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Integrating physicochemical and microbial characterization of red rice broth fermented over an 18-hour period augmented with metagenomic and metabolomic approaches

Madhumitha Hariharamohan, [a] Manali Chindarkar, [a] Hruta Sundar Swain, [b] N. Rajesh [c] and Vidya Rajesh *[a]

Fermentation enhances the nutritional properties of foods. Fermented water of Kerala red rice (*Oryza sativa* L. subsp. *indica*), traditionally consumed in South India remains underexplored scientifically. This study characterizes the nutritional, microbial, and metabolite profiles of Kerala red rice water (broth) after 18 hours of natural fermentation using biochemical assays, shotgun whole-genome metagenomic sequencing (Illumina NovaSeq X Plus), untargeted gas chromatography-mass spectrometry (GC-MS) metabolomics, and a phytase-mediated mineral release assay. Fermentation enhanced nutritional quality with increase in carbohydrates by 22.7%, protein by 163.52%, and free amino acids by 35.47% compared to unfermented controls. Phytase activity rose from negligible levels to 0.12 U mL^{-1}. Metagenomics identified 50 taxa, dominated by Proteobacteria (59.63%) and Firmicutes (40.12%), with ~34% of the community carrying phytase-encoding genes. Dominant genera included *Pantoea*, *Saccharibacillus*, and *Bacillus*. Fermentation also enhanced mineral release, with calcium, iron, and zinc in the fermented rice water showing increases of approximately 1190%, 566%, and 93%, respectively, relative to unfermented controls over a 360 min *in vitro* digestion period. These findings provide the first integrated insight bridging traditional dietary practice with modern analytical science.

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1. Introduction

Fermentation is a time-honored food processing method traditionally used to extend shelf life, enhance flavor, and improve food's digestibility and nutritional quality.[1,2] In recent years, fermented foods have attracted renewed scientific interest for their enrichment with probiotics, prebiotics, and postbiotics known to modulate gut microbiota, support immune function, and regulate metabolic health.[3] In South Asia, fermentation remains an integral culinary practice, particularly in rice-consuming regions where mildly acidic rice broths, produced by soaking cooked rice overnight, are consumed for hydration, digestive comfort, and thermoregulation.[4–6] Such traditional practices hold significant potential in addressing micronutrient malnutrition, or "hidden hunger", a pervasive global challenge affecting an estimated two billion individuals, particularly in low- and middle-income countries.[7] Given that iron and zinc deficiencies are especially prevalent and impair immune function, growth, and neurodevelopment,[8] there is an urgent need to identify sustainable, food-based solutions, such as these fermented rice preparations, to improve mineral bioavailability and intake.

Among regional varieties, Kerala red rice, also known as Palakkad matta rice (*Oryza sativa* L. subsp. *indica*) is a prominent whole-grain variety characterized by its red pericarp, coarse texture, and high bran retention. It is rich in dietary fiber, micronutrients, and antioxidants, and notably possesses a lower glycemic index.[9] Recognizing its unique properties, Palakkad matta rice was awarded Geographical Indication (GI) status in 2007.[10] While rich in nutrients, like many other cereals, this rice also contains the antinutrient phytate, which can inhibit mineral absorption by chelating essential minerals—including iron, zinc, calcium, magnesium, and manganese—into insoluble complexes that limit gastrointestinal absorption.[11–13] Natural fermentation serves as a traditional method to improve the nutritional profile by breaking down these complexes to increase mineral bioavailability (Fig. 1).

Recent work has explored probiotic diversity in traditional fermented rice products[14] and optimized fermentation for a few pigmented rice beverages.[15] However, there are no studies on

[a]*Department of Biological Sciences, Birla Institute of Technology and Science Pilani – Hyderabad Campus, Jawahar Nagar, Kapra Mandal, Medchal District, Telangana 500078, India. E-mail: vidya@hyderabad.bits-pilani.ac.in*

[b]*Department of Pharmacy, Birla Institute of Technology and Science Pilani – Hyderabad Campus, Jawahar Nagar, Kapra Mandal, Medchal District, Telangana 500078, India*

[c]*Department of Chemistry, Birla Institute of Technology and Science Pilani – Hyderabad Campus, Jawahar Nagar, Kapra Mandal, Medchal District, Telangana 500078, India*

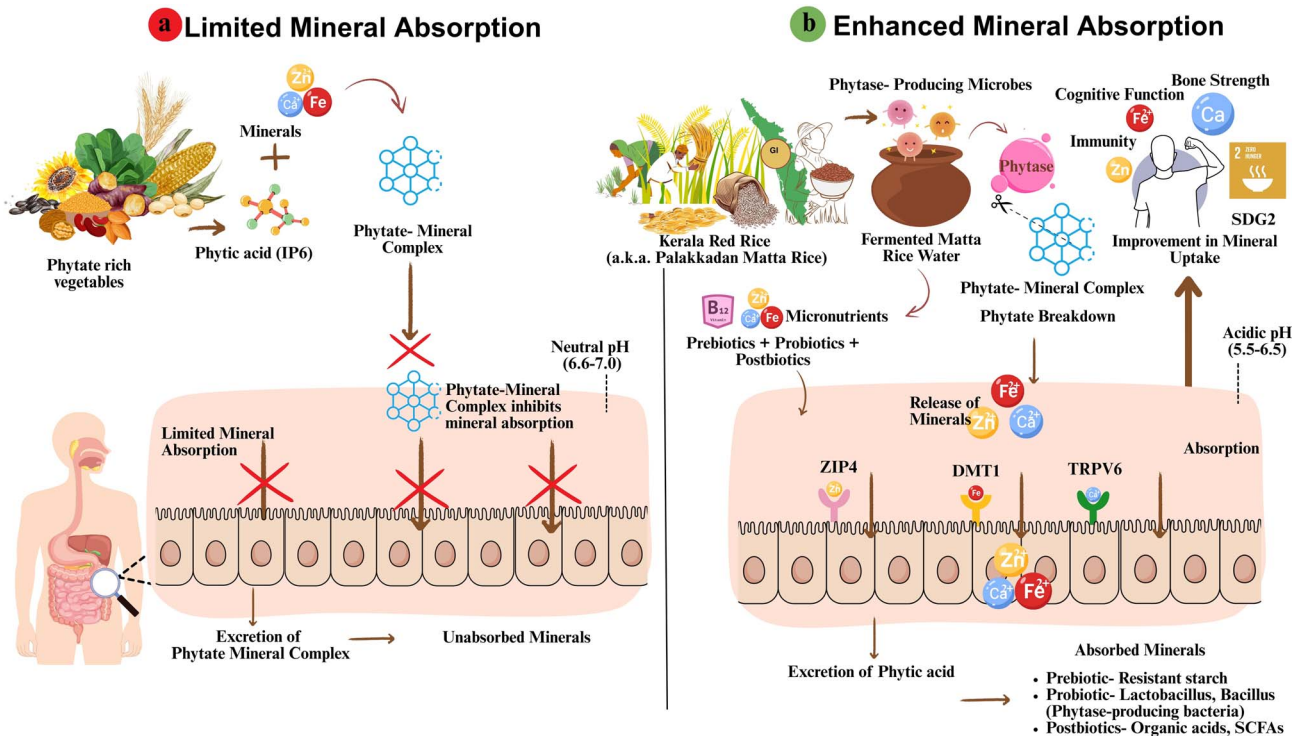

Fig. 1 Schematic representation of mineral bioavailability during fermentation of Kerala red rice (*Oryza sativa* L. subsp. *indica*). (a) In the unfermented state, phytic acid binds Fe^{2+}, Zn^{2+}, and Ca^{2+}, limiting their bioavailability. (b) During fermentation, phytase-producing microbes degrade phytic acid, releasing bound minerals. Acidic pH and organic acids enhance mineral solubility, facilitating uptake *via* proposed intestinal transport pathways (ZIP4 for Zn^{2+}, DMT1 for Fe^{2+}, and TRPV for Ca^{2+}).[73,74] Prebiotics, probiotics, and postbiotics contribute to gut health, with outcomes including improved bone strength, cognitive function, immunity, and sustainable nutrition (SDG2). Historical, geographical, and cultural contexts of red rice fermentation underscore its relevance.[75]

Kerala red rice with respect to its microbial diversity, enhanced nutritional bioavailability and metabolite profiling. Moreover, prior studies on fermented rice beverages have largely focused on fermentation optimization, with limited comprehensive metagenomic characterization of microbial diversity and metabolic functionality.[14,15] The biochemical landscape of Kerala red rice fermentation remains poorly characterized, with no prior untargeted GC-MS metabolomic profiling to identify diverse chemical features (alcohols, organic acids, amino acid derivatives). Most critically, empirical data on the kinetic release of minerals (Ca, Fe, Zn) from phytate complexes are absent. This study addresses these gaps by integrating shotgun whole-genome metagenomics (WGS) for functional annotation of microbial attributes, untargeted GC-MS for metabolites, and a validated phytase-mediated mineral release assay (FTIR/XRF/SEM-EDX confirmed) to quantify mineral liberation over 360 minutes.

Our study is based on the hypothesis that natural fermentation of Kerala red rice water induces growth of specific phytase-producing microbial communities, which through secretion of endogenous phytases and creation of an acidic environment, synergistically promote hydrolysis of antinutrient phytate complexes, significantly enhancing bio-accessibility of essential minerals. Fermentation thus transforms a simple soaked rice water broth into a nutritionally superior beverage for populations at risk of micronutrient malnutrition.[16–18] The hypothesis was tested and validated in this study by focusing on the specific objectives listed below: (1) assess nutritional changes by quantifying macro- and micronutrient shifts after 18 hours of natural fermentation; (2) characterize microbial communities *via* WGS, focusing on phytase-encoding genes and probiotic-associated taxa; (3) validate phytase induction and mineral release by measuring phytase activity and correlating it with phytate–mineral complex degradation and calcium/iron/zinc release; and (4) profile the functional metabolites using untargeted GC-MS metabolomics.

2. Materials and methods

2.1 Sample preparation and fermentation

One hundred grams (100 g) of raw Kerala red rice were rinsed thrice with distilled water and cooked in 300 mL of water for 30 min. After cooling to ambient temperature (~25 °C), the cooked rice was immersed in 700 mL of filtered drinking water (1 : 7 w/v) and transferred to a traditional earthenware pot. The mixture underwent fermentation at room temperature (25 °C) for 18 h. The 18 h fermentation duration was chosen to align with traditional overnight soaking practices, promote microbial growth, and to induce the optimal acidification phase (pH < 5.5), which activates phytase enzymes required for mineral liberation. Samples of the liquid fraction (rice water) were collected at 0 h (before fermentation as control) and 18 h

(fermented) for downstream analysis. The entire preparation and fermentation process were performed in three independent biological replicates ($n = 3$), each consisting of a separate fermentation batch. The work was planned and executed at BITS Pilani – Hyderabad Campus, Telangana, India.

2.2 Nutritional profiling of fermented red rice water

Nutritional profiling assessed macronutrients (total carbohydrates, proteins, fats, and amino acids) and micronutrients (elemental profiling) before and after 18 h of fermentation. All measurements are reported on a wet-weight (as-sampled) basis, except ash, which is reported on a dry-weight basis.

2.2.1 Macronutrient analysis. Total carbohydrates: quantified using the phenol–sulfuric acid method.[19] One millilitre of sample was reacted with 5% phenol and 98% sulfuric acid. Absorbance was measured at 490 nm using a UV-vis spectrophotometer (Beckman Coulter DU® 730) and concentrations were derived from a glucose calibration curve ($R^2 > 0.99$).

Proteins and free amino acids: total protein content was determined *via* the Lowry method[20] using Bovine Serum Albumin (BSA) as the standard, with absorbance recorded at 660 nm (Beckman Coulter DU® 730). Total free amino acids (TFAA) were quantified using the ninhydrin assay:[21] samples were heated with ninhydrin reagent in a boiling water bath for 15 min, cooled, and measured at 570 nm against a glycine standard curve (Beckman Coulter DU® 730).

Crude fats: determined gravimetrically using a modified liquid–liquid extraction. Five milliliters of sample were extracted with *n*-hexane (1 : 2 w/v) in three cycles. The organic phase was evaporated under nitrogen using a Rotary evaporator (Heidolph Laborota 4001), and the residual fat was weighed.

2.2.2 Micronutrient and elemental profiling. Ash content: determined by incinerating 5 g of dried sample (10 mL wet weight) in a Muffle furnace (550 °C) by dry ashing until constant weight. Values reported as mg g^{-1} on a dry-weight basis.

Elemental analysis by ICP-MS: concentrations of Na, K, Mg, Ca, Mn, Zn were quantified using Inductively Coupled Plasma Mass Spectrometry (ICP-MS) by acid digestion.[22,23] Instrument: iCAP Rqplus (Thermo Fisher Scientific) with iSC-65 autosampler. Samples were pre-filtered (0.22 μm) and diluted overnight in 2% HNO_3. External calibration was performed using Multi-Element Standard IV (Supelco, Sigma-Aldrich, Cat. No. 1113550100) across five levels (0.05–10 mg L^{-1}), with internal standard correction for matrix effects. All assays were performed in triplicate at BITS Pilani – Hyderabad Campus, Telangana, India. Calibration linearity: Zn ($R^2 = 0.996$), Ca ($R^2 = 0.997$), Fe ($R^2 = 0.998$).

2.3 Microbial community profiling (metagenomics)

Whole-genome shotgun (WGS) metagenomic sequencing was outsourced to MedGenome Labs Ltd, Bangalore, India, for microbial profiling. The sample (FRW_2024_01) was sequenced on the Illumina NovaSeq X Plus platform (151 bp, paired-end), generating 3.72 GB of data before adapter trimming (Q 30% > 92%, mean $Q = 39.26$, GC content = 47.67%). Adapter trimming was performed using fastq-mcf (v1.04.803), followed by *de novo* assembly using MEGAHIT (v1.2.9), producing 9211 contigs (≥1000 bp; largest contig = 904 399 bp; N50 = 11 330 bp) [SI 2B MedGenome Report, page 5, Table S2]. ORF prediction was executed *via* Prodigal (v2.6.3), filtering for ORFs ≥200 bp (63 728 total genes, 25 432 genes ≥200 bp). Species-level identification, taxonomic classification (phylum, genus, species), and SEED pathway functional annotation were performed using MEGAN6. (SI 2A, Fig. S1 and 2B). Diversity indices (Shannon, Simpson, Richness, Evenness) were calculated in-house from the MedGenome species abundance data and integrated SEED and BV-BRC databases. (SI 2C).

2.4 Phytate-bound mineral release and phytase assay

All assays were performed at BITS Pilani – Hyderabad Campus, Telangana, India.

2.4.1 Synthesis of phytate–mineral complexes. Phytate–Ca, phytate–Zn, and phytate–Fe complexes were synthesized by mixing sodium phytate (Sigma-Aldrich) with $CaCl_2 \cdot 2H_2O$, $ZnSO_4 \cdot 7H_2O$, and $FeSO_4 \cdot 7H_2O$, respectively in molar ratios: 1 : 1 for all three metals.[24] Solutions were stirred at ~25 °C for 1 h, filtered through Whatman no. 1 filter paper, washed with deionized water, and vacuum-dried. These complexes model mineral phytates resulting from natural food-borne phytic acid (myo-inositol hexakisphosphate).

2.4.2 Complex validation. Analytical tools like FTIR, XRF and SEM-EDX were used for analysis of phytate metal binding, elemental presence, and structural and spatial features respectively.

FTIR: Bruker ALPHA II spectrometer (Bruker), spectral range 4000–500 cm^{-1}, resolution 4 cm^{-1}, 40 ATR direct scans, automatic focus, automatic background, powder sample preparation, baseline-corrected. Key focus region: 1040–1080 cm^{-1} (phosphate–metal region).

XRF: PANalytical Epsilon 1 (Malvern Panalytical), system default voltage and current, count time 30 seconds, sample preparation: direct sample in a pressed thin mylar film.

SEM-EDX: FEI Apreo LoVac SEM (Thermo Fisher Scientific) with EDX detector, 15× kV acceleration voltage, 10× magnification.

2.4.3 Release kinetics. 50 mg of each complex was incubated in 10 mL of water, 0 h fermented rice water, and 18 h fermented rice water (25 °C) for comparative analysis. Two controls were maintained: (1) unfermented control (0 h rice water); (2) reagent-only blank (deionized water) for background. Aliquots were collected at 15, 60, 120, and 240 min for water and 0 h fermented rice water, with an additional 360 min for 18 h fermented rice water and mineral release was studied.

2.4.4 Elemental quantification of Zn, Ca and Fe was done by ICP-MS. Samples (1 : 10 diluted in 2% ultrapure HNO_3) were analyzed by ICP-MS (iCAP Rqplus, Thermo Fisher Scientific, iSC-65 autosampler). Calibration: ICP Multi-Element Standard IV (Supelco, Sigma-Aldrich, 0.05–10 mg L^{-1}). Linearity: Zn ($R^2 = 0.996$), Ca ($R^2 = 0.997$), Fe ($R^2 = 0.998$). All assays in triplicate.

2.4.5 Phytase activity assay in fermented red rice water. Phytase activity was measured by phosphorus release from sodium phytate using Fiske–Subbarow phosphate assay.[25] The 1 mL

reaction mixture contained 500 μL of 2.5 mM sodium phytate (0.1 M sodium citrate buffer, pH 3.0) and 500 μL enzyme sample (final substrate: 1.25 mM). After 15 min at 50 °C, the reaction was stopped with 10% TCA. Color was developed using an ammonium molybdate–acetone–sulfuric acid reagent and measured at 660 nm. Calibration used KH_3PO_4 (0–1000 nmol range, R^2 = 0.996). One unit (U) was defined as 1 μmol of phosphorus liberated per minute (U ml^{-1} and nkat mL^{-1}; 1U = 16.67 nkat).

2.5 Statistical analysis

All experiments were conducted in triplicate ($n = 3$) independent biological replicates, and results are presented as mean ± standard deviation (SD). For fermentation data (0 h *vs.* 18 h), statistical significance was evaluated using a paired *t*-test, with a significance threshold of $p < 0.05$. For mineral release studies across multiple time points (15, 60, 120, 360 min), multiple independent *t*-tests comparing each time point to baseline (0 h) were performed using the "Multiple *t*-tests (one per row)" option in GraphPad Prism, with statistical significance set at $p < 0.05$. No correction for multiple comparisons was applied as each time point was compared independently to the same baseline. All statistical analyses were carried out using GraphPad Prism (version 8.4.2).

2.6 Untargeted metabolomic profiling

Untargeted gas chromatography-mass spectrometry (GC-MS) metabolomic profiling was outsourced to MedGenome Labs Ltd, Bangalore, India. Raw GC-MS data were processed through the vendor pipeline (SI 4A). Metabolites were extracted using a modified Bligh–Dyer method and derivatized with MTBSTFA/acetonitrile/triethylamine (3 : 1 : 1, v/v/v) at 60 °C for 1 h. Samples were analyzed using an Agilent 5975 GC-MS system (splitless mode; helium carrier; EI ionization, 70 eV) and cross-validated on a Shimadzu GCMS-QP2020NX. Spectral matching against NIST17.L library was performed using the LibSearch algorithm.[25–31] Data processing thresholds: spectral match ≥40%, retention time 2–20 min, peak area ≥10 000 units. Redundant features filtered by CAS number and retention time overlap. After quality control, 80 chemical features were detected and tentatively annotated.

3. Results and discussion

3.1 Nutrient analysis

The nutritional composition of Kerala red rice water was evaluated before and after 18 h of natural fermentation to assess changes in macro- and micronutrients (Fig. 2). All measurements are reported on a wet-weight (as-sampled) basis, except ash, which is reported on a dry-weight basis.

3.1.1 Macronutrients. All measured macronutrients increased relatively to unfermented controls (Fig. 2). Carbohydrates increased by 22.7% (from 15.14 mg L^{-1} to 18.59 mg L^{-1}). This increase may reflect amylolytic activity of microbes (*e.g. Lactococcus*, *Saccharibacillus*, *Pantoea*), which are known to potentially hydrolyze resistant starch, yielding oligosaccharides and dextrins.[32] This suggests enhanced carbohydrate bioavailability and provision of prebiotic substrates for gut microbiota.[32,33] Protein increased by 163.52% (114.67 mg L^{-1} to 302.17 mg L^{-1}), and total free amino acids (TFAA) increased by 35.47% (from 43.19 mg L^{-1} to 58.51 mg L^{-1}). These increases align with the potential action of microbes like *Pantoea*, *Saccharibacillus*, and *Bacillus* identified in metagenomic data of the present study. The observed rise in TFAA and detection of amino acids like leucine *via* GC-MS may be indicative of protein breakdown during fermentation. Fat increased by 19.23% (wet weight, from 0.26 to 0.31 mg g^{-1}), likely reflecting microbial disruption of plant cell walls, liberating bound lipids, and incorporation of microbial membrane lipids. This aligns with occasional observations in rice bran biomass.[34] Ash increased by 66.46% (dry weight, from 31.6 mg g^{-1} to 52.6 mg g^{-1}), suggesting improved mineral availability *via* enzymatic degradation of phytate–mineral complexes.[35–43] This trend corroborates ICP-MS data and supports enhanced mineral availability. These findings align with reports on modified nutrient profiles in other fermented cereals,[44–49] thus corroborating improved macronutrient availability.

3.1.2 Micronutrients. Micronutrient concentrations increased compared to unfermented controls (Fig. 2): sodium by 49.49% (from 3.39 mg L^{-1} to 5.08 mg L^{-1}), potassium by 36.88% (8.77 mg L^{-1} to 12.01 mg L^{-1}), magnesium by 185.19% (from 1.08 mg L^{-1} to 3.10 mg L^{-1}), calcium by 296.4% (from 0.89 to 3.55 mg L^{-1}), and manganese by 1054.96% (from 0.015 mg L^{-1} to 0.17 mg L^{-1}). The increases in Na, K, Mg, Ca, Mn mirror earlier reports on lactic acid fermentation, where microbial activity and pH shifts facilitate breakdown of phytic acid complexes, liberating cations into the aqueous phase.[35,41,43] The pronounced Ca and Mn surges support pH-mediated release from the bran layer. Traditional fermented rice beverages enriched with zinc and calcium have shown significant micronutrient increases ($p < 0.05$) during fermentation, corroborating our findings.[50] Micronutrients including calcium, magnesium, potassium, sodium, and iron are enhanced in fermented rice water through breakdown of anti-nutritional factors by lactic acid bacteria, consistent with increased bioavailability reported in cereal fermentation.[51] Zinc quantity decreased by 40.92%, probably due to selective mineral uptake or redistribution by the microbes for their metabolism. This decline in soluble zinc is consistent with findings where aqueous zinc concentrations decrease as microbial populations peak, likely reflecting selective microbial sequestration and complexation with antinutrients like phytate.[39] However, if phytate was degraded during fermentation, this could theoretically enhance zinc bio-accessibility despite the lower absolute concentration.[39,52,53] Collectively, these findings confirm that 18 h of fermentation improves solubility and potential bioavailability of key micronutrients in Kerala red rice water, consistent with nutritional benchmarks from previous cereal fermentation studies.

3.2 Microbial community composition and functional insights

Shotgun whole-genome metagenomic sequencing of fermented Kerala red rice water was performed by MedGenome Labs Ltd, Bangalore, India, on an Illumina NovaSeq X Plus Platform (2 ×

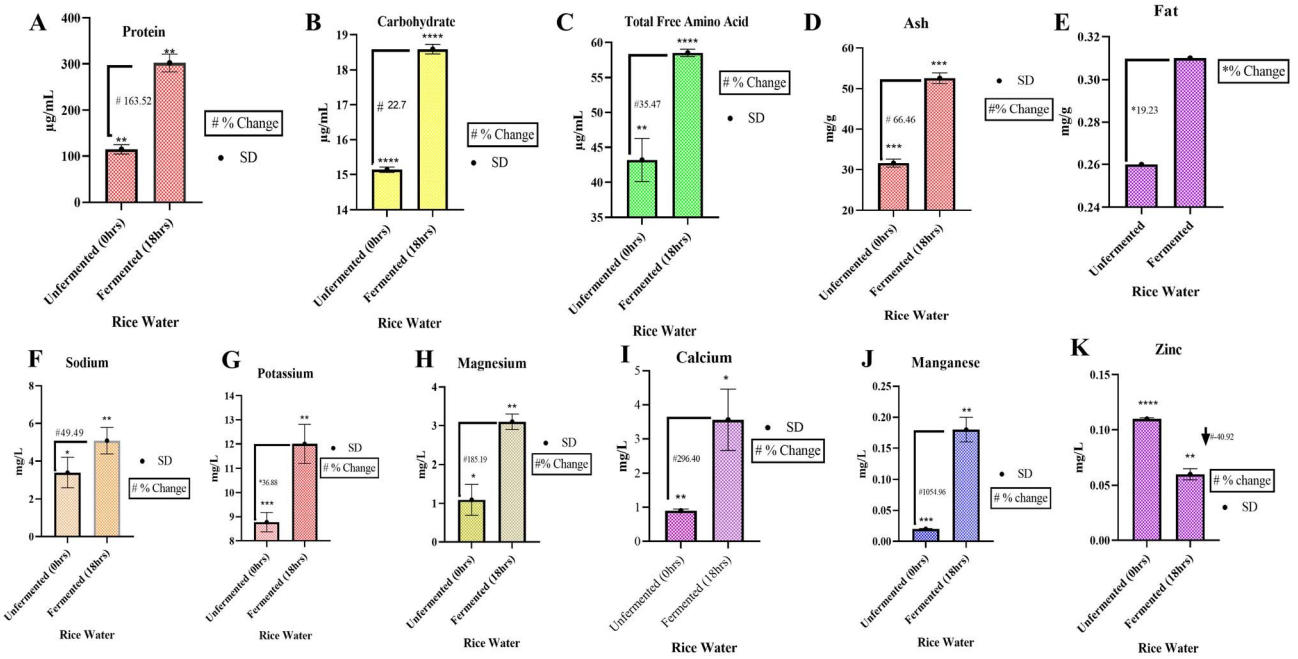

Fig. 2 Nutrient composition changes in Kerala red rice water following 18 h fermentation. Panels (A–K) macronutrient and micronutrient concentrations at 0 h (unfermented) *vs.* 18 h (fermented). (A) Carbohydrates, (B) Protein, (C) Total free amino acids (TFAA), (D) Fat, (E) Ash, (F) Sodium, (G) Potassium, (H) Magnesium, (I) Calcium, (J) Manganese, (K) Zinc. Data: A, B, C, E: $n = 3$ (paired t-test, $p < 0.05$); D (Fat): $n = 1$; F–K: $n = 3$ technical (multiple t-tests, $p < 0.05$). GraphPad Prism v8.4.2. Raw data: SI 1A.

151 bp, paired-end). Read quality control was performed using FastQC and fastq-mcf tool (v1.04.803) for adapter trimming, followed by *de novo* assembly using MEGAHIT (v1.2.9). The assembly yielded 2.58 GB of high-quality data after adapter trimming (mean Q = 39.26, Q 30% > 92%, GC content = 47.67%), producing 9211 contigs (≥1000 bp; largest contig = 904 399 bp; N50 = 11 330 bp). Gene prediction was executed *via* Prodigal (v2.6.3). Taxonomic classification, and functional profiling were performed using MEGAN6 with integrated SEED and BV-BRC databases. Microbial diversity indices (Shannon, Simpson, Richness, Evenness) were calculated from the species abundance data in-house. No contamination was detected after screening (SI 2A, Fig. S1, 2B and 2D–F).

Species-level analysis identified 50 microbial taxa (SI 2C), dominated by Proteobacteria (59.63%) and Firmicutes (40.12%) with a minor viral contribution from Uroviricota (0.25%). Major species included *Saccharibacillus* sp. O23 (13.89%), *Cronobacter sakazakii* (11.94%), and *Saccharibacillus* sp. O16 (8.89%). Approximately 25% of bacterial reads remained unclassified. The list of microbes along with the top 10 genera is shown in Fig. 3. The relative abundance of individual species and rank-abundance curve showing dominance by a few abundant species with a long tail of rarer members is shown in SI 2A and Fig. S2.

Functional annotation revealed strong fermentative potential (68%), with high representation of amylase producers (70%), and phytase produces (34%), antimicrobial traits (30%), vitamin biosynthesis (B1, B2, B9; 54%), and probiotic-associated species (10%) Fig. 3. These are *in silico* predictions; active metabolic expression requires validation *via* proteomics or metabolomics. The presence of phytase-encoding genes (34% of community) aligns with measured phytase activity (0.12 U mL^{-1}) and supports the observed mineral release (see Results and discussion, Section 3.3).

Alpha diversity (Shannon = 3.1; Simpson = 0.934, Pielou's evenness = 0.794) reflected moderate-high diversity. Comparison with published datasets (Table 1) shows fermented Kerala red rice water ranks above Assamese rice beverage (1.3–2.2), kimchi (∼1.8), and Indian buttermilk (∼2.5–3.5), is comparable to kombucha (>3.0), and is below idli (4.3–5.7):

Assamese rice beverage < kimchi < Indian buttermilk < Kerala red rice water ≈ kombucha < idli.

The dominance of lactic acid bacteria (LAB) in our fermented Kerala red rice water aligns with earlier reports of LAB in spontaneously fermented pigmented rice water.[59] The rank-abundance curve (SI 2A and Fig. S2) shows a "long tail" of rare taxa, consistent with Chakkaravarthi *et al.* (2025), who reported rare taxa contributing to polyphenol bioconversion in pigmented rice fermentation.[60] Several low-abundance taxa (*Stenotrophomonas*, *Massilia*, *Franconibacter*, *Salmonella*) were detected at ∼0.2. Overall, fermented Kerala red rice water is microbially diverse and functionally versatile, with predicted capacity of starch hydrolysis, phytate degradation, and vitamin biosynthesis. Functional predictions required experimental validation *via* proteomics or targeted metabolomics.

3.3 Phytate-bound mineral bioavailability

Phytic acid (myo-inositol hexakisphosphate)–metal (phytate) complex formation was validated through complementary

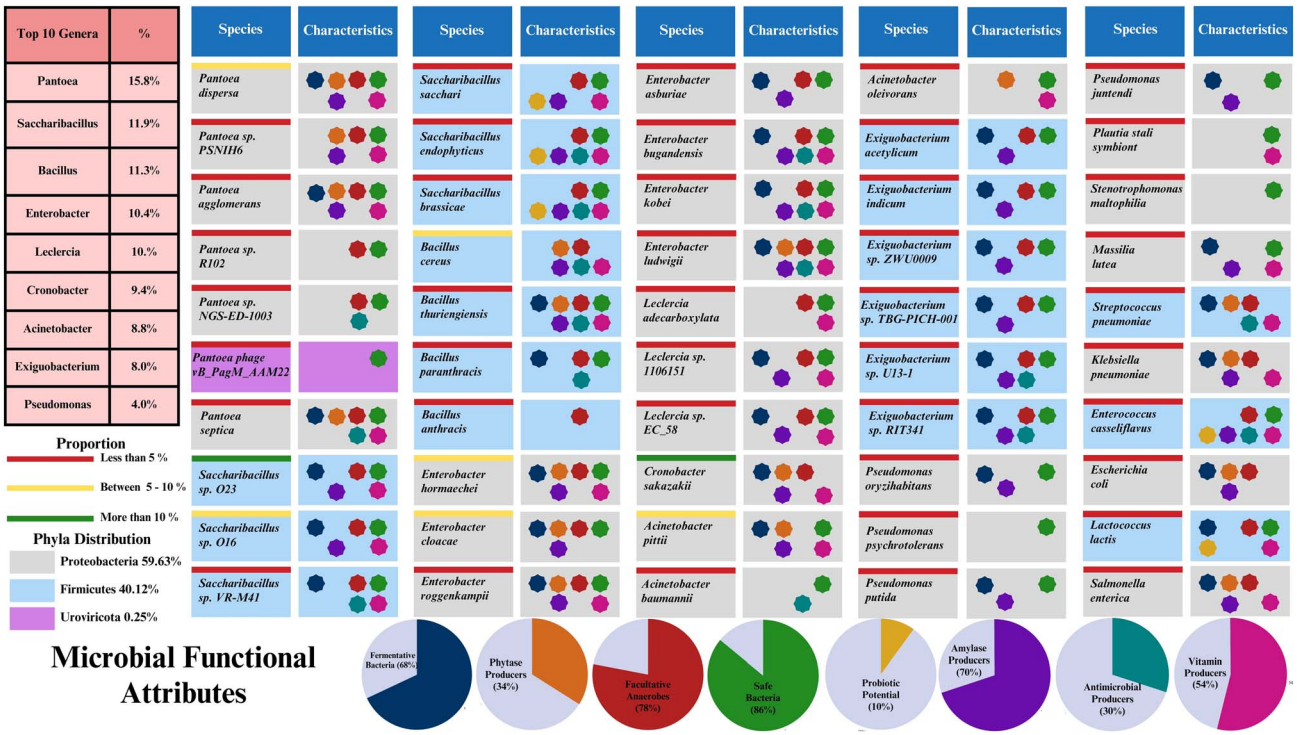

Fig. 3 Taxonomic composition and functional attributes of microbes in fermented Kerala red rice water (*Oryza sativa* L. subsp. *indica*). Fifty microbial taxa are displayed, where cell background colors denote phylum-level assignment: grey = Proteobacteria, blue = Firmicutes, and light purple = Uroviricota. Overlay bars indicate relative abundance categories: red (<5%), yellow (5–10%), and green (>10%). The top 10 genera are highlighted. Eight pie charts illustrate functional attributes, with sector size corresponding to the proportion of taxa associated with each attribute.

physicochemical and imaging analyses. Synthetic phytate–mineral complexes (Ca, Fe, Zn at 1 : 1 molar ratio) were used as controlled model proxies to simulate the anti-nutritional effects of phytic acid in cereal/legume-based diets. This standardized approach isolates the phytate–mineral interaction, eliminating interference from varying endogenous mineral levels in raw grains and providing mechanistic clarity to quantify mineral release dynamics during fermentation.

3.3.1 Phytate–mineral complex validation. Fourier-transform infrared (FTIR) spectroscopy (spectral range 4000–500 cm^{-1}; resolution 4 cm^{-1}; 40 ATR direct scans; powder sample preparation; automatic focus, automatic background, and automatic baseline correction) revealed characteristic

Table 1 Alpha diversity metrics of fermented Kerala red rice (*Oryza sativa* L. subsp. *Indica*) water after fermentation compared with selected fermented foods[a]

Fermented food	Shannon index (H')	Simpson's index ($1 - D$)	Species richness (S)/(operational taxonomic units [OTUs])	Pielou's evenness (J)	References
Fermented red rice water (*Oryza sativa* L. subsp. *indica*)	3.11	0.93	50	0.79	Present study (species-level relative abundance)
Kimchi (Korea)	~1.8 ± 0.7	—	~20–30	Moderate	54
Idli batter (India)	4.3–5.7	~0.95	~30–35	High	55
Buttermilk (India)	~2.5–3.5	—	~20+	Moderate-high	56
Kombucha (China, global)	>3.0	~0.96	~25–40	High	57
Fermented rice beverage (Assam, India)	1.3–2.2	—	~10–20	Low-moderate	58

[a] Diversity metrics include Shannon index (H'), Simpson's index ($1 - D$), species richness (S), and Pielou's evenness (J). Values for comparator foods were compiled from published metagenomic studies conducted at comparable sequencing depth. "—" indicates data not reported.

shifts in phosphate-associated vibrational bands—P=O stretching (1200–900 cm^{-1}) and P–O–M coordination (900–750 cm^{-1})—confirming metal–phosphate interactions (Fig. 4). The key focus region was 1040–1080 cm^{-1} (phosphate–metal region). A reduced –OH stretching band (3300–3500 cm^{-1}) further indicated hydroxyl group participation in binding, consistent with the metal-specific coordination environments.

X-ray fluorescence (XRF) (system default voltage and current; 30 s count time; pressed thin mylar film; powder sample preparation; automatic baseline correction) confirmed elemental incorporation of phosphorus, calcium, iron, and zinc into phytate complexes (SI 3 and Fig. S3). Quantitative profiles (Fig. 5) showed stable phosphorus retention alongside divalent metals.

Scanning electron microscopy (SEM) using an FEI Apreo LoVac SEM (Thermo Fisher Scientific) with EDX detector at 15× kV acceleration voltage and 10× magnification revealed distinct morphologies reflecting differences in ionic radius and coordination chemistry: calcium–phytate formed dense spherical granules (Fig. 6A), iron–phytate produced compact nodules (Fig. 7A), and zinc–phytate showed angular, plate-like structures (Fig. 8A). Energy-dispersive X-ray spectroscopy (EDX) and elemental mapping further corroborated the co-localization of phosphorus and metals within these complexes (Fig. 6B, C, 7B, C, 8B and C).[61]

3.3.2 Mineral release kinetics. Release kinetics were assessed by incubating 50 mg of each complex with 10 mL of water, 0 h fermented Kerala red rice water, and 18 h fermented Kerala red rice water (25 °C), with aliquots collected at 15, 60, 120, and 240 min for water and 0 h controls, and at 360 min for 18 h fermented rice water (see Methods, Section 2.4.3.). Fermented samples showed significantly enhanced mineral bioavailability compared to unfermented controls: calcium

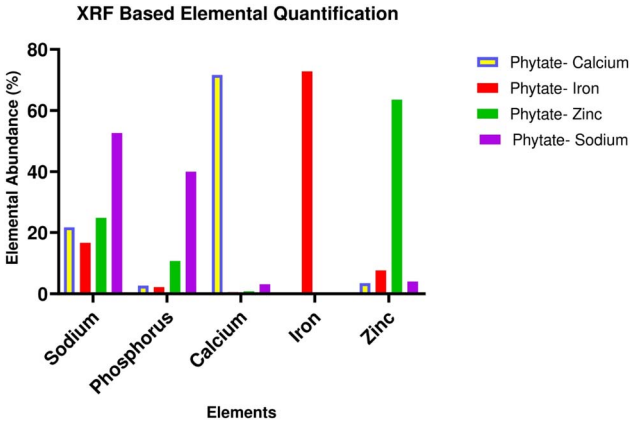

Fig. 5 Elemental composition of phytate–mineral complexes from XRF analysis. Relative abundance (%) of sodium, phosphorus, and mineral cations (Ca, Fe, Zn), confirming incorporation into phytate complexes.

increased by 1190% (fermented: 19 689 ppb at 15 min → 21 642 ppb at 360 min *vs.* unfermented: 1661 ppb at 15 min → 9816 ppb at 240 min), iron increased by 566% (fermented: 7113 ppb at 15 min → 38 153 ppb at 360 min *vs.* unfermented: 1294 ppb at 15 min → 3955 ppb at 240 min), and zinc increased by 93% (fermented: 448 ppb at 15 min → 1157 ppb at 360 min *vs.* unfermented: 421 ppb at 15 min → 600 ppb at 240 min). These increases correspond to 11.9×, 5.7×, and 1× fold-changes, respectively, demonstrating substantial improvement in mineral bioavailability following fermentation (SI 1B). Calibration plots confirmed linearity (R^2 = 0.952 Ca, 0.9798 Fe, 0.947 Zn), validating quantification accuracy. The release order (Ca > Fe > Zn) mirrors known stability constants of phytate–metal complexes, where calcium dissociates more readily, while

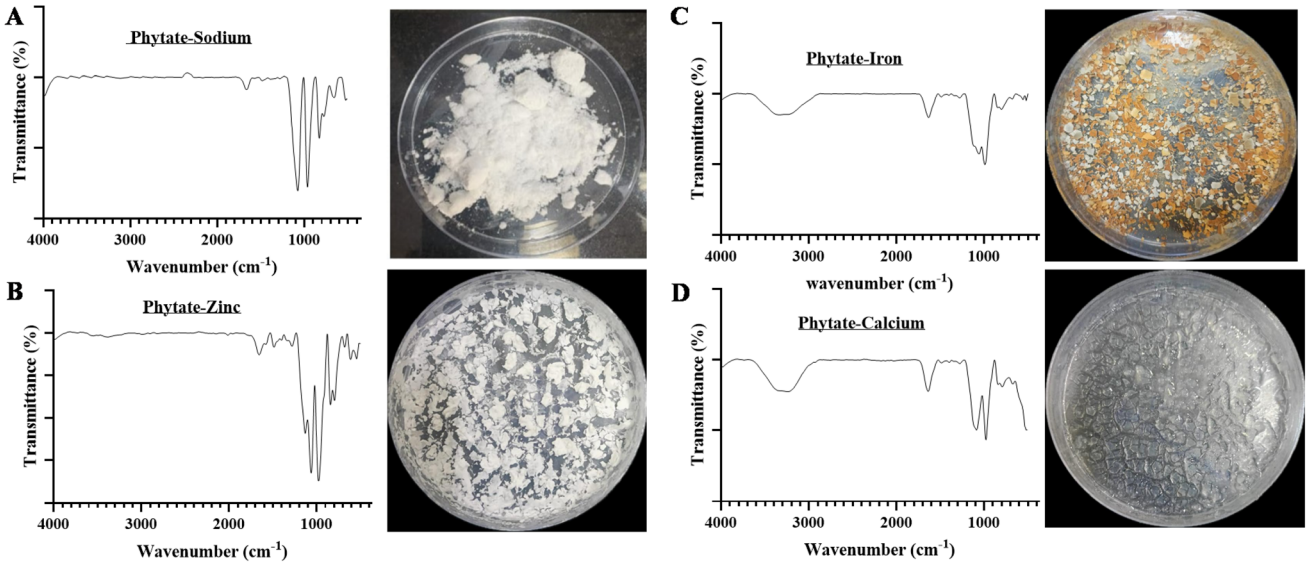

Fig. 4 Structural and spectroscopic characterization of phytate–mineral complexes. (A) Phytate–sodium, (B) Phytate–zinc, (C) Phytate–iron, and (D) Phytate–calcium complexes. FTIR spectra show characteristic shifts in the P=O stretching region (1200–900 cm^{-1}) and P–O–M vibration region (900–750 cm^{-1}), consistent with metal–phosphate coordination. Reduced –OH band intensity (3300–3500 cm^{-1}) indicates hydroxyl group participation in binding.

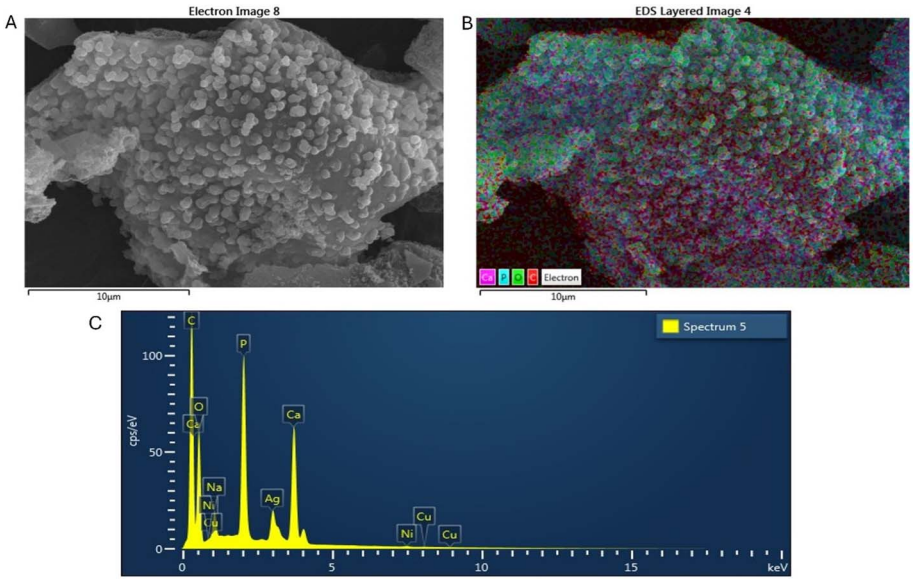

Fig. 6 SEM and EDX of calcium–phytate complex. (A) SEM: densely packed spherical granules. (B) EDX mapping: elemental distribution. (C) EDX spectrum: C, O, P, Ca detected.

iron and zinc form stronger coordination bonds that resist hydrolysis (Fig. 9).[62]

The binding affinity and release of the minerals are not governed by phytate alone but are modulated by matrix ligands in the fermented water. Organic acids (lactic, acetic, succinic) introduced competing ligands that form soluble mineral complexes, effectively out-competing phytate or lowering pH to favor dissociation.[63,64] Additionally, red rice polyphenols and proteins can form ternary complexes (protein–mineral–phytate) resistant to digestion;[65] fermentation acts as a biological "reset," with microbial metabolites (*e.g.*, citrate, malate) promoting transition from insoluble bound states to bioavailable ionic states.[24]

Microbial phytase activity emerged as a key drive, rising from negligible levels in unfermented samples to 0.12 U mL^{-1} after fermentation. This enzymatic dephosphorylation facilitated mineral liberation. Functional gene profiling supported this, with ~34% of the community carrying phytase-encoding genes. Representative taxa included *Bacillus*, *Enterobacter*, *Pantoea*, *Saccharibacillus*, and *Lactococcus*, all known for acid-tolerant phytase production in cereal fermentations.[24,66–69] While these taxa likely contributed to enhanced mineral release, specific

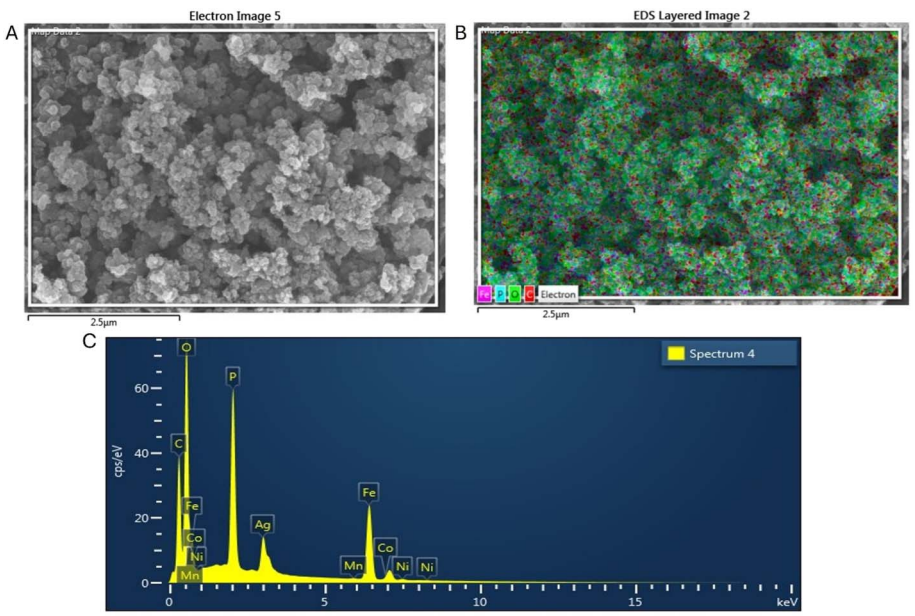

Fig. 7 SEM and EDX of iron–phytate complex. (A) SEM: nodular morphology. (B) EDX mapping: elemental localization. (C) EDX spectrum: C, O, P, Fe detected.

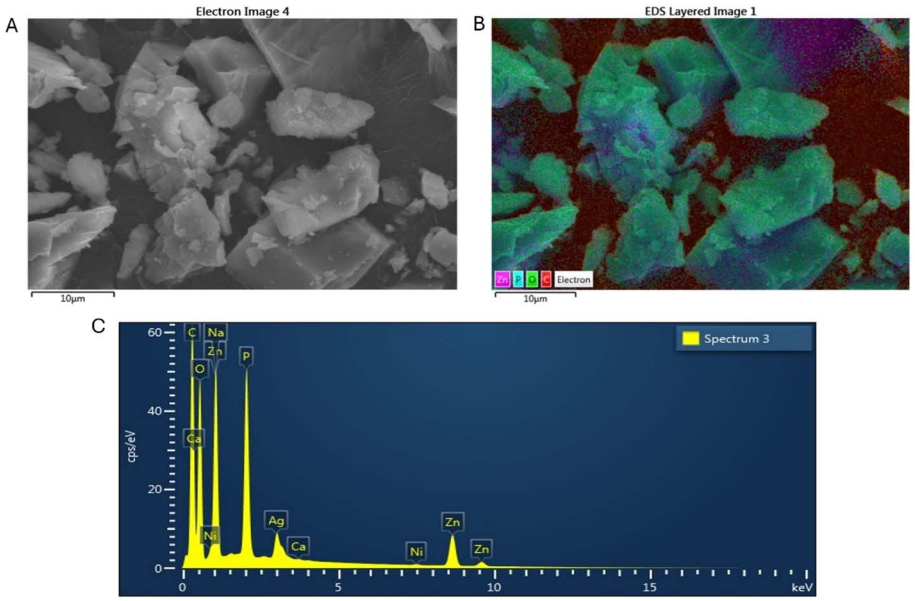

Fig. 8 SEM and EDX of zinc–phytate complex. (A) SEM: angular, plate-like crystals. (B) EDX mapping: spatial elemental distribution. (C) EDX spectrum: C, O, P, Zn detected.

roles of individual species require targeted functional validation.

Taken together, the integrated FTIR, XRF, SEM-EDX, release kinetics, and phytase activity analyses provide convergent evidence that fermentation transforms Kerala red rice water from a phytate-rich, mineral-inaccessible substrate into a beverage with improved mineral accessibility. These findings are consistent with reports from other cereal systems (millet, wheat, and sorghum) where fermentation enhances mineral bioavailability through phytate degradation.[24,68]

3.4 Metabolomics profiling

Untargeted gas chromatography-mass spectrometry (GC-MS) was employed to profile the metabolite landscape of fermented Kerala red rice water. Metabolites were extracted from 500 µL aliquots using a modified Bligh–Dyer method (chloroform : methanol : water, 1 : 1 : 1, v/v/v), followed by centrifugation at 12 000 rpm (4 °C) for 15 min. The supernatant was vacuum-dried *via* SpeedVac and derivatized with MTBSTFA/acetonitrile/triethylamine (3 : 1 : 1, v/v/v) at 60 °C for 1 h to enhance volatility. Primary analysis was conducted on an Agilent 5975 GC-MS system, with validation on a Shimadzu GCMS-QP2020NX.

Raw data processing initially detected 82 chemical features, tentatively annotated against the NIST17.L spectral library using LibSearch algorithm (SI 4B). To ensure high identification confidence and rigorous artifact filtering, sequential quality control filters were applied: duplicate removal, match score threshold ≥85%, signal-to-noise ratio >10, retention time window 2–20 min, and minimum peak intensity 10 000. This curation effectively filtered common GC-MS artifacts, including organosilicons.

The final metabolite set encompassed alcohols, organic acids, amino acid derivatives (including leucine derivatives),

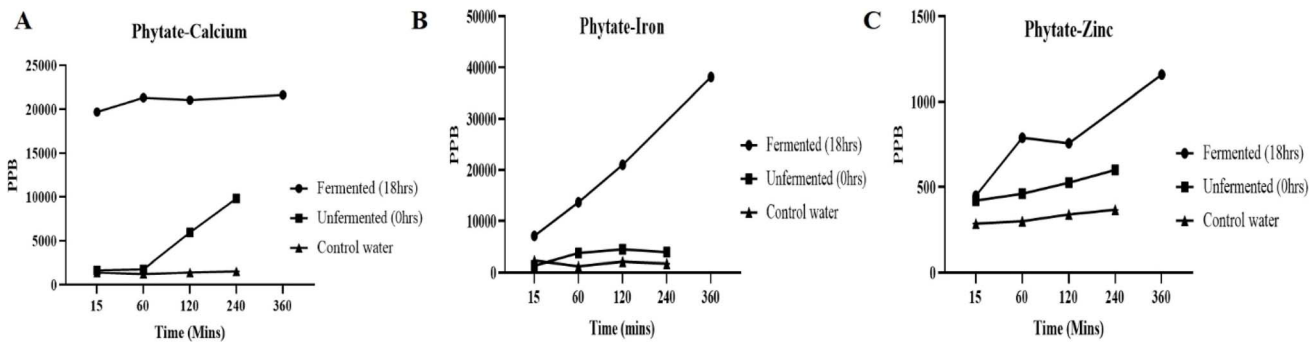

Fig. 9 Mineral release kinetics from phytate–mineral complexes. Panels (A–C) time-course release of (A) calcium, (B) iron, and (C) zinc from synthetic phytate complexes incubated in 18 h fermented Kerala red rice water (● solid lines) *vs.* 0 h unfermented rice water (○ dashed lines). Data represent mean ± SD ($n = 3$ biological replicates). Statistical significance ($p < 0.05$ *vs.* respective 0 h baseline) determined by multiple *t*-tests comparing each time point to baseline (GraphPad Prism v8.4.2). Error bars: mean ± SD. Time points: 0, 15, 60, 120, 240- or 360-min. Raw data: SI 1B.

thiols, heterocycles, indicating microbial biochemical activity.[70–72] Signature molecules like leucine derivatives and organic acids align fermented Kerala red rice water with other traditional cereal-based beverages.[59,60]

4. Conclusions

This work presents the first comprehensive molecular characterization of naturally fermented Kerala red rice (*Oryza sativa* L. subsp. *indica*) water, integrating nutritional, metagenomic, metabolomic, and mineral bioavailability analyses. After 18 hours of fermentation, the biochemical profile was restructured: the microbial community was enriched with taxa such as *Pantoea*, *Saccharibacillus*, and *Bacillus*, which are known for proteolytic and phytase-producing potential; phytase activity rose to 0.12 U mL^{-1}; from synthetic phytate complexes was quantified, with calcium increasing by 1190% (11.9× fold-change), iron by 566% (5.7× fold-change), and zinc by 93% (1× fold-change) compared to unfermented controls, demonstrating substantial improvement in mineral bioavailability following fermentation. Untargeted metabolite profiling detected compound classes including alcohols, organic acids, amino acid derivatives (*e.g.*, leucine), consistent with microbial biochemical transformation observed in other cereal fermentations.

These findings demonstrate enhanced mineral accessibility and metabolic enrichment, though the interpretation is based on a single fermentation condition (18 h, 25 °C) and *in vitro* mineral release measurements rather than human absorption trials. To build on these results, future work could directly quantify enzymatic activities, explore a range of fermentation times and temperatures to identify optimal conditions, and conduct *in vivo* or clinical studies to validate mineral bioavailability and physiological outcomes. Standardizing production protocols will also support reproducibility and potential scale-up. Together, fermented Kerala red rice water emerges as a culturally rooted, low-cost nutritional strategy with measurably improved mineral accessibility (Ca, Mg, Fe) and enriched bioactive metabolite profiles, representing a promising candidate for further investigation as a functional food to address mineral shortfalls in cereal-based diets.

Author contributions

Madhumitha Hariharamohan: investigation, formal analysis, data curation, writing – original draft, and visualization. Manali Chindarkar: methodology, validation, formal analysis, writing – review & editing, and visualization. Hruta Sundar Swain: investigation, data curation, methodology, validation, formal analysis, visualization, and writing – review & editing. N. Rajesh: validation, resources, and supervision. Vidya Rajesh: conceptualization, methodology, validation, resources, writing – review & editing, visualization, supervision, and project administration. All authors have agreed to the final version of the manuscript.

Conflicts of interest

The authors declare no conflicts of interest.

Data availability

The complete dataset generated and analyzed during this study is available from the corresponding author upon reasonable request.

Supplementary information (SI) is available. See DOI: https://doi.org/10.1039/d6ra00382f.

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