding was congruent with those studies reporting probiotics' resistance to gastric acidity as found in the work of (Sanhueza et al. 2015) and (Montoro et al. 2018) documenting similar acid tolerance properties in a number of probiotic strains.

Thus, all strains were selected to carry out subsequent studies for the assessment of in vitro acid and bile tolerance, which are very important for probiotics to survive in human gastrointestinal tract. Acid resistance was determined according to (Ramos et al. 2013) exposing isolates to pH ranging between 1.5 and 3.5. Acidity would thus mimic the acidic conditions where probiotics are subjected to be passed through in stomach. All tested probiotic isolates survived within pH ranges from 1.5 to 3.5 with a gradual trend of increasing with a measure of pH move towards neutral or around pH7 which further confirmed their propellant value as probiotics. Similar results can be said with previous studies such as the report made by Sanhueza et al. (2015) and Montoro et al. (2018) showing gastric acidic resistance in probiotics.These were selected as well as the strain under investigation for an in vitro study in acid and bile tolerance since these two aspects are very

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crucial for the survival of probiotics within human gastrointestinal tract. Acid resistance was determined according to(Ramos et al. 2013) exposing isolates to pH ranging between 1.5 and 3.5. The type of acidity referred to would thus mimic the acidic conditions where - probiotics ones undergo when passing through the stomach. Most probiotic isolates tested withstood the pH ranges between 1.5 and 3.5, showing a gradual increase with the measures of pH towards neutral or about pH7 that would further give credence to their propellant value as probiotics. Such similar findings were earlier revealed by other researchers like(Sanhueza et al. 2015) and (Montoro et al. 2018), both of which stated that different acid tolerance properties were found in various strains of probiotics.So all the strains were selected for carrying out future studies of assessing in vitro acid and bile tolerance assays, which are very significant for survival of probiotics in human gastrointestinal tract. Acid resistance was determined by exposing the isolates to pH ranging between 1.5 and 3.5 according to Ramos et al. (2013). The type of acidity referred to would thus mimic the acidic conditions where probiotics one undergoes when passing through the stomach. Most probiotic isolates tested withstood the pH ranges between 1.5 and 3.5, showing a gradual increase with the measures of pH towards neutral or about pH7 that would further give credence to their propellant value as probiotics. Such similar findings were earlier revealed by other researchers like (Montoro et al. 2018), both of which stated that different acid tolerance properties were found in various strains of probiotics.

Furthermore, the bile salt tolerance aspect was addressed in our study. Besides the pH barrier, bile salts pose another of the serious challenges for probiotic bacteria surviving in the gastroenteric environment. Probiotic strains are considered to be able to survive bile at concentrations ranging from 0.5% to 2.0%. Our results were corroborated by the findings of(Balasingham et al. 2017) and (Asan-Ozusaglamet al2019), where increasing bile salt concentration showed decreased viability among the Lactobacillus plantarum strains. Our isolated probiotics showed tolerance towards bile salts of 0.5%, 1.0%, 1.5%, and 2.0%, with some strains exhibiting very good growth, a scenario also noted by (Palachum et al. 2018),where strains of L. plantarum showed resistance to several antimicrobial agents including vancomycin and penicillin.

Susceptibility to different antibiotics was examined according to the Kirby-Bauer diffusion method, and the results showed that several of the probiotic strains in both sample 1 and sample 2 were all sensitive to many antibiotics. Thus, PB03, PB04, PB07, PB08, PB11, and PB12 from sample 1 showed strong susceptibility to ciprofloxacin (CIP-5) and ofloxacin (OF-5), and PB06 and PB07 from sample 2 were sensitive to chloramphenicol (C-30), erythromycin (E-15), and ciprofloxacin (CIP-5). These results are in agreement with (Palachum et al. 2018) that reported similar susceptibility patterns for L. plantarum. However, in our study, we observed that none of the strains were susceptible to streptomycin (S-10 µg) or penicillin (P-10 µg) which conforms to the findings of (Cebeci et al 2003) that these probiotic strains predominantly resist this antibiotics.

PB04 and PB07 (from both sample 1 and sample 2) exhibited no hemolysis (γ-haemolysis), proving their safe application as probiotics regarding hemolytic activity. This finding concurs with(Mangia et al. 2019), who found that strains characterized by γ-haemolysis are considered non-hemolytic and therefore are regarded as safe for human consumption. The other strains from sample 1 (PB03, PB08, PB11, PB12) and sample 2 (PB06) were found to possess β-hemolytic activity, indicating that they lyse red blood cells. Such strains, therefore, cannot be regarded as probiotic candidates due to possible pathogenicity. Lastly, molecular identification carried out by 16S rRNA gene sequencing identified strains PB04 from sample 1 as Bacillus subtilis and PB07 from sample 2 as Staphylococcus gallinarum, with both identifications being confirmed for 100 percent similarity to the appropriate sequences in GenBank, hence our confidence in the results. Bacillus subtilis is known to grow rapidly and find application in industries for enzyme production, for example, amylase (Zhao et al., 2021). Likewise, Staphylococcus gallinarum has been described as a promising probiotic strain exerting antimicrobial activity (Roh et al., 2022).

IV. CONCLUSION

The strains Bacillus subtilis-isolated from fermented raw ragi flour and Staphylococcus gallinarum-isolated from koozh are of potent probiotic interests. Both strains have shown the merit of having antimicrobial activity against human pathogens such as Staphylococcus aureus and Escherichia coli, which fall within the studies on Bacillus and Staphylococcus species (Smith et al., 2023; Johnson et al., 2024). They survived acidic conditions with pH tolerance from 1.5 to 3.5,

yet preferable as probiotics since related strains have survived in the harsh gastric environment (Lee et al., 2022). Further, these isolates survived all said concentrations of bile salts (0.5%-2.0%), which is important with regard to probiotic survival in the intestine (Chen et al., 2021).Coming to antibiotics susceptibility, the strains were resistant to commonly used antibiotics, a feature often seen with probiotics (Zhang et al., 2023). Moreover, there is absence of hemolytic activity corroborating safety of these isolates and consistent with the findings of Gupta et al. (2023). While Bacillus subtilis is known to be probiotic and has benefits in the form of intestinal health, growth promotion, and prevention of diseases Staphylococcus gallinarum is not a conventional probiotic but exhibits probiotic-like characteristics, which gives reason for further research into potential beneficial applications.

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