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Microbial diversity and probiotic potential of fermented traditional rice varieties: Characterization and functional evaluation

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ABSTRACT

This study focuses on the characterization of probiotic diversity derived from two fermented traditional rice varieties, Kattuyanam and Mappillai Samba. The rice samples were processed, lyophilized, and subjected to microbiological analysis. Molecular identification was performed using metagenomics analysis. Probiotic efficacy was evaluated through acid and bile salt tolerance tests, antibiotic susceptibility assays (agar well diffusion), glucose fermentation, and adhesion assays on stainless steel plates. Metagenomic analysis confirmed the dominance of the Firmicutes phylum in both samples, with Lactobacillaceae as the most prevalent family. Antibiotic susceptibility tests revealed varying resistance patterns, with notable resistance to penicillin, streptomycin, and ampicillin, while susceptibility to tetracycline, rifampin, and erythromycin varied between samples. The combination of rice varieties displayed increased susceptibility compared to individual strains. In vitro probiotic efficacy tests demonstrated acid and bile salt tolerance, with the Kattuyanam and Mappillai Samba 1:1 (KRMR) showing the highest survival rates under acidic (80.287±0.328%) and bile salt (78.419±0.285%) conditions. Glucose fermentation tests indicated homofermentative behavior in all isolates, producing lactic acid without gas accumulation. Additionally, adhesion assays revealed that KRMR had the highest adherence to stainless steel (39.333±1.52%), indicating its potential for gut colonization. These findings highlight the probiotic potential of traditional rice-fermented microbes for applications in gastrointestinal health and functional foods.

Contribution/Originality: This study combines metagenomic profiling and culture-based functional assays to assess and compare the probiotic potential of Kattuyanam and Mappillai Samba rice in combination. By highlighting distinct microbiological and functional characteristics, the integrated analytical approach provides new perspectives on their potential applications in the development of functional foods.

1. INTRODUCTION

Fermented food is an edible product developed through bacteria on raw or cooked plant or animal ingredients, either naturally or by incorporating certain cultures (Fuloria et al., 2022). The antibacterial properties of the lactic acid bacteria found in fermented foods have positive health advantages. Owing to their intriguing advantageous qualities, lactic acid bacteria (LAB) are frequently utilized as starter cultures, probiotics, and microbial cell factories for the synthesis of bioactive compounds, such as lactic acid, which has antibacterial characteristics and inhibits the

growth of harmful microbes (Jeyagowri et al., 2023). Probiotics, whether in the form of supplements or food products, have emerged as the most important ingredient in the era of functional foods. Probiotics have long been a crucial element and a lucrative target due to their possible health advantages (Hamad et al., 2022). According to scientific research, certain probiotics can help with a number of gastrointestinal conditions, including inflammatory bowel disease, diarrhea, allergic diseases, non-alcoholic fatty liver disease, gastrointestinal disorders, obesity, type 2 diabetes, various cancers and their side effects, immune system health, metabolic health, dental health, and brain health (Maftei et al., 2024). Widespread rice consumption, particularly across Asia, is linked to micronutrient deficiencies in vulnerable populations. Although whole grain and fortified rice products offer potential remedies, cultural traditions and regional preferences often limit their acceptance. Rice fermentation emerges as a promising alternative, leveraging microorganisms such as lactic acid bacteria (LAB) to enhance the rice's nutritional profile. This process effectively reduces antinutritional compounds and increases antioxidant content, offering a natural and culturally adaptable solution to improve nutrient intake (Sivakumar, Adebo, Gieng, & Feng, 2024).

Lactic acid bacteria (LAB), such as certain strains of *Lactobacilli* and *Bifidobacteria*, can ferment rice and break down its anti-nutritional components, increasing the bioavailability of micronutrients and minerals like calcium, potassium, and iron by several thousand percentage points. This process can improve the nutritional value of rice-based foods. It is also known to safeguard the microflora, which has anti-carcinogenic, anticancer, and anti-cholesterol properties and inhibits harmful germs. People who are lactose intolerant may be able to safely ingest it (Fawzi et al., 2022). Henceforth, the major highlight of our study is to characterize and compare the probiotic populations present in fermented rice (Kattuyanam and Mapillai Samba) individually as well as in combination by high-throughput metagenomic sequencing. Further, the fermented rice varieties were subjected to in vitro evaluation of probiotic efficacy.

2. MATERIALS AND METHOD

2.1. Formulation of Sample

Two rice varieties, namely Kattuyanam (KR) and Mappillai Samba (MR), were procured from a local store in Chennai, Tamil Nadu, and were authenticated by the Siddha Central Research Institute, Chennai. The different samples, i.e., Kattuyanam (KR), Mappillai Samba (MR), and Mappillai Samba: Kattuyanam (1:1) (KRMR), were processed according to specific parameters, including quantity, rice-to-water ratio, cooked rice-to-water ratio, cooking duration, and fermentation hours. The bacterial growth curve of the samples was analyzed. The biomass of the fermented sample was calculated, and the samples were lyophilized (Subaratinam, Vidya, & Suganthi, 2025).

2.2. Isolation of Probiotics

The lyophilized samples were dispersed in saline (0.8% NaCl) and diluted up to 10^{-5} dilutions. An aliquot of 100 µl from 10^{-3} and 10^{-5} dilutions was pour-plated on MRS agar medium and incubated for 24 hours at 37 °C. After incubation, the plates were examined. The size, color, elevation, margin, opacity, surface, and texture of the colonies were observed. These colonies were then purified through streaking (De Man, Rogosa, & Sharpe, 1960). The colony-forming unit (CFU/ml) was calculated using Equation 1.

$$\text{CFU/ml} = \frac{Number\ of\ colonies \times Dilution\ factor}{Volume\ of\ loaded\ in\ the\ culture\ plate} \quad (1)$$

2.3. Identification of Bacteria

2.3.1. Morphological Identification

2.3.1.1. Gram Staining

Gram staining was performed on a glass slide. The glass slide was covered with a loopful of bacterial suspension, which was subsequently fixed using the heat fixation procedure. After applying crystal violet stain, the slide was left

alone for 30 to 60 seconds. After that, water was used to rinse the slide. The slide was then stained for one minute with Gram's iodine and then rinsed with water. The washed slide was then washed with 95% ethanol for 10-20 seconds before being rinsed with water. Lastly, safranin was added and allowed to sit for a minute and again rinsed with water. The slide was reviewed under a light microscope. Gram-positive bacteria will appear blue or purple following Gram staining, while gram-negative bacteria would appear pink or red (Coico, 2006).

2.4. Metagenomics

DNA was extracted using the CTAB technique, and target regions (16S rRNA/18S rRNA/ITS) were amplified with Phusion® High-Fidelity polymerase. After purification (TianGen DP214) and gel verification, amplicons were used to create PCR-free sequencing libraries (NEBNext® Ultra™II FS, E7430L). Libraries were pooled, sequenced on Illumina platforms, quality-checked using Bioanalyzer, and quantified using Qubit and qPCR. Read assembly (FLASH), quality control (fastp), chimera elimination (UCHIME/vsearch), OTU clustering (Uparse), and taxonomic annotation (Silva, Unite) were all part of the bioinformatics process. QIIME, R, Python, and Perl were used to conduct community comparisons, diversity analyses (alpha, beta), association studies (Spearman, CCA/RDA/dbRDA), and functional predictions (PICRUSt, Tax4Fun, BugBase, FunGuild, FAPROTAX).

2.5. Antibiotic Susceptibility

The agar well diffusion method was used to analyze the effects of antibiotics on the cell-free supernatant of KR, MR, and KRMR bacterial cultures. 100 µl of overnight-grown culture was evenly distributed across the surface of nutrient agar plates using a sterile cotton swab. Following drying, wells were created on the solidified agar surface, and 100 µl of antibiotics were added to the respective wells. The plates were incubated for 24 to 48 hours at 37°C. Using the mentioned antibiotic discs, resistance was determined by the disc diffusion method, and calipers were used to measure the diameters of the inhibition zones (Zhang et al., 2016).

2.6. Invitro Probiotic Efficacy

2.6.1. Acid Tolerance and Bile Salt Tolerance

The bile salt tolerance of samples was tested using the methodology by Vinderola and Reinheimer (2003). In brief, 0.2 ml of inoculum suspension (10^8 CFU/ml) was mixed with 10 ml of MRS broth containing bile salt (0.3%) and broth without bile salt as a control, and the mixture was then incubated. The ability of isolates to tolerate acidic pH (3.5) values was assessed using methodology by Victor, François, Marie, Alberto, and Florence (2011). After incubation, optical density (OD) was measured at 560 nm with the control at two different time intervals for acid tolerance (3 and 6 hrs) and bile salt tolerance test (3 and 24 hrs) The percentage of survival rate was calculated using Equation 2.

$$\%\ of\ Survival\ rate = \frac{Sample\ OD}{Control\ OD} \times 100 \qquad (2)$$

2.6.2. Bacterial Adhesion

An overnight-grown bacterial culture (500 µl) and 450 µl of fresh MRS broth were added to a fresh tube along with a sterile stainless-steel plate and incubated at 37°C for 24 hours. After incubation, the stainless-steel plate was rinsed with sterile 1% peptone water. The bacteria adhered to the steel plate was removed by vortexing for three minutes in sterile peptone water. The number of bacterial colonies on the MRS agar plates was counted following a 24-hour incubation period at 37°C (Mulaw, Sisay Tessema, Muleta, & Tesfaye, 2019).

2.6.3. Glucose Fermentation

To identify the homo- and heterofermentative characteristics of the fermented culture, modified MRS broth with 1% glucose was used to quantify CO_2 generation from glucose. 50 µl of 1% overnight culture were added to fresh MRS broth with inverted Durham tubes and incubated at 37°C for five days. The gas production in Durham tubes after incubation confirms the generation of CO_2 from glucose (Mulaw et al., 2019).

3. RESULTS AND DISCUSSION

3.1. Probiotic Isolation

The probiotic culture was isolated from two traditional rice varieties, namely Kattuyanam and Mappillai Samba (Table 1). Five distinct colonies with a total colony count of 1.88×10^7 cfu/ml were observed in Kattuyanam. On the other hand, four distinct colonies with a total colony count of 1.74×10^7 were observed in Mappillai Samba rice. Table 2 lists the different colony morphologies of all nine isolates. The majority of microbes were cream-white in color with a smooth surface. Other physiological traits of microbes varied from one another (Table 2).

Table 1. Isolation of probiotics and colony morphology.

S.no	Sample	Dilution	No of colonies	Cfu/ml	No of distinct colonies
1	Kattuyanam	10^{-5}	188	1.88×10^7	5
2	Mappillai Samba		174	1.74×10^7	4

Table 2. Colony morphology of isolates.

S.no	Isolate code	Colony morphology					
		Size	Form	Edge	Surface	Texture	Color
1	KR -1	Large	Round	Raised	Entire	Smooth	Cream colored
2	KR-2	Medium	Round	Convex	Wavy	Smooth	Cream white
3	KR-3	Medium	Round	Raised	Entire	Smooth	White
4	KR-4	Small	Ovoid	Convex	Entire	Smooth	White
5	KR-5	Medium	Round	Raised	Entire	Smooth and glistening	Light cream colored
6	MR-1	Large	Round	Raised	Entire	Smooth	Cream colored
7	MR-2	Small	Ovoid	Convex	Entire	Smooth	White
8	MR-3	Small	Round	Raised	Entire	Smooth	Light cream colored
9	MR-4	Medium	Round	Raised	Entire	Smooth and glistening	White

3.2. Bacterial Identification

3.2.1. Morphological Identification

Totally five isolates from Kattuyanam and four isolates from Mappillai Samba were subjected to Gram staining to observe colony morphology (Supplementary fig. 1a & b). Gram-positive yeast cells were predominantly observed in the Kattuyanam sample, along with Gram-positive cocci. On the other hand, Gram-positive rods, cocci, and yeast cells were equally observed in Mappillai Samba.

3.2.2. Metagenomics Analysis

The metagenomics results indicated that the taxonomic assignment of the Bacteria domain was the most abundant within the Kattuyanam and Mappillai Samba fermented samples. In the bacteria domain, the Firmicutes phylum was predominantly found compared to other phyla, followed by the minimal presence of Proteobacteria (Figure 1a & 2a). Furthermore, the most abundant families were Lactobacillaceae within the sequence data. Consequently, Lactobacillus was the most prevalent at the genus level. The most abundant species in the Kattuyanam sample were *Liquorilactobacillus hordei* (16%), *Lactobacillus ruminis* (9%), *Fructilactobacillus sanfranciscensis* (8%), *Lactobacillus jensenii* (7%), *Weissella hellenica* (6%), *Lactobacillus helveticus* (6%), *Levilactobacillus_suantsaii* (5%), *Lactobacillus plantarum* (5%), *Periweissella_cryptocerci* (5%). Other less prevalent organisms include *Lactobacillus*

acidophilus, *Lactobacillus delbrueckii*, *Limosilactobacillus fermentum*, and *Loigolactobacillus coryniformis* (Figure 1b). Similar organisms were observed in the Mappillai Samba sample with minimal variation in abundance percentage (Figure 2b).

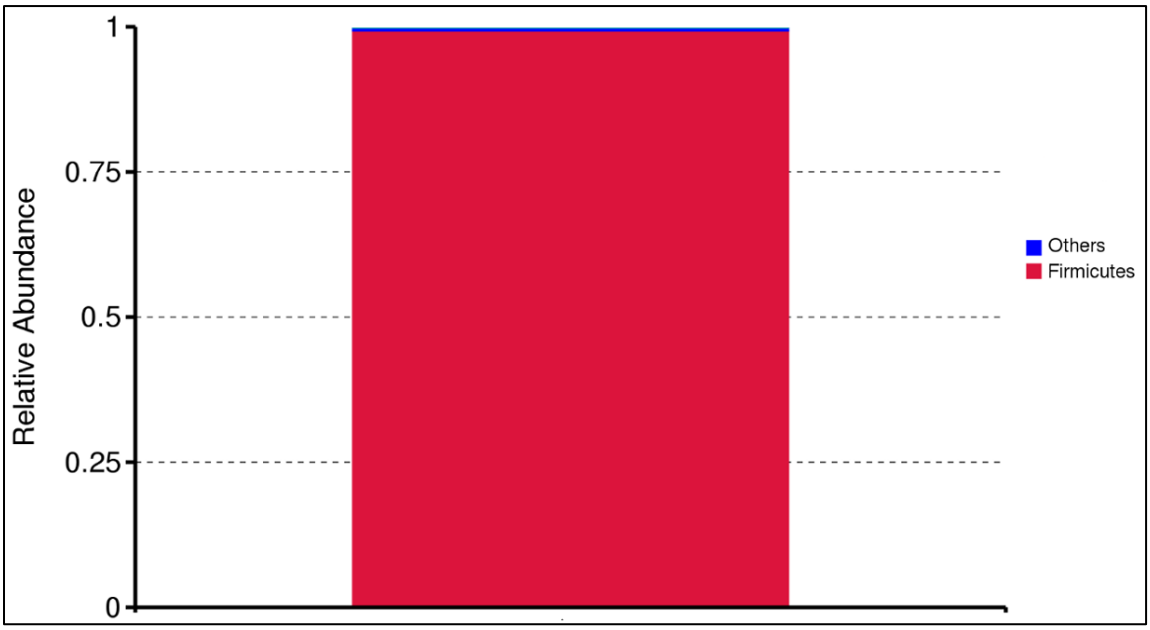

Figure 1a. Taxa relative abundance in the phylum (Kattuyanam).

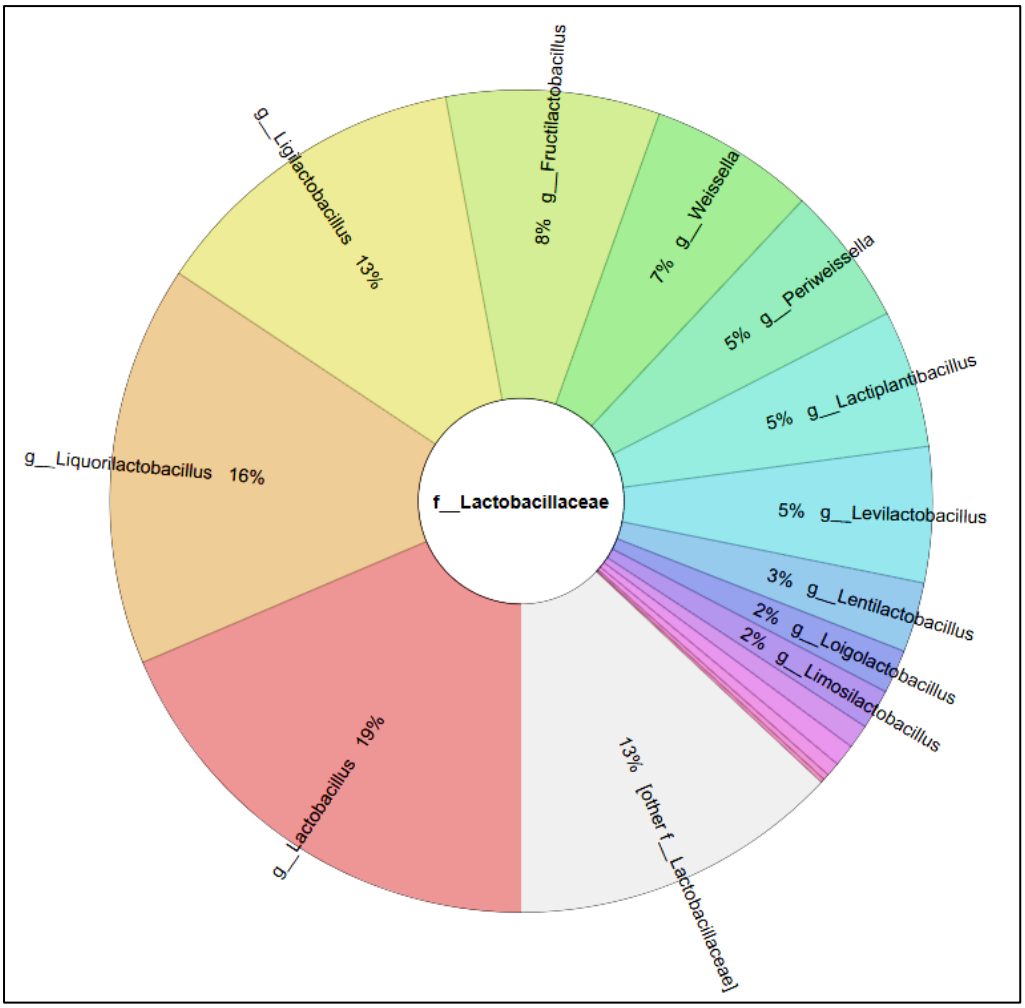

Figure 1b. Taxonomic distribution of microbial species within the Kattuyanam metagenome.

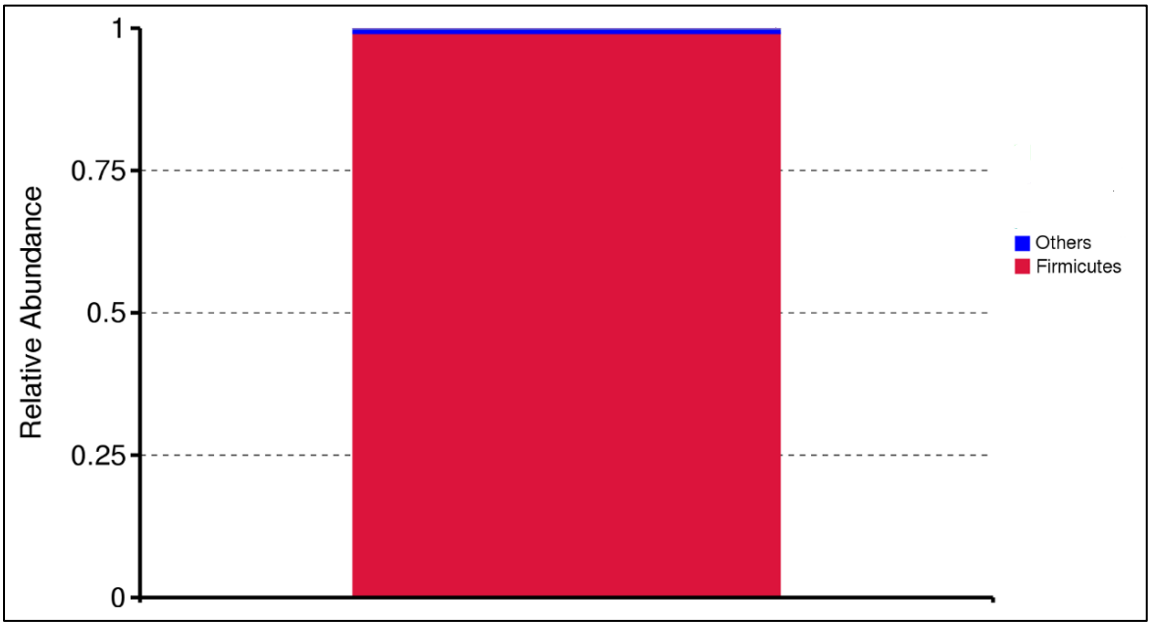

Figure 2a. Taxa relative abundance in phylum (Mappillai Samba).

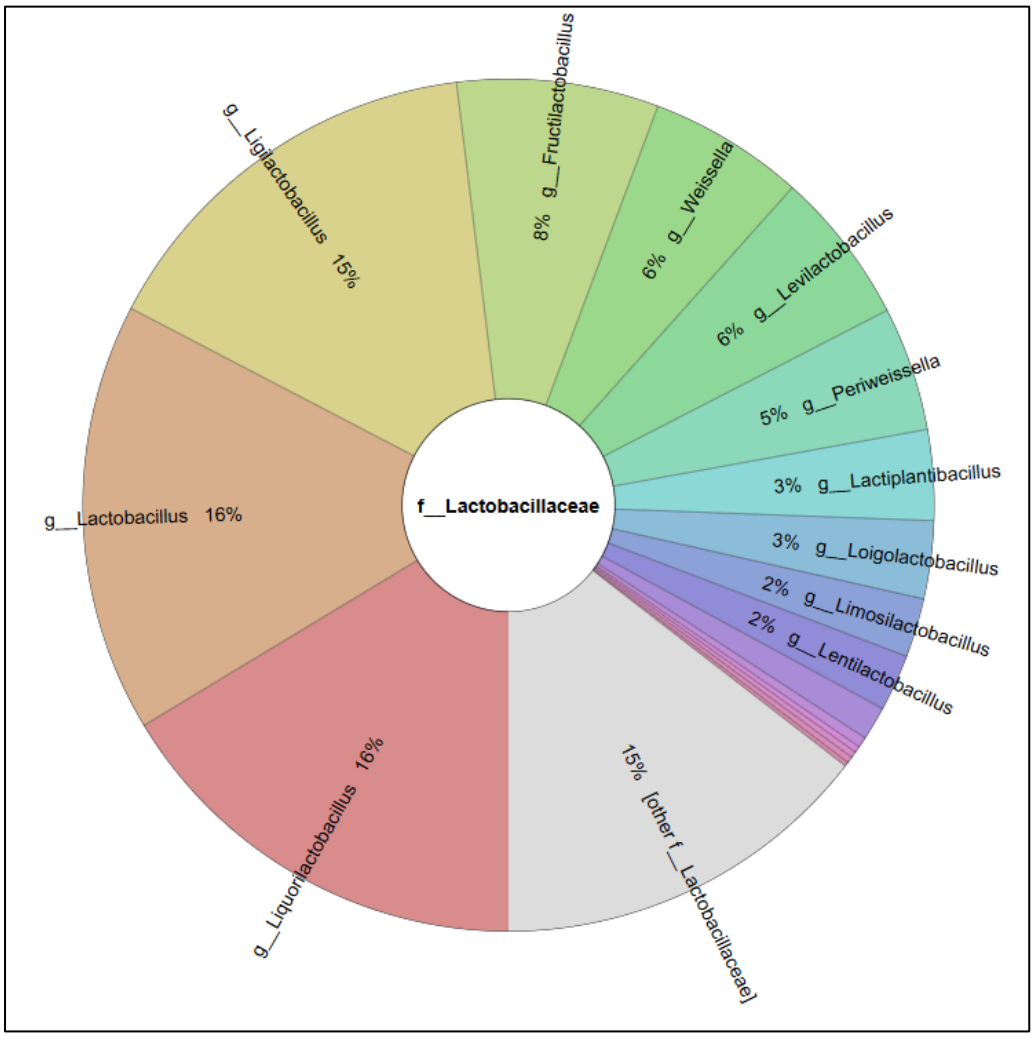

Figure 2b. Taxonomic distribution of microbial species within fermented Mappillai Samba.

3.3. Antibiotic Susceptibility Test

The antibiotic susceptibility testing on Mappillai Samba, Kattuyanam, and a mixed ratio in comparison to a panel of antibiotics provides useful insights into the interaction between these probiotics and antimicrobial drugs, as shown in Table 3. The antibiotics tested showed a variety of inhibitory effects on the tested samples (Figure 3). Some antibiotics, such as tetracycline and rifampin, caused one sample to have a larger zone of inhibition than the other. These changes could be due to differences in the strains' intrinsic resistance mechanisms or their ability to metabolize or counteract the antibiotic's effects. While both samples responded similarly to certain antibiotics such as erythromycin and ciprofloxacin, others showed significant differences in susceptibility. The mixed ratio of Kattuyanam and Mappillai Samba indicates a subtle trend toward increased susceptibility when the two samples were combined. Lactobacilli's antibiotic resistance can be viewed as a double-edged sword. On the one hand, probiotic bacteria are thought to benefit from intrinsic resistance since it allows them to withstand antibiotic treatment, preventing or treating drug-associated diarrhoea (Anisimova, Gorokhova, Karimullina, & Yarullina, 2022).

Table 3. Antibiotic susceptibility (ZOI).

S. NO	Antibiotics	Zone of inhibition (mm)		
		MR	KR	KRMR
1	Erythromycin (15µg/ml)	24.13±0.32	24.5±0.5	26.06±0.20
2	Tetracycline (30 µg/ml)	32.56±0.45	33.46±0.75	34±0.5
3	Penicillin (10µg/ml)	R	R	R
4	Cefotaxime (30µg/ml)	20.56±0.60	22.1±0.26	22.06±0.20
5	Rifampin (5µg/ml)	22.93±0.81	25±0.3	28.06±0.30
6	Ciprofloxacin (30µg/ml)	24±0.5	23.46±0.50	25.4±0.52
7	Streptomycin (10µg/ml)	R	R	R
8	Ampicillin (10µg/ml)	R	R	R

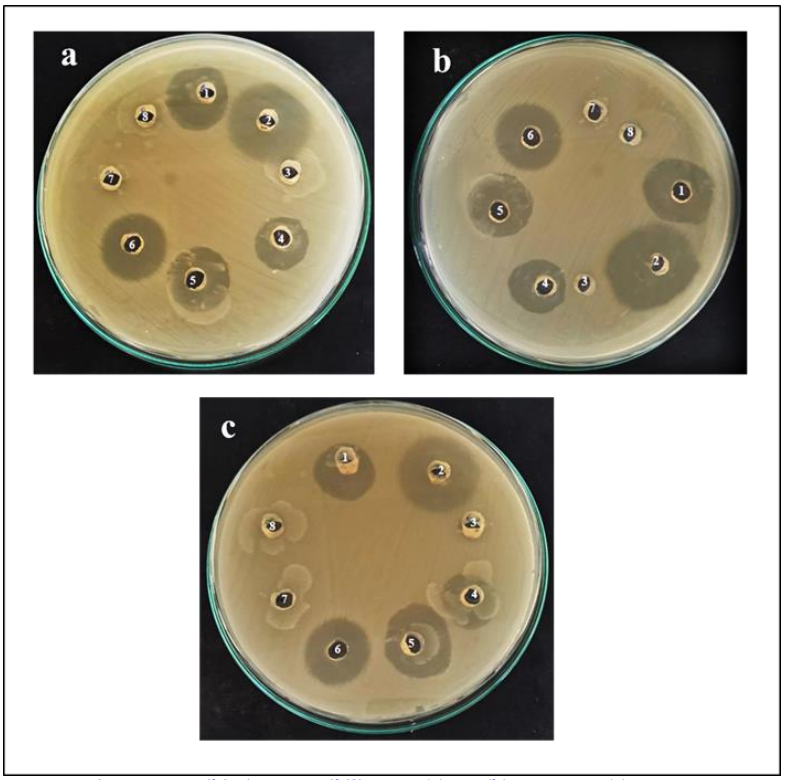

Figure 3. Antibiotic susceptibility test (a) KR (b) MR AND (c) KRMR.

3.4. In Vitro Probiotic Efficacy

3.4.1. Acid Tolerance and Bile Salt Tolerance

Bacteria that can withstand low pH and low bile salts in human gastrointestinal tracts are thought to have the potential probiotic characteristics (Lama & Tamang, 2022). Table 4 represents the percentage of survival rate in accordance with pH and bile tolerance. From the previous results obtained, it was evident that the selected samples with a tolerance rate of above 50% are said to be at an acidic pH (3.5) (Figure 4a) and bile (0.3%) (Figure 4b) salt-tolerant strain. In comparison, KRMR showed the highest survival rate of 80.287±0.328% (acid tolerance) and 78.419±0.285% (bile salt tolerance) after 6 and 24 hours, respectively. Bile tolerance is a crucial factor in strain selection since probiotics must survive, colonize, and remain active in the gut, allowing them to alter microbiota, engage with the immune system, and provide health benefits (Madushanka et al., 2025). Since probiotics are usually taken orally, they must be able to withstand the passage through the stomach and small intestine. Therefore, a key consideration in their selection is their capacity to withstand the acidic conditions of gastric juice in the stomach and the presence of bile salts in the small intestine (Leska, Nowak, Rosicka-Kaczmarek, Ryngajłło, & Czarnecka-Chrebelska, 2023). The results of the current study confirmed that the probiotic strains of a mixed ratio of Kattuyanam and Mappillai Samba (KRMR) have a better capability to survive in severe conditions of the gastrointestinal (GI) tract.

Table 4. Acid and bile salt tolerance.

S.no	SAMPLE	pH tolerance		Bile salt tolerance	
		3 h	6 h	3 h	24 h
1	KR	77.318±0.235	77.238±0.222	74.251±0.813	76.355±0.184
2	MR	74.758±0.553	73.889±0.463	72.826±0.725	74.902±0.294
3	KRMR	80.882±0.865	80.287±0.328	76.688±0.411	78.419±0.285

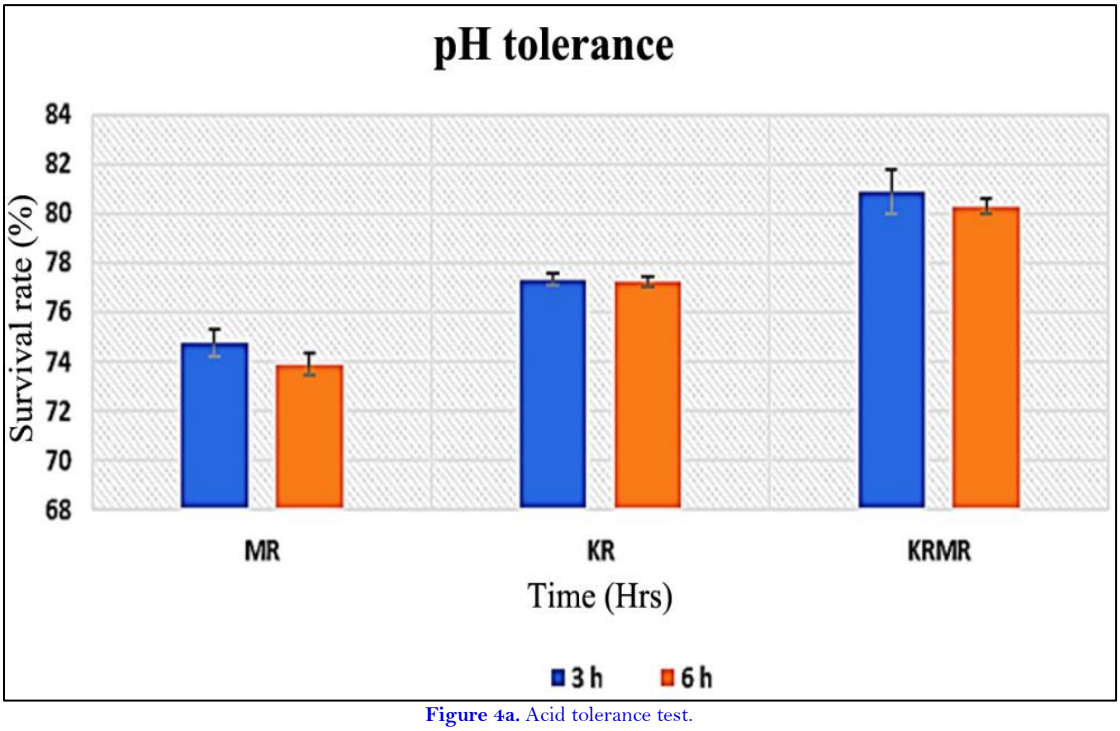

Figure 4a. Acid tolerance test.

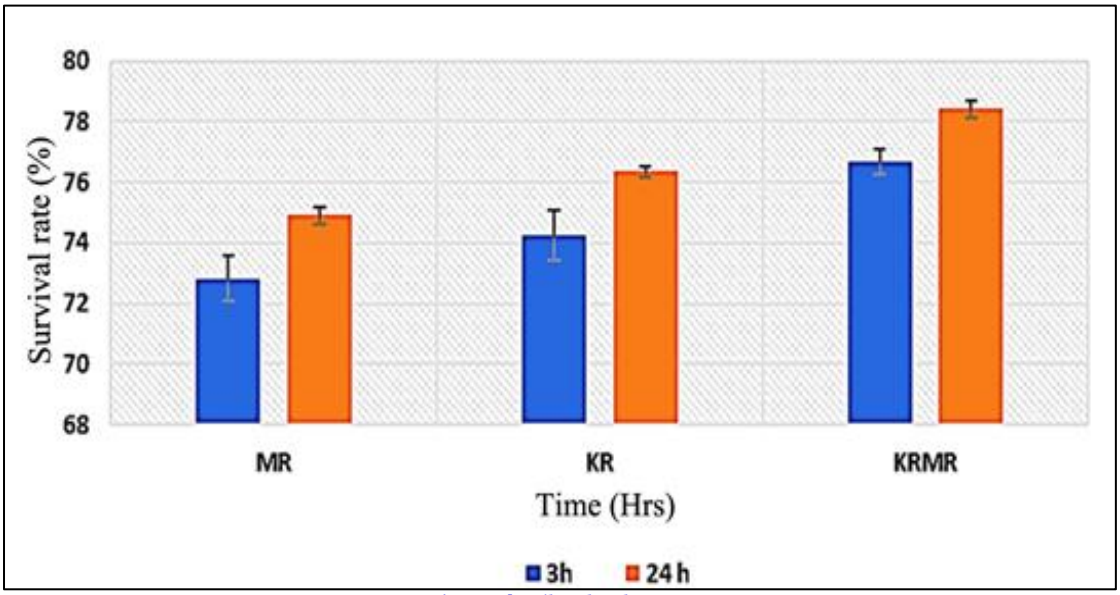

Figure 4b. Bile salt tolerance.

3.4.2. Glucose Fermentation and Gas Production

Glucose fermentation was performed in phenol glucose broth with Durham tubes inverted into the tubes to observe gas production. The figure represents the glucose fermentation of control, KRMR(A), KR(B), MR(C) (Figure 5). Acid production as a result of fermentation was observed in all three samples, as the color of the medium changed from red to yellow. No gas accumulation from glucose was observed. This confirmed that all three samples were homofermentative in nature. The primary by-product of this fermentation is lactic acid. According to previous research, Lactobacillus species from Ergo have been identified as being both homofermentative and heterofermentative equally (Jermen, Fassil, & Anteneh, 2015).

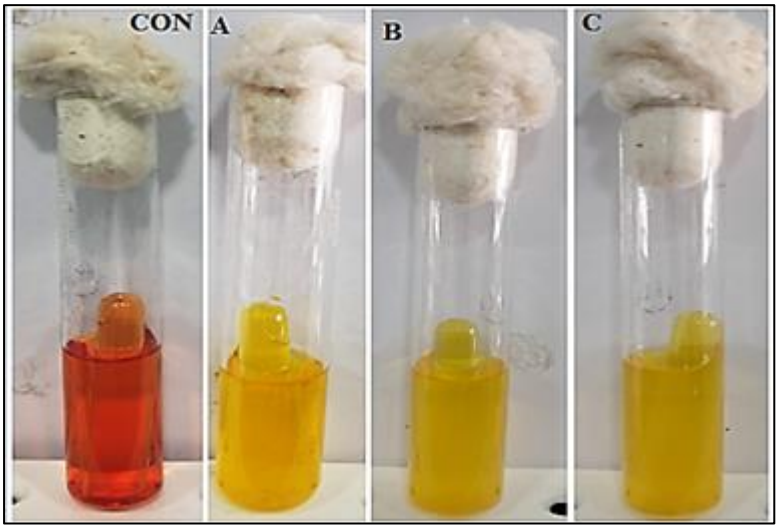

Figure 5. Glucose fermentation (a) MR (b) (KR) AND (c) KRMR.

3.4.3. Bacterial Adhesion to Stainless Steel

Adherence to the intestinal mucosa is one of the key properties of probiotic bacteria (Table 5). Probiotic adhesion is a critical factor because it directly impacts the effectiveness and viability of probiotics when they reach the human gastrointestinal tract. "KRMR" showed the highest adhesion at 39.333±1.52%, followed by "KR" at 22.667±2.08%, and "MR" at 18.367±1.19% (Figure 6 & 7). Higher adhesion percentage probiotics have a better chance of adhering to the intestinal lining, surviving the severe conditions of the gastrointestinal system, and providing long-term health

benefits. The Lactobacillus spp. LAB strain, isolated from Ethiopian fermented foods, showed a hydrophobicity ability of 32.75–36.30% (Mulaw et al., 2019). The capacity to adhere to a substrate is a necessary characteristic for probiotic selection since adhesion to host surfaces is required for efficient probiotic colonization in the host, and pathogen adhesion disruption is required for pathogen biocontrol (Ashraf & Shah, 2011). This is particularly important for probiotics intended for daily consumption, as their ability to establish and maintain a presence in the gut is crucial.

Table 5. Percentage of probiotic adhesion.

S.no	Sample	Probiotic adhesion %
1	KR	22.667±2.08
2	MR	18.367±1.19
3	KRMR	39.333±1.52

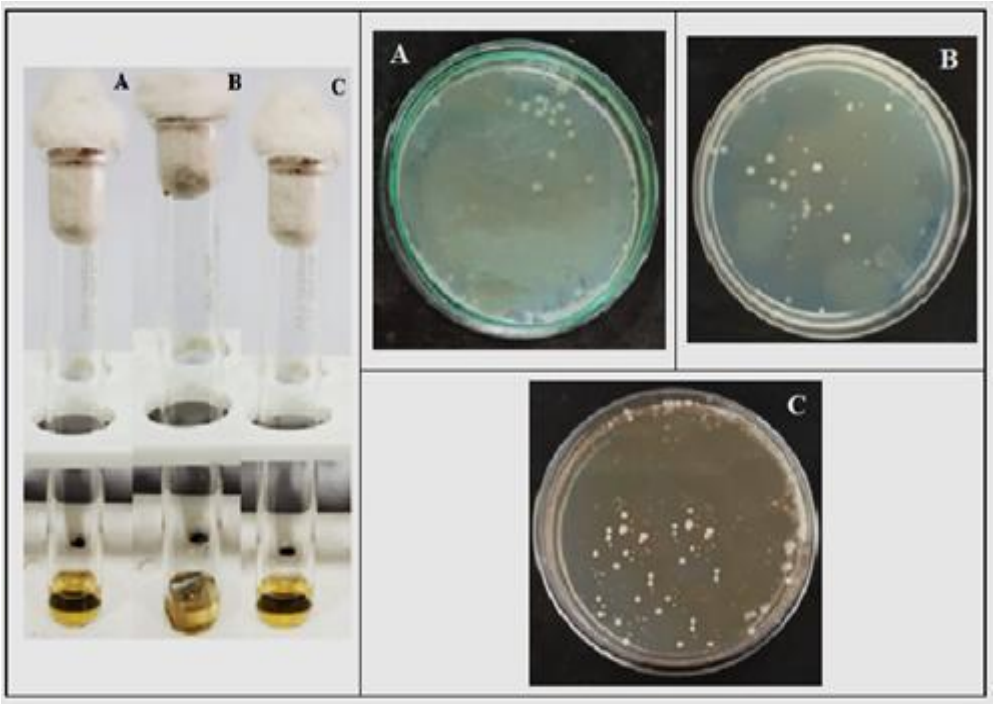

Figure 6. Bacterial adhesion to stainless steel plate (a) MR (b) (KR) AND (c) KRMR.

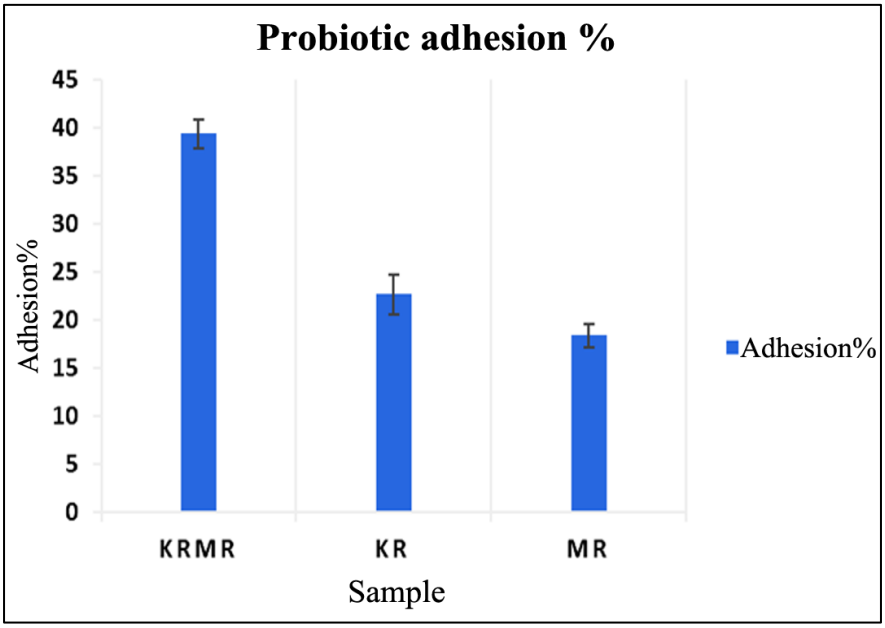

Figure 7. Percentage of probiotic adhesion to stainless steel plate.

4. CONCLUSION

This study successfully identified the presence of probiotic strains from two fermented traditional rice varieties. Metagenomic analysis identified Firmicutes as the dominant phylum, with *Lactobacillus* spp. being the most abundant genus. The KRMR sample, with a wide variety of probiotic strains, exhibited strong acid and bile salt tolerance, confirming their potential for gastrointestinal survival. Antibiotic susceptibility testing showed resistance to penicillin, streptomycin, and ampicillin, while displaying susceptibility to erythromycin, tetracycline, and ciprofloxacin. Glucose fermentation confirmed their homofermentative nature, producing lactic acid without gas accumulation. The adhesion study revealed that the KRMR mixed culture exhibited the highest adhesion, highlighting its superior probiotic potential. These findings suggest that the combination of rice varieties Kattuyanam and Mappillai Samba are promising candidates for functional food applications and gut health improvement. Rice fermentation naturally produces probiotics, which play an important role in boosting its bioactive characteristics. In addition to enhancing rice's overall nutritional value, this technique offers a solid basis for creating a variety of functional food products. These probiotic-enriched rice products can help increase dietary diversity and improve health benefits.

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Authors' Contributions: All authors contributed equally to the conception and design of the study. All authors have read and agreed to the published version of the manuscript.

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