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Exploring the Role of Traditional Cooking Methods in Boosting Probiotic Diversity in Fermented Rice (Oryza sativa). A Comprehensive Insight into its Nutritional Influence on Human Health

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Abstract

Objectives: The current study investigates the probiotic characteristics of naturally fermented rice prepared through three traditional cooking techniques: mud pot-cooked, pressure-cooked, and steel vessel-cooked. The objective is to evaluate how these methods influence microbial development, lactic acid bacteria (LAB) proliferation, and core biochemical parameters which contribute to the probiotic prospective of fermented rice. **Methods:** Rice was cooked using the three methods and subjected to spontaneous fermentation for 16 hours. pH, titratable acidity were measured at before (0 hrs) and after (16 hrs) fermentation to monitor fermentation progress. Total Viable count (TVC), Lactic acid bacteria (LAB), Yeast count, overall microbial diversity index were enumerated using selective methods respectively. Additionally, the production of significant organic acids like reducing sugars, total protein and total lipid content were analysed before and after fermentation. Enzymatic activities - amylase, protease, phytase and lactase were assessed to understand microbial metabolism and nutritional enhancement resulting during the process of fermentation. **Findings:** Food fermentation through pot cooking produced fermented rice with the richest microbial diversity including beneficial lactic acid bacteria *Lactobacillus plantarum*, *L. fermentum* and *Lactococcus garvieae*. Multiple selected bacterial isolates demonstrated beneficial probiotic properties in addition to their capability to tolerate pH and bile salts and show moderate hydrophobicity and pathogen-threatening capability. Pot-cooked rice samples revealed increased enzymatic activity together with higher lipid oxidation rates because they enabled active microbial fermentation. The microbial growth along with probiotic functionality remained lower in steel- and pressure-cooked rice samples because higher-pressure cooking can prevent substrate effectiveness or inhibit microbial settlements. The antibiotic sensitivity tests established the safety properties of selected bacterial isolates, yet the antimicrobial resistance

analysis uncovered possible antagonistic traits among *Bacillus pumilus* and *Citrobacter freundii*. The research demonstrates that traditional cooking techniques both protect rice nutritional components during pot preparation while creating suitable conditions for probiotic food development. Research indicates that pot-finished rice functions as an appealing natural probiotic dietary component with cultural roots. **Novelty:** This study represents a pioneering investigation into the 16-hour spontaneous fermentation of rice prepared through divergent traditional cooking methods, engaging a variety of utensils such as steel vessels, pressure cookers, and earthenware pots. This unique approach stands out as the first of its kind to compare the effects of these three distinct cooking methods. The results of this study reveal that rice cooked in an earthenware pot harbors the maximum growth of beneficial microbes and induces exemplary biochemical changes including the formation of hundreds of useful metabolites.

Keywords: Cooking method; Early fermentation; Nutritional values; Microbial population; Probiotic properties; Pot rice

1 Introduction

Fermentation is an age-old biotechnological process widely incorporated across cultures for its capacity to enhance the nutritional quality, digestibility, and functional properties of food[(1)]. The key important of this alteration is the activity of probiotics especially lactic acid bacteria (LAB) and bacillus species which in turn initiates the breakdown of complex substrates into metabolically active compounds includes organic acids, short-chain fatty acids (SCFAs) and bioavailable nutrients beneficially for human beings[(2)]. The fermentation process initiated by lactic acid producing bacteria is well known for its ability to enhance the shelf life, improves gut health and elevates the immunity by associating balanced intestinal microbiota population[(3)]. Due to changing lifestyle habits among modern consumers who are gradually seeking natural functional foods, fermented rice is one among the functional foods which attracts more population, as their dietary supplement options are deep-rooted in tradition and reinforced due to emerging exploration in-order to improve the nutritional quality of functional food.

Among the major food sources worldwide and throughout Asia, stands rice (*Oryza sativa*) because of the high carbohydrate content serves as a staple food, remarkably well known for its microbial fermentation[(4)]. Civilizations throughout South and Southeast Asia for generations have revered traditional fermented rice because of its double benefits for energy and digestion. Based on the scientific evidence it indicates that fermented rice demonstrates potential in sustaining gut health while providing probiotics together with reducing metabolic syndrome risks too[(5)]. The initial method of rice preparation fundamentally influences fermentation potential because it modifies the nutritional content and structural composition as well as microbial accessibility. The different rice cooking methods may influence the physical, chemical and metabolic changes of fermentation dynamics[(6)]. Pot cooking method leads to gradual gelatinization that occurs during traditional pot cooking because rice stays in low heat for a long time which in turn maintains nutrients while supporting better microbial fermentation and rice structure preservation. During pressure cooking, rice gets exposed to high temperature in combination with pressure which likely reduces heat sensitive nutrient content and creates unfavourable starch modifications for microbial development. On the other hand, steel vessel cooking provides a contemporary adaptation which combines these two conventional methods to offer moderate temperature control alongside better preservation of nutrients. The consequences of fermentation could be assessed through multiple indicators including changes in pH, titratable acidity, LAB count, enzymatic activities, and the generation of key biochemical metabolites such as SCFAs. These parameters directly influence the health-promoting characteristics of the final fermented product. LAB play a vital role in reducing pH and generating organic acids while secreting enzymes like amylase, protease, and phytase that break down complex biomolecules into more digestible forms. Additionally, the microbial metabolism of carbohydrates during fermentation results in a marked reduction in reducing sugar content and modulates total protein levels through proteolysis and peptide release[(7)].

Despite the long-standing cultural use of fermented rice, few studies have systematically compared how varying cooking techniques impact its fermentation efficiency and probiotic potential. Assumed the increasing preference for modern cooking appliances such as pressure cookers and stainless-steel vessels, there is a pressing need to understand how these methods affect the biochemical and microbiological transformations that occur during rice fermentation.

This study addresses this gap by evaluating naturally fermented rice (at 0 and 16 hours of fermentation) prepared using three cooking techniques-pot-cooked, steel vessel-cooked, and pressure-cooked. The key parameters including pH, Titratable acidity, reducing sugar, total protein content, LAB enzymatic activity (amylase, protease, phytase and lactase), and microbial acidification potential were analysed. Through this comparative analysis, this study focuses to identify the cooking method

most conducive to developing probiotic-rich fermented rice, thus contributing to the broader goal of promoting gut-friendly and functionally enriched traditional foods.

2 Methodology

2.1 Preparation of Rice Samples and Fermentation Process

Rice was cooked using three traditional and commonly employed methods: mud pot, steel vessel, and pressure cooker. For each method, 100 grams of raw rice was cooked with 200 mL of water under standardized conditions. Upon completion of cooking, the samples were cooled to room temperature[8]. Each cooked rice sample was then subjected to spontaneous fermentation by adding reverse osmosis (RO) water at a ratio of 1:5 (rice: water). The mixtures were incubated at controlled temperature of $30 \pm 1^{\circ}$C for 16 hours to promote natural early fermentation. After the fermentation period, all samples were stored at 4°C until further microbial analysis, molecular confirmation, pH, Titratable Acidity, production of organic acids, enzymatic and nutritional analysis were conducted.

2.1.1. Total Viable Count (TVC) of fermented rice samples

To assess the overall microbial load in fermented rice, samples cooked using three different methods (pot, pressure cooker, and steel vessel) were serially diluted using sterile saline. Appropriate dilutions were plated on Total Plate Count Agar (TCA) using the pour plate technique. Plates were incubated at 37°C for 24-48 hours. After incubation, colonies were counted and results expressed as colony-forming units per gram (CFU/g) of fermented rice samples respectively[9].

2.1.2. Lactic Acid Bacteria (LAB) Count of fermented rice samples

For LAB enumeration in the fermented rice samples, serial dilutions were prepared in sterile saline and plated on MRS (de Man, Rogosa and Sharpe) agar. The plates were incubated at 37°C for 48 hours. Colonies that developed were counted and reported as CFU/g, indicating the growth of lactic acid bacteria (LAB).

2.1.3. Total Yeast Count of fermented rice samples

Yeast populations in the fermented rice were determined by plating serial dilutions on Potato Dextrose Agar (PDA) supplemented with chloramphenicol (100mg/L) to suppress bacterial growth. Plates were incubated at 28°C for 48-72 hours. Yeast colonies were identified based on morphology and recorded as CFU/g of sample.

2.1.4. Shannon Diversity Index (H')

To evaluate microbial diversity in fermented rice, colony morphotypes observed on selective media (PCA for total bacteria, MRS for LAB, PDA for yeast) were documented. The relative abundance of each morphotype was calculated. Shannon Diversity Index (H') was computed using the formula:

$$\mathbf{H'} = -\sum(\mathbf{pi} \times \mathbf{ln\ pi})$$

where pi is the proportion of each morphotype relative to the total colony count. This index provided an estimate of microbial richness and diversity across different cooking methods[10].

2.2 Measurement pH of fermented rice sample before and after Fermentation

To determine the acidity level of the fermented rice samples, 10 grams of each cooked rice type (mud pot-cooked, steel-cooked, and pressure-cooked) were homogenized thoroughly in 100 mL of sterile distilled water (1:10 w/v ratio). The mixture was blended until uniform and allowed to stand briefly for particulate settling. The pH of the supernatant was measured using a calibrated digital pH meter, ensuring the electrode was rinsed with distilled water between measurements to prevent cross-contamination and false reading. The readings were recorded in triplicates for accuracy.

2.2.1. Analysis of Titratable Acidity of fermented rice sample before and after Fermentation

Titratable acidity was assessed by preparing a homogenized rice suspension (1:10 w/v) with sterile distilled water. A 10 mL aliquot of this homogenate was titrated against 0.1 N sodium hydroxide (NaOH) using phenolphthalein as an acid-base indicator. The endpoint of titration was marked by the persistent appearance of a faint pink colour. The volume of NaOH consumed was used to calculate the percentage of titratable acidity, expressed in terms of lactic acid equivalents, which reflects the metabolic activity of LAB present in the fermented samples.

2.2.2. Short-Chain Fatty Acids (SCFA) Analysis via Spectrophotometry of fermented rice sample before and after fermentation

To quantify short-chain fatty acids such as acetic acid, propionic acid, and butyric acid, fermented rice samples were first homogenized with distilled water in a 1:10 (w/v) ratio. The homogenates were centrifuged at 10,000 rpm for 15 minutes at 4°C to collect the supernatant. A known volume of this supernatant was mixed with ferrous ammonium sulfate (0.2%) and perchloric acid (2%) in equal parts and heated at 60°C for 10 minutes in a water bath to allow colour development. After cooling to room temperature, the absorbance was measured at 540 nm using a UV-Visible spectrophotometer. Standard calibration curves were prepared using known concentrations of acetic acid ranging from 0.5 to 20 μmol/ml. The SCFA concentrations in the samples were calculated and expressed in μmol/g of fermented rice.

2.2.3. Estimation of Reducing Sugar Content of fermented rice sample before and after Fermentation

Reducing sugar content was estimated using the dinitro salicylic acid (DNS) method. Rice samples from both pre- and post-fermentation stages were homogenized with distilled water in a 1:10 (w/v) ratio and centrifuged at 8,000 rpm for 10 minutes to extract the supernatant. A 1 mL aliquot of the supernatant was mixed with 1 mL of DNS reagent in a test tube and boiled in a water bath for 10 minutes to develop a reddish-brown colour. After cooling to room temperature, the absorbance was measured at 540 nm using a spectrophotometer. A standard curve was prepared using glucose solutions of known concentrations ranging from 0.2 to 2 mg/ml. The sugar content was calculated and expressed as mg of glucose equivalents per gram of sample.

2.2.4. Estimation of Total Protein of rice sample before and after Fermentation

Total protein content was determined by the Lowry method. Samples were prepared by homogenizing 1 g of rice (both pre- and post-fermentation) in 10 mL of phosphate-buffered saline (PBS) and centrifuging at 10,000 rpm for 15 minutes. A 0.5 mL aliquot of the supernatant was mixed with 2.5 mL of alkaline copper reagent and incubated at room temperature for 10 minutes. Then, 0.25 mL of Folin-Ciocalteu reagent (1N, diluted 1:1 with water) was added, followed by incubation in the dark for 30 minutes. The absorbance was recorded at 660 nm using a UV-visible spectrophotometer. A standard curve was constructed using bovine serum albumin (BSA), and protein content was expressed as mg/g of sample.

2.2.5. Analysis of Total lipid content in fermented rice sample before and after Fermentation

The total lipid content was estimated using a spectrophotometric method based on the Folch extraction technique [11]. The cooked and fermented rice samples (1 g) were homogenized in a chloroform–methanol mixture (2:1, v/v) to extract lipids. The homogenate was filtered and centrifuged at 3000 rpm for 10 minutes to separate the organic layer containing the lipids. This organic phase was collected, and the solvent was evaporated under reduced pressure to obtain the lipid residue. The dried lipid extract was then re-dissolved in a known volume of chloroform. For quantification, phosphomolybdic acid reagent was added to the lipid solution, and the resulting mixture was analysed spectrophotometrically at 535 nm. A standard calibration curve was prepared using known concentrations of oleic acid, and the lipid content in the sample was calculated using the Beer-Lambert law and expressed as μg/100 g of sample.

2.3 Analysis of Enzyme Activities of Fermented rice samples

2.3.1. Amylase Activity

Amylase activity was evaluated based on the amount of reducing sugars released from starch. Fermented rice extracts were incubated with 1% soluble starch in phosphate buffer (pH 6.8) at 37°C for 30 minutes. The reaction was stopped by adding DNS reagent, followed by boiling for 5 minutes. After cooling, the absorbance was read at 540 nm. A glucose standard curve was used to determine the amount of reducing sugar released. One unit (U) of amylase activity was defined as the amount of enzyme that releases 1 μmol of glucose per minute under assay conditions [12].

2.3.2. Protease Activity

Protease activity was measured using casein as the substrate. A 0.5 mL aliquot of fermented extract was incubated with 1 mL of 1% casein solution in 50 mM phosphate buffer (pH 7.0) at 37°C for 30 minutes. The reaction was terminated by adding 2 mL of 10% trichloroacetic acid (TCA), and the mixture was centrifuged at 10,000 rpm for 10 minutes. The absorbance of the clear supernatant was measured at 280 nm. A tyrosine standard curve was used for calculation. One unit of protease activity was defined as the amount of enzyme that liberates 1 μg of tyrosine per minute [13].

2.3.3. Phytase Activity

Phytase activity was determined by quantifying the release of inorganic phosphate from sodium phytate. A reaction mixture containing 0.5 mL of rice extract, 0.5 mL of 5 mM sodium phytate, and 1 mL of 100 mM acetate buffer (pH 5.5) was incubated at 37°C for 30 minutes. The reaction was stopped by adding 1 mL of 10% TCA. Then, 1 mL of the reaction mixture was mixed with 4 mL of colour reagent (ammonium molybdate and ferrous sulphate) and incubated at room temperature for 10 minutes. Absorbance was read at 700 nm. A standard curve of inorganic phosphate was used to quantify the released phosphate. One unit of phytase activity was defined as the amount of enzyme that liberates 1 μmol of phosphate per minute [14].

2.4 Molecular and Biochemical identification of pot rice colonies

2.4.1. Molecular Confirmation by Sanger Sequencing

DNA was isolated from the selected LAB colonies using a commercial DNA extraction kit. The 16S rRNA gene was amplified using universal primers. The PCR product was purified and sent for Sanger sequencing. The obtained sequences were analysed using BLAST against the NCBI database for taxonomic confirmation [15]. Subsequently, the sequences were submitted to the NCBI database as a BioProject.

2.4.2. Gram Staining

To confirm the presence of Gram-positive lactic acid bacteria (LAB), colonies isolated from each fermented rice sample were subjected to Gram staining. A thin bacterial smear was prepared on a clean glass slide, heat-fixed, and then sequentially stained with crystal violet, iodine solution (as mordant), decolorized with alcohol, and counterstained with safranin. After drying, slides were observed under a bright-field microscope at 1000x magnification using oil immersion. Gram-positive bacteria appeared violet, while Gram-negative bacteria appeared pink. Cellular morphology such as rods or cocci was also noted.

2.4.3. Methyl Red and Voges-Proskauer (MR-VP) Tests

Bacterial isolates were inoculated into MR-VP broth and incubated at 37°C for 48 hours. For the Methyl Red test, a few drops of methyl red indicator were added, and the development of a red colour indicated strong acid production through mixed-acid fermentation. For the Voges-Proskauer test, reagents (alpha-naphthol and potassium hydroxide) were added to a separate aliquot of the same broth, and a red colour formation indicated the presence of acetoin, a neutral end product of fermentation.

2.4.4. Catalase Test

The catalase activity of isolated bacterial strains was evaluated by placing a drop of 3% hydrogen peroxide (H_2O_2) on a clean glass slide, followed by adding a loopful of freshly cultured bacterial colony. Immediate effervescence or bubble formation indicated the breakdown of H_2O_2 into water and oxygen, confirming the presence of catalase enzyme. LAB are typically catalase-negative, and this test helps differentiate them from other aerobic or facultative anaerobic organisms.

2.4.5. Motility Test

Motility nature of bacterial isolates was evaluated using semisolid motility agar medium. A sterile needle was used to stab inoculate the centre of the agar tube, and the tubes were incubated at 37°C for 24 hours [16].

2.4.6. H_2S Test

In order to quantify the amount of hydrogen sulphide (H_2S) developed, bacterial cultures were scattered over peptone iron agar slants and incubated for 24 to 48 hours at 37°C. A black precipitate called ferrous sulphide is created when H_2S combines with ferric ions in the medium. Positive H2S generation was indicated by the development of blackening at the butt of the slant or along the stab line [16].

2.4.7. Biochemical enzymatic hydrolytic activities of pot rice colonies

The hydrolytic activities of pot rice colonies were biochemically determined using certain agar medium to identify the isolates' capacity to break down lipids, casein, and starch. Isolates were streaked onto starch agar plates and cultured for 24 to 48 hours at 37°C to hydrolyse the starch. Following incubation, plates were flooded with iodine solution; the enzymatic breakdown of starch resulted in a clear zone surrounding the colonies, indicating positive starch hydrolysis. Skim milk agar plates were used to test for casein hydrolysis. Similar settings were used for the spot-inoculation and incubation of the microbial isolates. Positive casein hydrolysis and caseinase activity were indicated by a distinct halo surrounding the colonies. Spirit blue agar or tributyrin agar were used to measure lipid hydrolysis. To measure lipid hydrolysis, spirit blue agar or tributyrin agar were used. Lipase

activity was established by the development of a clear zone surrounding the growth on tributyrin agar following inoculation and incubation.

2.5 Probiotic properties of pot rice colonies

2.5.1. Acid Tolerance Test

Acid tolerance was tested by suspending bacterial isolates in MRS broth that had been modified to an acidic pH (2.5-4.0) with hydrochloric acid. The cultures were incubated at 37°C for 1–2 hours. After incubation, viable counts were assessed by plating them on MRS agar. The survival rate under acidic conditions gave insights on probiotic potential, particularly survival in the stomach environment [17].

2.5.2. Bile Salt Tolerance

Bacterial strains were added to MRS broth enriched with 0.3% bile salts and cultured at 37°C to assess bile salt tolerance. The optical density at 600 nm (OD600) was measured periodically to observe growth. The strain's capacity to withstand stress in bile and replicate tiny intestinal settings was validated by sustained or elevated OD600 values [18].

2.5.3. Hydrophobicity and Auto-Aggregation

Bacterial suspensions were mixed with equal amounts of n-hexane to test for hydrophobicity. After phase separation and vortexing, the aqueous layer's OD600 was measured. Hydrophobicity rose when the OD decreased. To assess OD600 at 0 and 2-4 hours, bacterial suspensions were incubated at 37°C without stirring to induce auto-aggregation. The capacity of the cells to aggregate, a critical probiotic property, was indicated by a gradual drop in OD [19].

2.6 Antimicrobial Activity against Pathogens

To assess antimicrobial activity, LAB strains were spot inoculated on MRS agar in close proximity to known human pathogenic bacteria, such as Staphylococcus aureus and E. coli. After a 24-hour incubation period at 37°C, the zones of inhibition surrounding the LAB colonies were assessed. Greater zones showed more potent antibacterial qualities, perhaps because of the synthesis of organic acid or bacteriocin [20].

2.7 Antibiotic Susceptibility Test

The Kirby-Bauer disk diffusion method was used to identify the isolates' profile of antibiotic resistance. On Mueller-Hinton agar plates, bacterial cultures were evenly distributed, and discs impregnated with antibiotics were positioned on top. Zones of inhibition were evaluated following a 24-hour incubation period at 37°C [21].

2.8 Statistical analysis

The data collected will be analytically analyzed using SPSS software. Results will be presented as mean $\pm$ standard deviation (SD). A one-way analysis of variance (ANOVA) was performed to determine significant differences before and after fermentation of rice samples. A p-value of less than 0.05 ($p < 0.05$) will be considered statistically significant. Graphs and bar charts will be generated to visually represent the data, and all experiments will be conducted in triplicates to ensure accuracy and reproducibility.

3 Results and Discussion

The present study investigated the Total microbial diversity, pH Dynamics, Nutrient content and Enzymatic Activities of naturally fermented rice prepared through three different cooking methods -earthen pot-cooked, steel vessel-cooked, and pressure-cooked- by analysing various biochemical and enzymatic parameters at 0 and 16 hours of spontaneous fermentation.

3.1 Total count of *Lactobacillus* population in cooked rice samples before and after Fermentation

Based on the results represented in Table 1, the Total Viable Count (TVC) was significantly higher in pot-cooked rice, documented at 7.5×10^7 CFU/g, followed by steel-cooked rice with 5.2×10^7 and pressure-cooked rice with 4.2×10^6 CFU/g. Similarly, the LAB count, which serves as an indicator of potentially beneficial microbial populations, was highest in pot-cooked

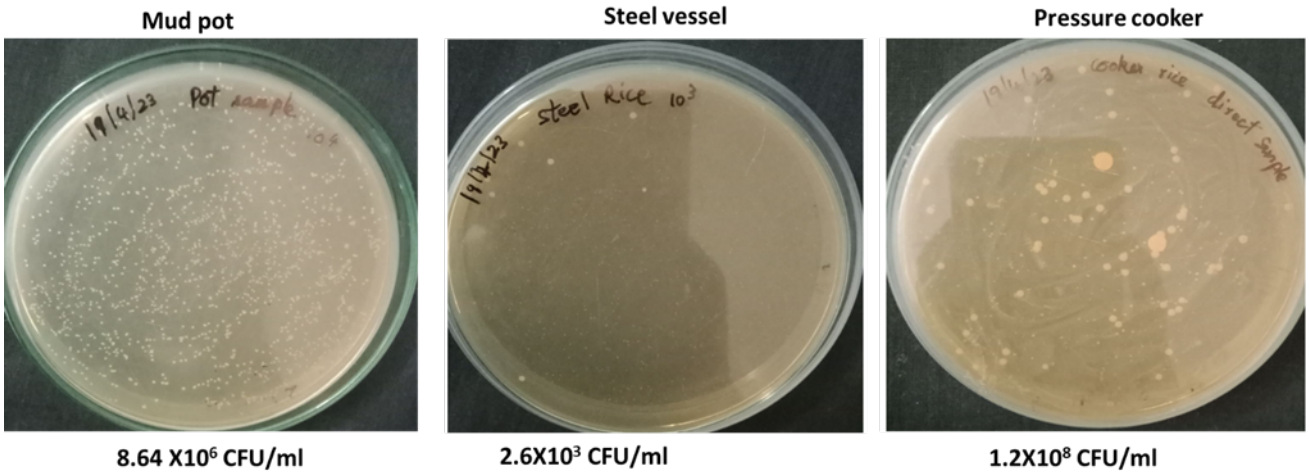

Fig 1. Total plate count of fermented rice samples on MRS agar plate

rice (6.9×10^7 CFU/g), moderate in steel-cooked rice (3.5×10^7 CFU/g), and lowest in pressure-cooked rice (1.8×10^6 CFU/g). The yeast population, known for its role in food spoilage and fermentation, followed a comparable trend: pot-cooked rice (2.3×10^7 CFU/g), in steel-cooked rice (1.2×10^6 CFU/g) and in pressure-cooked rice (5.0×10^4 CFU/g).

These results highlighted that pot-cooked rice harbors a greater abundance of microbial life, which may be attributed to its relatively lower cooking temperature and longer post-cooking cooling period, conditions more favourable for microbial survival or post-cooking colonization. Pressure cooking, which subjects rice to high temperature and pressure, was effective in minimizing microbial load, confirming its efficacy as a sterilizing cooking method.

The Shannon Diversity Index (H′) provided further insights into the microbial ecology of the samples. Pot-cooked rice exhibited the highest microbial diversity with a Shannon index of 0.9982, suggesting a relatively balanced and rich community of microorganisms. Steel-cooked rice showed moderate diversity (H′ = 0.7368), while pressure-cooked rice had the lowest diversity (H′ = 0.65), reflecting both reduced species richness and dominance of certain microbial groups. [22'23] demonstrate through their work that fermented rice bran along with sour rice shows increasing promise as a carrier of beneficial probiotic strains. Probiotic attributes of *Lactiplantibacillus plantarum* EM and *Weissella confusa GCC_19R1* have been shown to be promising through tests that evaluated both survivability and functional health benefits

Therefore, Cooking methods significantly influence the microbial load and diversity in rice. Pressure cooking effectively reduces microbial risks, while traditional pot-cooking retains higher microbial diversity, including beneficial LAB. The choice of cooking method should align with the desired balance between food safety and microbial functionality.

Table 1. Total Microbial diversity of fermented rice samples

Parameter	Pot-Cooked Rice	Steel-Cooked Rice	Pressure-Cooked Rice
Total Viable Count (TVC)(CFU/g)	7.5 x 10^7	5.2 x 10^7	4.2 x 10^6
Lactic Acid Bacteria (LAB)(CFU/g)	6.9 x 10^7	3.5 x 10^7	1.8 x 10^6
Yeast Count (CFU/g)	2.3 x 10^7	1.2 x 10^6	0.5 x 10^5
Shannon Diversity Index (H')	0.9982 (Moderate to high microbial diversity)	0.7368 (Moderate microbial diversity)	0.65 (Lower microbial diversity)

3.2 pH and Acidification Potential of fermented rice samples

The initial pH values at 0 hours were highest in pressure-cooked rice (5.8), followed by steel-cooked (5.6), and pot-cooked rice (5.2). After 16 hours of fermentation, a significant reduction in pH was observed across all samples, with the most pronounced acidification in pot-cooked rice (pH 4.2), indicating robust microbial fermentation. The steel-cooked rice reached a pH of 4.8, while the pressure-cooked rice showed a relatively modest decrease to 5.2. The extent of acidification reflects the

metabolic activity of lactic acid bacteria (LAB), with pot-cooked rice offering the most favourable substrate conditions for LAB proliferation and organic acid production.

3.3 Titratable acidity of fermented rice samples

The titratable acidity of rice samples was evaluated at 0 hours and after 16 hours of fermentation. The initial titratable acidity values were observed as 0.065% in pot-cooked rice, 0.071% in steel-cooked rice, and 0.029% in pressure-cooked rice. After 16 hours of fermentation, there was a notable increase in acidity across the samples. The highest rise in titratable acidity was observed in pot-cooked rice, which reached 0.55%, and pressure-cooked rice, which reached 0.39%. It's interesting to note that steel-cooked rice only slightly increased, reaching a final acidity of 0.042%. Fermentation of rice by lactic acid bacteria (LAB) results in a marked drop in pH due to the production of lactic and acetic acids. The fermentation duration of 6–24 hours under specific conditions leads to a pH decrease from 5.0–6.1 down to 3.7–4.5 based on the LAB strain selection. The acidic environment attained through this process functions as an important method to halt spoilage microorganisms while lengthening product shelf stability. The same acidification behaviour is seen in rice-based batters combined with beverages because of LAB's high acid-generating capacity [24, 25, 26].

The observed increase in titratable acidity after 16 hours of fermentation suggests strong microbial metabolism, particularly the generation of organic acids like lactic acid. Pot-cooked rice showed the greatest increase in acidity (from 0.065% to 0.55%), indicating a favorable habitat for fermentative microorganisms, most likely due to its porous texture and moisture retention, both of which encourage microbial growth. In contrast, pressure-cooked rice, which had the lowest beginning acidity (0.029%), increased to 0.39% during fermentation. This mild increase could be related to partial denaturation of rice proteins and the breakdown of complex carbohydrates during pressure cooking, which provide fermentable substrates for microbial action.

Table 2. Analysing the pH Dynamics, Titratable acidity Nutrient content and Enzymatic Activities of Fermented Rice Samples

Parameters	Pot-Cooked Rice	Steel-Cooked Rice	Pressure-Cooked Rice
pH			
pH (0 hrs)	5.2	5.6	5.8
pH (after 16 hrs)	4.2	4.8	5.2
Titrable acidity			
Titrable acidity (0 hrs)	0.065	0.071	0.029
Titrable acidity (16 hrs)	0.55	0.042	0.39
Reducing Sugar content			
Before Fermentation (mg/g)	25.9	17.63	14.96
After Fermentation (mg/g)	3.96	5.2	7.56
Total Protein content			
Before Fermentation (mg/g)	11.25	6.48	6.23
After Fermentation (mg/g)	9.25	8.47	8.52
Total Lipid content			
Before Fermentation (g/100 g)	4.23	3.96	4.9
After Fermentation (g/100 g)	3.15	2.81	3.98
Enzymatic activity			
Amylase Activity (U/g)	11.26	8.96	7.85
Protease Activity (U/g)	21.6	16.8	12.5
Phytase Activity (U/g)	8.56	6.9	5.8

The acidity of steel-cooked rice very slightly increased (from 0.071% to 0.042%), which may indicate a limited microbial activity, or suppressive conditions brought on by deeper cooking or thermal inactivation of microbiota. The increase in titratable acidity in pot and cooker rice indicates a successful fermentation process with active acidogenic bacteria, potentially enhancing the preservation, safety, and nutritional value of the rice. These findings underline the role of cooking method in modulating fermentative microbial behaviour and acid production in rice-based fermented foods.

3.4 Nutrient content of fermented rice samples

The initial reducing sugar content was highest in pot-cooked rice (25.9 mg/g), suggesting better retention or availability of fermentable sugars due to minimal nutrient loss. Steel-cooked and pressure-cooked rice showed lower levels at 17.63 mg/g and 14.96 mg/g respectively. After 16 hours, all samples exhibited a marked reduction in sugar levels due to microbial utilization. Pot-cooked rice had the lowest residual sugar (3.96 mg/g), indicating the highest microbial metabolic activity, followed by steel-cooked (5.2 mg/g) and pressure-cooked rice (7.56 mg/g). The decline in sugar content supports the efficient carbohydrate metabolism by LAB, essential for fermentation progression.

Total protein content before fermentation was again highest in pot-cooked rice (11.25 mg/g), compared to steel-cooked (6.48 mg/g) and pressure-cooked (6.23 mg/g). Post-fermentation, a mild decrease was observed in pot-cooked rice (9.25 mg/g), possibly due to proteolytic breakdown into peptides and amino acids, enhancing digestibility. Interestingly, steel-cooked and pressure-cooked rice exhibited an increase in protein levels (8.47 mg/g and 8.52 mg/g, respectively), which may result from microbial biomass contribution or increased protein extractability due to enzymatic action. These findings imply that pot-cooked rice not only preserves protein better but also allows for optimal proteolytic activity during fermentation.

However, the total lipid content of the rice samples showed a noticeable decrease after fermentation across all three types. Initially, the lipid content was 4.23 g/100 g, 3.96 g/100 g, and 4.90 g/100 g in the respective samples. After fermentation, these values decreased to 3.15 g/100 g, 2.81 g/100 g, and 3.98 g/100 g. This reduction in lipid levels can be attributed to the metabolic activity of fermenting microorganisms, which utilize lipids as an energy source. The breakdown of lipids by microbial lipases during fermentation likely contributed to this decline. The extent of reduction varied slightly between samples, possibly due to differences in microbial composition, initial lipid availability, or cooking method. Overall, the results indicate that fermentation effectively reduces lipid content in cooked rice samples, which may influence the nutritional and functional properties of the final product. Throughout fermentation the biochemical elements within rice undergo substantial changes. The early fermentation time leads to increased sugar levels mainly thanks to amylase enzymes that break down complex starches into basic sugars. The metabolism of microbes during extended fermentation consumes the sugar content leading to decreased levels in rice The protein content remains steady during fermentation, yet the digestion process improves because complex proteins convert into small peptides and amino acids. Fermentation generally results in minimal changes in the lipid composition of food materials. The functional value of fermented rice receives enhancement from certain microbial actions which include lipids becoming more available through liberation of bound compounds and beneficial lipid metabolite production. The stabilization of lipids through LAB fermentation takes place indirectly by decreasing lipid destruction processes. Through fermentation rice becomes a better food product because it gains improved nutritional attributes with added functional qualities for better bioavailability and health benefits [27, 28].

3.5 Enzymatic Activities of fermented rice samples

Pot-cooked rice demonstrated superior enzymatic activity across all parameters: based on the results obtained amylase activity was highest in pot-cooked rice (11.26 U/g), indicating active starch degradation and glucose release, supporting LAB metabolism. Steel-cooked and pressure-cooked rice showed lower activities at 8.96 U/g and 7.85 U/g, respectively. Protease activity is responsible for protein hydrolysis, was also greatest in pot-cooked rice (21.6 U/g), followed by steel-cooked (16.8 U/g), and pressure-cooked (12.5 U/g). This highlights efficient proteolysis and potentially improved amino acid bioavailability. Phytase Activity, critical for breaking down ant nutritional phytates, showed a similar trend as pot-cooked rice (8.56 U/g) > steel-cooked (6.9 U/g) > pressure-cooked (5.8 U/g). Therefore, enhanced phytase activity in pot-cooked samples suggests better liberation of bound minerals and nutrients, further improving the functional quality of the rice.

The collective data suggest that pot-cooked rice provides the most favourable conditions for spontaneous fermentation, as evidenced by lower pH, greater sugar consumption, and superior enzymatic activities. The steel vessel-cooked rice showed intermediate performance, while pressure-cooked rice lagged in nearly all tested parameters, likely due to thermal degradation of nutrients and altered rice matrix properties. The findings underscore that the method of rice preparation critically affects its fermentability, biochemical transformations, and probiotic potential. This study reinforces the value of traditional cooking practices, particularly pot-cooking, in enhancing the functional and health-promoting attributes of fermented rice. It offers a scientific basis for promoting culturally rooted, cost-effective dietary interventions aimed at improving gut health and nutrition through naturally fermented foods.

3.6 Molecular and biochemical confirmation of fermented pot cooked rice samples

Totally, ten bacterial colonies were isolated from pot cooked rice showed higher population of probiotics. Based on the Table 2, the bacterial organisms were identified biochemically

and confirmed through molecular sequencing. The strains *Lactococcus garvieae*, *Lactobacillus plantarum*, and *Lactiplantibacillus fermentum* revealed strong properties of probiotics among several Lactic Acid Bacteria (LAB). The strains displayed valuable characteristics including motility inhibition and their lack of catalase enzyme together with casein hydrolysis enzyme activity that doctors believe benefit gastrointestinal wellness. Furthermore, the presence *Lactobacillus fermentum* demonstrated two more advantageous properties through production of antimicrobial peptides and strengthened gut barrier feature.

The probiotic potential of *Bacillus pumilus* and *Bacillus altitudinis* was confirmed through the assessment of their strong enzymatic activities which included starch and casein hydrolysis. Therefore, extensive research evaluation of safety and efficacy will enable these species to become promising probiotic candidates. The broad category Enterobacteriaceae contains *Citrobacter freundii*, *Enterobacter cloacae*, *Escherichia coli* and *Enterobacter hormaechei* that exist commonly in fermented food yet contribute to understanding microbial ecosystem diversity. The LAB group demonstrates strong potential as probiotics, so these isolates become excellent choices to enhance gut health and beneficial microbe diversity.

Table 3. Biochemical identification of pot cooked Fermented Rice Samples

Pot rice colonies	Bacterial species	Gram Stain	MR TEST	VP TEST	Citrate test	Motility test	H_2S test	Catalase	Starch hydrolysis	Caesin hydrolysis	Lipid hydrolysis
Colony1	*Lactococcus garvieae*	(+) VE	(-) VE	(-) VE	(-) VE	NON MOTILE	(-) VE	(-) VE	(-) VE	(+) VE	(-) VE
Colony2	*Citrobacter freundi*	(-) VE	(-) VE	(+) VE	(+) VE	MOTILE	(+) VE	(+) VE	(-) VE	(-) VE	(-) VE
Colony3	*Bacillus pumilus*	(+) VE	(-) VE	(+) VE	(+) VE	MOTILE	(-) VE	(+) VE	(+) VE	(+) VE	(-) VE
Colony4	*Enterobacter clocae*	(-) VE	(+) VE	(+) VE	(+) VE	MOTILE	(-) VE	(+) VE	(+) VE	(-) VE	(-) VE
Colony5	*Bacillus altititudinis*	(+) VE	(+) VE	(+) VE	(+) VE	MOTILE	(-) VE	(+) VE	(+) VE	(-) VE	(-) VE
Colony6	*Lactobacillus plantarum*	(+) VE	(-) VE	(-) VE	(-) VE	NON MOTILE	(-) VE	(-) VE	(-) VE	(-) VE	(-) VE
Colony7	*Lactiplantibacillus fermentum*	(+) VE	(+) VE	(-) VE	(-) VE	Non MOTILE	(-) VE	(-) VE	(-) VE	(-) VE	(-) VE
Colony8	*Escherichia coli*	(-) VE	(-) VE	(+) VE	(+) VE	Motile	(-) VE	(+) VE	(-) VE	(-) VE	(-) VE
Colony9	*Lactobacillus fermentum*	(+) VE	(+) VE	(-) VE	(-) VE	NON MOTILE	(-) VE	(-) VE	(-) VE	(-) VE	(-) VE
Colony10	*Enterobacter hormaechei*	(-) VE	(+) VE	(+) VE	(+) VE	Motile	(-) VE	(+) VE	(-) VE	(-) VE	(-) VE

(+) – Positive; (-) – Negative

3.7 Probiotic characteristics of Fermented rice samples

The hydrophobicity and auto-aggregation properties of microbial colonies isolated from three types of fermented rice pot-cooked were determined and represented in Table 4. Hydrophobicity is an important indicator of microbial adhesion to intestinal mucosa, a key attribute for probiotic functionality. Whereas acid tolerance is critical for probiotic strains to survive gastric conditions. Alkaline pH tolerance, though less emphasized in probiotic evaluation, reflects the survivability in the upper intestinal tract and food systems.

The probiotic potential of the bacterial isolates from pot-cooked fermented rice was evaluated based on various probiotics parameters including hydrophobicity, autoaggregation, pH tolerance, bile salt tolerance, and NaCl tolerance. Among the isolates, *Escherichia coli* showed the highest hydrophobicity (64.72%) and substantial auto aggregation ability (56.7%), suggesting strong adhesion potential. However, due to its opportunistic pathogenic nature, its probiotic utility is limited and must be approached with caution.

Prominent Lactic Acid Bacteria (LAB), including *Lactobacillus plantarum*, *Lactiplantibacillus fermentum*, and *Lactobacillus fermentum*, demonstrated moderate to good hydrophobicity (ranging from 3.58% to 11.17%) and notable autoaggregation

Table 4. Probiotic characters of Fermented rice samples

Mud Pot rice	Hydrophobicity and auto aggregation (%)	pH-tolerance test		Bile salt tolerance test			NaCl (salt)tolerance test		
		pH 3	pH10	0.5%	1%	1.5%	0.5%	1%	1.5%
Lactococcus garvieae	0.67%	23.19	58.51	10.2	10.4	10.6	12.98	11.28	14.58
Citrobacter freundi	2.86%	21.2	43.2	10.1	10.1	10.08	12.66	9.81	9.81
Bacillus pumilus	6.82%	31.6	60.94	12.8	11.3	118.5	15.01	17.98	13.06
Enterobacter clocae	7.35%	15.56	37.76	10.1	10.6	93.8	13.27	9.66	9.74
Bacillus altititudinis	10.46%	28.01	23.82	10.7	12.8	13.4	52.7	60.69	53.32
Lactobacillus plantarum	3.58%	20.82	12.7	89.5	10.6	99.1	31.01	16.04	29.74
Lactiplantibacillus fermentum	11.17%	22.13	34.9	13.15	12.6	18.9	15.3	16.31	17.87
Escherichia coli	64.72%	56.7	23	63.56	67.89	70.4	26.8	29.6	31.9
Lactobacillus fermentum	8.57%	73	27.02	56.5	56.9	67.9	28.04	29.3	36.3
Enterobacter hormaechei	10.71%	52	22.8	67	68.1	69.5	28.97	30.6	35.2

(12.7%–34.9%), indicating fair cell surface adhesion properties which are desirable in probiotics. These LAB strains also exhibited good survival at extreme pH values (pH 3 and pH 10), simulating gastric and intestinal environments, with *L. fermentum* showing the highest acid tolerance (73% at pH 3). Bile salt and NaCl tolerance are important for gut survivability. *Lactobacillus plantarum* displayed excellent bile salt tolerance (89.5% at 0.5% bile), and *Lactobacillus fermentum* also showed consistent tolerance across varying concentrations. *Lactiplantibacillus fermentum* demonstrated good overall stress tolerance, suggesting its robust probiotic potential.

Bacillus pumilus and *Bacillus altitudinis*, though not traditional probiotics, showed strong tolerance to environmental stresses. *B. altitudinis* exhibited high NaCl tolerance (up to 60.69%), and *B. pumilus* had good bile and salt resistance, indicating their potential as next-generation probiotics or functional microbes in food applications. *Enterobacter species* and *Citrobacter freundii*, though showing some tolerance traits, are generally not preferred as probiotics due to potential pathogenicity. Their presence, however, reflects the microbial diversity of fermented rice. Overall, *Lactobacillus fermentum, Lactiplantibacillus fermentum, and Lactobacillus plantarum* stand out as the most promising probiotic candidates from pot-cooked fermented rice, due to their survivability under stress conditions, ability to adhere, and overall probiotic characteristics.

3.8 Antibiotics resistant analysis of pot cooked fermented rice samples

The antibiotic sensitivity profile of bacterial isolates from pot-cooked fermented rice revealed significant variations in resistance and susceptibility patterns (Table 5). Most isolates, including *Lactococcus garvieae*, *Citrobacter freundii*, *Bacillus pumilus*, *Enterobacter clocae*, and *Lactobacillus fermentum*, exhibited complete resistance (Nil zones of inhibition) to all tested antibiotics (Ampicillin, Streptomycin, Ciprofloxacin, Fluconazole, and Penicillin), indicating a potential for intrinsic or acquired resistance mechanisms. On the other hand, *Bacillus altitudinis* showed moderate sensitivity, with inhibition zones of 12 mm for Ampicillin, 16 mm for Streptomycin, 20 mm for Ciprofloxacin, and 10 mm for Penicillin, suggesting partial susceptibility, particularly to Ciprofloxacin. Notably, *Lactobacillus plantarum* and *Lactiplantibacillus fermentum*, key probiotic candidates, demonstrated significant sensitivity to the antibiotics tested. *L. plantarum* showed strong inhibition zones: 18 mm (Ampicillin), 20 mm (Streptomycin), 19 mm (Ciprofloxacin), and 14 mm (Penicillin), suggesting it remains sensitive to common antibiotics, which is considered a safe probiotic trait, reducing the risk of contributing to antibiotic resistance gene pools. Additionally, *Lactiplantibacillus fermentum* had good sensitivity, exhibiting inhibitory zones of 9 mm for penicillin, 17 mm for streptomycin, 19 mm for ciprofloxacin, and 11 mm for ampicillin. This sensitivity profile lends even more credence to its probiotic safety. Responses from *Escherichia coli* and *Enterobacter hormaechei* varied. E. coli was most susceptible to Ciprofloxacin (20 mm) and Streptomycin (25 mm), but less sensitive to Ampicillin (9 mm) and Penicillin (10 mm). Since opportunistic infections are known to exhibit partial resistance, *E. hormaechei* shown low to moderate sensitivity to all treatments. The multidrug resistance observed in other isolates calls for careful consideration before considering them for probiotic or food applications, but overall, Lactobacillus plantarum and *Lactiplantibacillus fermentum* stand out as the safest and most effective probiotic candidates due to their obvious sensitivity to antibiotics. Our study was compared with recent research conducted by[22] also highlights the

detection of vancomycin resistance genes among LAB isolated from traditional fermented rice water, indicating the potential for these bacteria to survive in the presence of clinically important antibiotics. The widespread occurrence of drug-resistant LAB in fermented rice creates food safety risks because it poses the potential for resistance genes to move among bacteria affecting human health transmission through food. Monitoring antibiotic resistance in fermented rice products becomes essential because their consumption contributes to spreading resistant bacteria throughout human gut microbiota [30, 31].

Table 5. Antibiotic sensitivity of Pot cooked Fermented Rice sample

	Ampicillin	Streptomycin	Ciprofloxacin	Fluconazole	Penicillin
Lactococcus garvieae	Nil	Nil	Nil	Nil	Nil
Citrobacter freundi	Nil	Nil	Nil	Nil	Nil
Bacillus pumilus	Nil	Nil	Nil	Nil	Nil
Enterobacter clocae	Nil	Nil	Nil	Nil	Nil
Bacillus altititudiNils	12mm	16mm	20mm	Nil	10mm
Lactobacillus plantarum	18mm	20mm	19mm	Nil	14mm
Lactiplantibacillus fermentum	11mm	17mm	19mm	Nil	9mm
Escherichia coli	9mm	25mm	20mm	Nil	10mm
Lactobacillus fermentum	Nil	Nil	Nil	Nil	Nil
Enterobacter hormaechei	9mm	16mm	16mm	Nil	6mm

3.9 Antimicrobial resistant analysis of pot cooked fermented rice samples against pathogens

Different inhibitory profiles were found in the antibacterial activity of bacterial isolates from pot-cooked fermented rice against common pathogens (*Candida albicans, Escherichia coli, and Streptococcus aureus*). With inhibition zones of 11 mm against *S. aureus* and *E. coli* and 8 mm against *Candida albicans*, *Citrobacter freundii* demonstrated broad-spectrum antibacterial activity and a moderately antagonistic impact. With inhibition zones of 13 mm (*S. aureus*), 11 mm (*E. coli*), and 10 mm (*C. albicans*), *Bacillus pumilus* also showed antimicrobial activity against all three test species, indicating that it is a promising candidate with remarkable antibacterial potential.

Selective inhibition was observed in *Lactobacillus fermentum* and *Lactococcus garvieae*. While *L. fermentum* demonstrated considerable inhibition against *S. aureus* (10 mm), *E. coli* (10 mm), and *Candida albicans* (7 mm), suggesting weak to moderate antibacterial characteristics, *L. garvieae* produced a 10 mm zone against *E. coli* only. On the contrary, *Lactobacillus plantarum*, *Lactiplantibacillus fermentum*, *Bacillus altitudinis*, *Enterobacter hormaechei*, and *E. coli* (isolated from fermented rice) did not exhibit any inhibition against the tested pathogens, indicating a lack of direct antimicrobial effect under the tested conditions. Therefore, *Bacillus pumilus* and *Citrobacter freundii* showed the most promising antimicrobial activity among the isolates, while certain probiotics like *L. plantarum* and *L. fermentum* may exert their probiotic effects through other mechanisms such as competitive exclusion or acid production rather than direct antimicrobial action. Based on the study using rice bran fermented with *Lactiplantibacillus plantarum* EM exhibits strong antimicrobial activity (100–400 AU/mL) against foodborne pathogenic bacteria such as *Bacillus cereus* and spoilage fungi like *Aspergillus fumigatus*, highlighting its potential as a functional food with health-promoting properties [23]. A study conducted by [17] revealed that traditional rice-based fermented beverages in India also identifies the presence of beneficial lactic acid bacteria (LAB), including *Lactobacillus plantarum*, which contribute to the antimicrobial effects observed during fermentation. These findings emphasize the dual nature of fermented rice products: while they offer natural antimicrobial benefits, careful monitoring and quality control are essential to prevent the spread of antimicrobial resistance through the food chain.

4 Conclusion

This study provides conclusive evidence that cooking methods significantly affect the microorganisms and biochemical attributes in addition to the probiotic features of spontaneously fermented rice. Food fermentation through pot cooking produced fermented rice with the richest microbial diversity including beneficial lactic acid bacteria *Lactobacillus plantarum*, *L. fermentum* and *Lactococcus garvieae*. From the results, other bacterial isolates demonstrated beneficial probiotic properties in addition to their capability to tolerate pH and bile salts and show moderate hydrophobicity and pathogen-threatening capability. Pot-cooked rice samples revealed increased enzymatic activity together with higher lipid oxidation rates because they enabled

active microbial fermentation. The microbial growth along with probiotic functionality remained lower in steel- and pressure-cooked rice samples because higher-pressure cooking can prevent substrate effectiveness or inhibit microbial settlements. The antibiotic sensitivity tests established the safety properties of selected bacterial isolates, yet the antimicrobial resistance analysis uncovered possible antagonistic traits among *Bacillus pumilus* and *Citrobacter freundii*. In comparison to other research done [24] this research is first-of-its-kind study where more emphasis is given on the different cooking methods that are widely used in Indian homes. Eventually it demonstrates that traditional cooking techniques both protect rice nutritional components during pot preparation while creating suitable conditions for probiotic food development. Pot-fermented rice emerges as a beneficial dietary component which extends from traditional culture while delivering natural probiotic benefits. Mud pot-cooked and fermented rice emerges as a beneficial dietary component which extends from age-old yet slowly disappearing traditional culture while delivering natural probiotic benefits. It is evident that conventional methods of cooking still stand tall when it comes to nutrition retention and in providing better health prospects. This concludes that utilizing earthenware pots may hold promise for evolution of natural probiotics through cost-effective and culturally pertinent techniques. Additional studies employing animal models and clinical research trials need to prove the health benefits and functional food potential of this product.

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