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Evaluation of some effective potentialities of newly formulated rice fermented food using *Elephantopus scaber* L. rhizome as herbal starter

Papan K. Hor[1], Kuntal Ghosh[3], Suman Kumar Halder[1], Subhadeep Mondal[2] & Keshab Chandra Mondal[1]*

[1]Department of Microbiology, Vidyasagar University, Midnapore-721 102, West Bengal, India
[2]Centre for Life Sciences, Vidyasagar University, Midnapore-721 102, West Bengal, India
[3]Department of Biological Sciences, Midnapore City College, Bhadutala, Paschim Medinipur-721 129, West Bengal, India

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Traditionally, fermented food and beverages are prepared by adding a mixture of plant residues as a starter or source of microbes. Most of the conventional fermented foods use a local starter which contains a mixture of herbs or old ferment or otherwise cereal dust-coated tablet. In this study, we have made an attempt to prepare a rice-based fermented food with the herbal starter (0.5% w/w) of *Elephantopus scaber* L. rhizome, and also examined its microbial and nutrient profiles. The food product is fortified with organic acid and titratable acidity of 0.58% and also contained an excellent source of microbes (LAB and *Bifidobacterium* sp.). The fermented food contains significant amount of fat, protein, minerals, vitamins, oligosaccharide, unsaturated fatty acids (ω3, ω6, ω7 and ω9) and a pool of free amino acids. The presence of phytochemical contents in the fermented rice was also exhibited significant effects against commercially available free radicals (DPPH, ABTS, FRAP and OH^-radicals). Thus, food-grade microbes containing newly formulated fermented food would provide essential macro-and micro-nutrients to the individuals and convey the sustainability of good health. Therefore, the mentioned plant part would be used as an effective starter for aiding rice-based food products.

Keywords: Antioxidant, Backslopping, *Bifidobacterium* sp. Bull's tongue, CAZymes, Elephant's Foot, Ironweed, LAB, Macro and micronutrients, Rice fermentation

Fermented foods and beverages are an essential part of the traditional estate on the Indian subcontinent, and fermentation is the first indication of the human-microbes relationship[1]. Fermentation enhances the shelf life, aroma, taste, texture, and nutritive value of the food due to starter culture. The ethnic methods of starter culture for different fermented food preparation include backslopping (a method of inserting a small portion of the previous batch of fermented food into the start of a new batch of food to be fermented), using the particular vessels, stirring a small amount of oldest ferment and adding of an active microorganism containing specific essential products[2]. Biological resources of local food crops such as rice, corn, wheat or sorghum were used to prepare traditional Indian fermented foods and beverages with the help of the starter culture method. Rural women with their indigenous knowledge are mostly practiced for small to midscale production units of traditional fermented food preparation and sell in the local market apart from their consumption for living maintenance of their family[3].

Worldwide, the use of wild plant residues directly as a starter material was increased day by day to prepare different types of fermented foods and beverages. Recently, it has been suggested that the rhizome of wild herbs contains many effective bacteria as endophytic organisms and the tribal people have the artisanal knowledge to select and use them as a starter, which could participate in the multi-stage and multi-species fermentation process for the preparation of diverse type fermented foods/ beverages[4]. Das *et al*.[5] unfolded the mechanism of starter preparation with the help of different plant parts by the tribes with their unique starter culture cum flavouring and stability agent to carry out fermentation. This is known as '*bakhar*' or '*rice beer cake*' (with a size of 4.5-7.0 cm or sometimes 1.5-2.0 cm in diameter). Boiled rice is fermented with '*bakhar*' in earthen pots at room temperature (35°C) for 4-5 days to prepare '*haria*' in central, eastern, and north-eastern India. The presence of microbes in the starter worked interdependently to complete the process of fermentation[5]. The hydrolytic activity of aerobic microbes sets the environments for the growth of

*Correspondence:
Phone: +91 3222 276554 (ext. 477); Fax: +91 3222 275329
E-Mail: mondalkc@gmail.com; ORCID: 0000-0003-4446-9137

yeast and molds. In the beginning, fermentation of '*haria*' is facilitated by the molds that decomposed the boiled rice, followed by the growth of lactic acid bacteria, yeasts, and *Bifidobacterium* sp., at the latter stage of fermentation[6]. The native peoples believe that this beverage can protect them from many gastro-intestinal ailments (particularly dysentery, diarrhoea, amebiosis, acidity, vomiting), skin, hair, eye and cardiac diseases[6,7]. During the preparation of another traditional rice-based alcoholic beverage ('*Chhang*'), a similar type of microbial diversity was also noted. '*Balma*' is the starter used for this fermentation process was also prepared from the wild herbs, namely, leaves of *I. cuspidate*, *M. biflora*, *O. vulgare*, *T. linearis*, and roots of *Rubus* sp., It was mainly prepared by '*bhutiya*' tribe of Siliguri (West Bengal), Uttarakhand, India[8,9]. '*Chhang*' is also prepared from barley in the region of Himachal Pradesh where traditional inoculums ('*phab*') is used for the preparation[10].

Fermentation with the help of wild herb (*Echinophora sibthorpiana*) was also recognized during the preparation of '*tarhana*' (Turkish soup), a popular wheat-based fermented food in Turkey[5]. Nephroprotective activity along with antioxidant properties was also observed by *Pisonia aculeata* L. (folk medicinal plant of India) leaf extract against cisplatin induced nephrotoxicity and renal dysfunction in experimental rodents[11]. It was also reported that, raw and parboiled whole red rice consumption possess antidiabetic potential with the antioxidant improving ability, and could be utilized as dietary supplements in diabetes management[12]. The native people, with their experience, selected different wild plant parts as starter and then added a considerable amount with the concerned substrate for the preparation of many fermented foods/beverages. The use of individual plant residues for food fermentation has not been undertaken to date.

In this study, we explored the probability of single plant residue for preparing nutrient-rich functional food by fermentation of boiled rice. We prepared a rice-based fermented food using the root (rhizome) of *Elephantopus scaber* L. (local name *Dekhopatra*), a well-known ethnomedicinal plant[7]. This plant residue was also used for the preparation of many rice-based fermented food and beverages. Further, we evaluated the microbial composition, nutrient profiles, and the *in vitro* antioxidant activity of the formulated fermented rice.

Material and Methods

Chemicals

All the chemicals used in the phytochemical and antioxidant study are of analytical grade and were procured from standard companies. Most of the chemicals used in the analysis were acquired from the HiMedia Laboratories, Mumbai, India. Standard vitamins, minerals, and organic acids were commercially obtained from Sigma Aldrich, USA.

Plant collection and preparation of fermented food

The recognized plant *Elephantopus scaber* L. commonly called Elephant's Foot, Bull's tongue or Ironweed, and its root were collected from the natural lateritic forest of the Jangalmahal area of West Bengal, India. The rhizome parts were washed repeatedly with sterile water, dried under sunlight, and then powder form was made by mixer grinder. Whole grain rice (*Oryza sativa*) was purchased from the local market and cooked for 1 h. Then 0.5% (w/w) of plant residues were added directly with the boiled rice and kept in the pot at room temperature (dark place) for fermentation process at 37ºC for 4-5 days[4]. The fermented rice product was considered as a test sample (T). One control sample (C) was also prepared, for which sterilization was done just after adding the starter with the boiled rice that makes it microbes free. For the phytochemicals and antioxidant experiment, clear watery extracts were prepared. Food samples were dried in a food dryer at 55°C for 10 h, and then dissolved into sterile distilled water (0.1%, w/v) by homogenization and centrifuged at 1700 g for 10 min. The collected supernatants were used for analysis.

Microbial composition of fermented food

The quantity of superior microbes in the fermented food was itemized on the basis of colony forming units (cfu) using strain specific selective media (HiMedia, India)[13] . In short, for the microbial count, 1.0 g of fermented rice was mixed with 10 mL of phosphate buffer saline (pH 7.2) and used as stock. Total aerobic and anaerobic bacteria were estimated with the help of Plate count agar (PCA) and Wilkins chalgren agar and incubated at 37°C. Yeast and mold were analyzed using yeast and mould agar and Potato Dextrose Agar (PDA) media, respectively, and incubated at 30°C. The group of lactic acid bacteria (LAB) and *Bifidobacterium* sp. were enumerated in Rogosa SL agar (supplemented with 0.132% acetic acid) and Bifidobacterium agar supplemented with Bifidobacterium selective supplement, respectively,

and plates were incubated in a CO_2 incubator (5% CO_2), at 37°C. The unit of the microbial load was expressed as log10 cfu/g of wet content.

pH and titratable acidity

For pH analysis, both the samples ('C' and 'T') were homogenized with sterile distilled water in a ratio of 1:10, followed by shaking for 5 minutes and allowed to settle for 10 min. Then the pH of the sample was measured by a glass probe digital pH meter (ELCO, India)[4].

Titratable acidity was determined by the standard titration procedure of AOAC (2005)[14]. The fermented and unfermented samples were titrated by 0.1 M NaOH using phenolphthalein [0.1%, (w/v) in 95% ethanol] as an indicator. The percent of titratable acidity was calculated as a percent (%, w/v) of lactic acid according to the following formula:

Total titratable acidity (% of lactic acid) = mL of 0.1 N NaOH×0.009×100/sample amount (g)

0.009 is the conversion parameter of lactic acid, i.e., 1 mL 0.1 M sodium hydroxide standard solution corresponds to 0.009 g lactic acid and 100 is used to calculate the TTA in percentage.

Proximate analysis

The composition of fermented (T) and unfermented (C) foods was estimated by proximate analysis[14]. The moisture, protein, fat, carbohydrate, and ash content of the samples were analyzed using the standard methods of Association of Official Analytical Chemists (AOAC) procedures (2005). Details of the methods are as follows:

Moisture contents were analyzed by weight difference method (Method No. 925.10) (AOAC, 2005); Ash contents were enumerated by combustion (Method No. 930.05) (AOAC 2005); Crude protein (Nx6.25) contents were estimated following the Kjeldahl method (Method No. 978.04) (AOAC, 2005); Crude fats were determined following the Soxhlet extract method (Method No.930.09) (AOAC 2005); Fiber contents weremeasured by method no. 962.09 (AOAC 2005) and Total carbohydrate contents were calculated from the above parameters, and the total caloric value was evaluated using the "Atwater factor".

Organic acid content

According to the method described by Hor *et al*.[4], water/salt-soluble extracts of both samples were prepared with slight modification[4]. Briefly, 5 g of samples were diluted with 15 mL of 50 mMTris-HCl (pH 8.8), kept at 4°C for 1 h, then centrifugation were done at 17,000 g for 20 min. With the help of a 0.22 um pore size filter, the supernatant containing the water/salt-soluble fraction was filtrated. Then, the extracts were analyzed by reverse-phase HPLC using an Agilent HPLC system (Agilent Technology, 1200 infinity series) equipped with a Zorbax SB-C18 column. The elution was carried out at 60°C with a flow rate of 0.5 mL/min using 10 mM H_2SO_4 as a mobile phase. UV-VWD (Variable Wavelength Detector) with the wavelength of 210 nm and run time of 30 min was applied for detection of organic acid contents.

Determination of B-group of vitamins

Water-soluble vitamins present in the fermented and unfermented products were determined following the methods of Hor *et al*.[4]. The extracted hydro-soluble vitamins were analyzed by reverse-phase HPLC using an Agilent HPLC system (Agilent Technology, 1200 infinity series) equipped with a Zorbax SB-C18 column and the mobile phase was 0.05 M KH_2PO_4 (pH 2.5) and acetonitrile. The temperature was kept at 15°C, and a flow rate was maintained at 1 mL/min. The effluent from the column was monitored by a variable wavelength UV detector (204 nm).

Volatile compound analysis

Five grams of both samples were mixed with 20 mL dichloromethane to extract major alcoholic volatile constituents and then analyzed through gas chromatography (Agilent Technology, USA) furnished with a manual injector and a flame ionization detector (FID)[15]. For this purpose, a capillary column, HP 5 (30 mt × 0.25 mm i.d., 0.25 µm film thicknesses) was used. The injector and detector temperature was set to 250°C. The temperature of the oven was established at 50°C for 5 min, then rise from 50 to 220°C, at 3°C/min, and finally settled at 10 min at 220°C. Nitrogen was used as a carrier gas, and the split vent was set to 13 mL/min[4]. Evaluation of volatile components was performed with the help of Chem Station software.

Free mineral content

Firstly, fermented and unfermented rice samples (5 g) were dissolved in 25 mL of deionized distilled water and then homogenized, followed by centrifugation at 10,200 g for 10 min. From the water extracts, the contents of free minerals (calcium, magnesium, iron, zinc and manganese) were

measured by atomic absorption spectrophotometer (AAS) [Shimadzu Analytical (India) Pvt. Ltd][16].

Analysis of free amino acids by HPLC

The concentration of amino acids present in both products was measured by slightly modifying the method described by Das *et al*.[17]. Different commercially available amino acid standards (SRL, India) were prepared using methanol at a ratio of 1 mg/mL (w/v) and filtered with 0.22 µm size syringe-driven filter. Samples were kept in a hydrolysis tube and mixed with 6 mL HCl containing 0.1% phenol, followed by hydrolysis of the total preparation at 110°C for 24 h in a vacuum. The residual acid was dried off in a vacuum oven during the hydrolysis process. Then both the samples were suspended in 100 mMHCl and passed through a 0.22 µm size syringe driven filter for proper filtration. For derivatization of amino acids, firstly, a mixture was prepared with the composition of 100 µL sample, 900 µL of borate buffer (1 M, pH 6.2), and 1 mL of fluorenyl-methyl-oxycarbonyl chloride. This was kept for 2 min, and then 4 mL of *n*-pentane was added, followed by vortexing for 4 to 5 min. After filtration, the lower layer was collected and used for injection through a 0.22 µm size syringe-driven filter. The upper layer was separated and discarded.

Analysis of free amino acids was carried out in an HPLC system (Agilent Technology, 1200 infinity series) equipped with a Zorbax SB-C18 column (5 µm beads size; 4.0×250 mm). The injection volume was 20 µL, and the wavelength detector was set at 265 nm (UV range). The column oven temperature was maintained at 30°C. The mobile phase used was (A) acetate buffer-acetonitrile (9:1) and (B) acetate buffer-acetonitrile (1:9) with a flow rate of 1.0 mL/min.

Analysis of oligosaccharide components by TLC and HPLC

Investigation of oligosaccharides components in the fermented and unfermented food was employed from the Dreisewerd *et al*.[18] with some modification using thin layer chromatography (TLC). A commercially available Silica gel-made TLC plate (Merck, India) with a diameter of 0.2 nm was used as a stationary phase. A mobile phase was prepared using *n*-propanol/acetic acid/water in the ratio of 3:2:2 and kept in the TLC chamber. Then 5 µL of samples/standards were charged onto the bottom layer of the TLC plate in a marked position and dried properly with an air drier. Then the plate was transferred to the mobile phase (TLC chamber) and ran for 15 to 18 h. After that, 1% arsenal (50% H_2SO_4, v/v) was sprayed and kept for 15 min at 110ºC for colour development. The concentrations of malto-oligosaccharides in the fermented food samples were also estimated through HPLC described by Ghosh *et al*.[2]. At first, samples were diluted with 50 mMTris-HCl (pH 8.8) at the ratio of 1:3 (W/V) and kept at 4°C for 1 h followed by centrifugation at 8500×g for 20 min. Then with the help of 0.22 mm pore size filter, the collected supernatant was filtered and used for HPLC analysis (Agilent Technology, 1200 infinity series, USA). The Carbohydrate-NH_2 column was used for this purpose with the maintained temperature at 30°C. The mobile phase was prepared by acetonitrile and water at the ratio of 75:25 with a constant flow rate of 1 mL/min. Different concentrations of commercially available malto-oligosaccharides (Sigma) and glucose were used as standards for both the chromatography.

Analysis of fatty acids

At first, 5 g of control and test samples were mixed with hexane (30 mL) for 5 min and sonicated at room temperature for 5 min. The prepared mixtures were allowed to vortex for few times and then centrifuged for 5 min at 5950 g. The upper layer of hexane was collected and kept in another tube, and by following the previous process, the remaining part was extracted again with hexane. After complete collection, all the supernatants were appropriately evaporated under a stream of nitrogen. The dry product was dissolved again with 1 mL hexane and kept under the stream of nitrogen for extraction of free fatty acids. With the addition of triethylamine and 2-bromo-2'-aceto-phenone solutions, fatty acids were derivatized to fatty acid vinyl ester[19]. To complete the reaction, the reaction mixture was incubated for 15-20 min at 85ºC, followed by the addition of acetone. Using the RP-HPLC column (5 µm, 250 × 4.6 mm), the extracted fatty acid was separated, which was thermostated at 35°C. With the combination of methanol and water at the ratio of 75:25, the mobile phase was prepared. The flow rate was set to 1 mL/min, and the wavelength was set to 250 nm[19]. Different fatty acid standards (Sigma Aldrich) were also run.

Phytochemical analysis

Estimation of total phenolic content

Total phenolics content was estimated by following Folin-Ciocalteu[5]. Briefly, 1 mL food extracts, 4 mL of sodium carbonate solution (7%, w/v), and 5 mL of diluted Folin–Ciocalteu phenol reagent (1:10 distilled water) were mixed in separate test tubes and placed in

the dark for 1 h, and the absorbance was recorded at 725 nm against a reagent blank. The quantity of total phenolic components in extracts was expressed as gallic acid equivalent (GAE) and mg/g extracts.

Analysis of phenolic compounds by HPLC

The samples (500 mg) were extracted with 5 mL of methanol/water (80/20, v/v) by sonication at room temperature for 10 min. After that, centrifugation was done at 6800×g for 5 min, the supernatant was collected, and similarly, the extraction was repeated twice. By centrifugal evaporation, all the collected supernatants were evaporated to dryness. The accumulated residues were dissolved in 400 µL of methanol/water (80/20, v/v) and filtered through a 0.22 mm pore size membrane filter. For this purpose, 20 µL of the final filtered solution was injected into the HPLC system.

Here, LUNA-PFP (2) (3 µm, 150 mm × 4.6 mm) column thermostated at 35°C, was used to separate phenolic components. The mixture of methanol/water (10/90, v/v) containing 0.1% acetic acid was prepared and considered mobile phase A, while methanol containing 0.1% acetic acid served as mobile phase B. The gradient elution was performed as follows: 0.0 min, 5% B; 6.5 min, 25% B; 30.5 min, 37% B; 35.0 min, 55% B; 37.0 min, 95% B; 44.0 min, 95% B; 45.0 min, 5% B and 50.0 min; 5% B for re-equilibration of the column. The flow rate was set to 0.7 mL/min. Phenolic compounds were detected at 280 and 320 nm. For quantification, standards (Sigma-Aldrich, United States) of two subgroups of phenolic acid, viz., hydroxybenzoic acid (proto-catechuic acid and *p*-hydroxybenzoic acid) and hydroxycinnamic acid (*p*-coumaric acid, ferulic acid, and sinapic acid) experimented[5,10].

Estimation of total flavonoids content

The total flavonoid content was determined by the modifying method of Zhishen *et al*.[20]. Briefly, sample extracts of 0.5 mL were mixed with 0.1 mL of 5% $C_4H_4O_6KNa{\cdot}4H_2O$ [potassium sodium L-(+) tartrate] to estimate the flavonoid compounds. After 10 min, 0.1 mL of aluminium chloride (10%) was added to the mixture and made up to 3 mL with distilled water followed by incubation for 1 h at room temperature. Then absorbance of the reaction mixture was measured at 430 nm against a blank that contained 0.1 mL of distilled water in place of aluminium chloride. The flavonoids content was expressed as mg of quercetin equivalent (QUE)/g extracts.

Assay of *in vitro* antioxidant activity

DPPH (*2,2-diphenyl-1-picrylhydrazyl*) *scavenging activity*

As described by Ghosh *et al*.[2], the procedure was obtained to analyze antioxidant capacity in respect to the DPPH scavenging activity of the extracts collected from fermented and unfermented products. In short, food extracts (100 µL) were added to 1.9 mL of 0.1 mM 2,2-diphenyl-1-picrylhydrazyl (DPPH) and mixed repeatedly. After 15 min of incubation, absorbance was taken at 515 nm. Ascorbic acid was used as a positive control. The scavenging activity of the extracts against DPPH free radicals was expressed by using the following equation:

$$\text{Scavenging activity (\%)} = (1\text{-}A_{Sample}/A_{control}) \times 100 \quad \text{... (I)}$$

where A_{Sample} is the absorbance of the sample and $A_{control}$ is the absorbance of the control.

The inhibition curve was prepared against respective concentrations and from the graph; IC_{50} was calculated using the following equation:

$$IC_{50} = 50\text{-}C/m \quad \text{... (II)}$$

Here, values of C and m are obtained from the linear equation of the graph.

ABTS (*2,2-Azino-Bis-3-Ethylbenzothiazoline-6-Sulfonic Acid*) *Assay*

According to the methods of Halder *et al*.[21], ABTS radical-scavenging activity was assayed with necessary modifications. A stock solution (1:1 ratio) of potassium persulfate (2.45 mM) and ABTS (7.0 mM) was incubated at 25°C overnight before use. The stock solution was diluted with methanol to reach an absorbance of 0.7 ± 0.02 at 734 nm ($A_{control}$). About 0.9 mL of the ABTS/ persulfate mixture and 0.1 mL of aqueous extracts were mixed, and absorbance was taken immediately after 15 min at 734 nm. Here, TROLOX was used as positive control, and IC_{50} was calculated according to equations I and II.

Measurement of ferric reducing/antioxidant power (*FRAP*)

The reducing power of the food extracts was measured by mixing it with an equal volume of phosphate buffer (0.2 M, pH 6.6) and then incubating it at 50°C for 20 min with potassium ferricyanide (1%, w/v)[2]. The reaction was stopped by adding 0.5 mL of 5% TCA solution, and the mixture was centrifuged at 4530 g for 10 min. After that, the supernatant was mixed with 0.5 mL of 0.1% (w/v) aqueous ferric chloride solution, and the absorbance was measured at 700 nm. Ascorbic acid was used as a standard antioxidant, and IC_{50} was calculated according to equations I and II.

Hydroxyl (*OH*) *radical scavenging activity*

The hydroxyl radical scavenging activity was determined based on quantification of the degradation product of 2-deoxy ribose and condensation with thiobarbituric acid (TBA)[22]. Fenton reaction was prepared, contained with 2-deoxy-2-ribose (2.8 mM), KH_2PO_4 buffer (20 mM, pH-7.4), $FeCl_3$ (100 μM), EDTA (100 μM), H_2O_2 (1 mM) and ascorbic /acid (100 μM), from which hydroxyl ion was generated. 100 μL of food extract was with 1 mL reaction mixture and allowed for 1 h incubation at 37°C. After that, 1 mL of 5 % trichloroacetic acid (TCA) and 1 mL of 1% TBA were added and boiled for 10 min, then centrifuged at 4530 g for 5 min. The absorbance of the supernatant was measured at 532 nm against a concerned blank. For this purpose, mannitol was considered a standard antioxidant, and according to equations I and II, the IC_{50} value was calculated.

Statistical analysis

All the experiment was carried out in triplicate, and results were expressed as arithmetic mean (mean ± SD). One-way ANOVA analyzed the significant difference, and the t-test was done for all possible pairs with Sigma plot 11.0 (USA) statistical software.

Results

Total count of microbes

The microbial inhabitants in the fermented rice sample was examined and represented in Fig. 1. Use of herbal starter assembled the food with a group of characteristic microbes, including total aerobes (3.52±0.15 $\log_{10}$ CFU/g), total anaerobes (4.97±0.32 $\log_{10}$ CFU/g), LAB (5.62±0.19 $\log_{10}$ CFU/g), *Bifidobacterium* sp. (5.15±0.13 $\log_{10}$ CFU/g), yeast (4.12±0.17 $\log_{10}$ CFU/g) and mould (3.68±0.22 $\log_{10}$ CFU/g). In the control (C) sample, no notable changesof sensory characteristic, texture, aroma, and microbial growth were found due to sterilization.

pH, titratable acidity, and organic acid content

For acidity determination of any food, pH and titratable acidity (TA) are measured. It was noted that the fermented rice was comparatively more acidic (pH 4.25±0.14 and total titratable acidity0.58±0.04) than the unfermented one (pH 6.70±0.18 and total titratable acidity0.16±0.02), with a significant difference of P <0.001 (Table 1). The lactic acid and acetic acid content in both samples were also significantly different (P <0.001).

Proximate Composition

Proximate composition such as moisture, fat, protein, carbohydrate, and crude fiber of both samples were evaluated. In the fermented rice sample, the protein and fat content was distinctly higher than the unfermented rice (Table 1). On the other hand, the content of carbohydrates was lower in the fermented rice sample (61.35 g %) compared to unfermented (74.62 g %) rice. The quantity of crude fiber was significant, but the energy content was lower in the fermented sample. All the parameters regarding this analysis were significantly different (P <0.001) between the two samples.

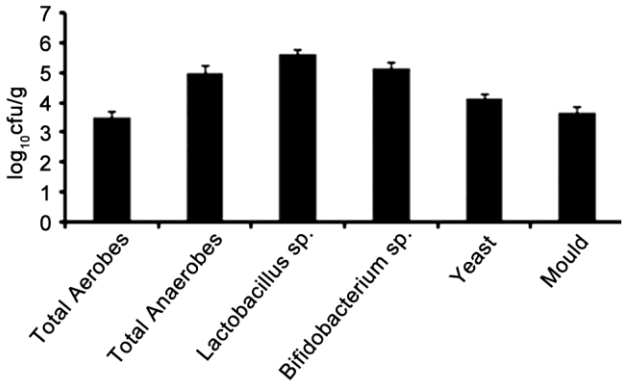

Fig. 1 — Dominant microbes in the 4th day of fermented rice were detected in $\log_{10}$ cfu/g, using plate assay technique.

Table 1 — Proximate composition, vitamin, mineral and volatile components in the unfermented\fermented rice sample

Parameters		Unfermented rice (C)	Fermented rice (T)
Proximate composition	pH	6.7±0.18[a]	4.25±0.14[b]
	Moisture (%)	11.23±1.32[b]	25.45±1.47[a]
	Protein (g%)	7.67±1.12[b]	13.22±0.96[a]
	Carbohydrate (g%)	74.62±1.54[a]	61.35±1.25[b]
	Fat (g%)	0.45±0.04[b]	1.75±0.06[a]
	Crude fiber (g%)	0.32±0.02[b]	1.06±0.04[a]
	Total energy (kcal/100 g)	365	292
Acidity	Titratable acidity (%)	0.16±0.02[b]	0.58±0.04[a]
	Lactic acid (mg/g)	-	0.35±0.02
	Acetic acid (mg/g)	-	0.21±0.02
Vitamins	Vitamin B12 (mg/g)	0.015±0.002[b]	0.52±0.04[a]
	Folic acid (mg/g)	-	1.47±0.28
	Riboflavine (mg/g)	-	0.054±0.004
	Thiamine (mg/g)	0.002±0.0001[b]	0.21±0.06[a]
	VitaminC(mg/g)	0.006±0.0002[b]	0.62±0.04[a]
Volatile compounds	Methanol (mL/g)	-	0.004±0.0001
	Propan -2-ol (mL/g)	-	0.050± 0.004
	Butan-1-ol (mL/g)	-	0.015±0.002
	Fatty Alcohol(mL/g)	-	0.42±0.006
Minerals	Calcium (ppm)	0.62±0.02[b]	5.45±0.25[a]
	Magnesium (ppm)	0.75±0.06[b]	4.86±0.28[a]
	Iron (ppm)	0.072±0.002[b]	0.19±0.04[a]
	Zinc (ppm)	0.023±0.004[b]	0.64±0.04[a]
	Manganese (ppm)	0.0015±0.0002[b]	0.21±0.02[a]

[Data are presented as mean±SD. Values in same row with different superscript (alphabets) indicate significant difference (P <0.05)]

Analysis of vitamins, minerals, and volatile compounds

The content of vitamins such as vitamin B12, folic acid, riboflavin, thiamine, and vitamin C was present in higher quantity in the fermented rice compared to unfermented rice (Table 1) and more appropriate than the recommended daily allowance (RDA) level for Indian people. The content of folic acid, vitamin C, and vitamin B12 were markedly improved in the fermented rice. The amounts of vitamin B12, folic acid, riboflavin, thiamine, vitamin C in the fermented rice were 0.52±0.04, 1.47±0.28, 0.054±0.004, 0.21±0.06, 0.62±0.04 mg/g, respectively (Table 1).

The deficiency of micronutrients is a common problem in developing countries. The mineral content in food products was enriched with the process of fermentation. Minerals including calcium, magnesium, iron, zinc, and manganese in the newly formulated fermented rice were estimated. The quantities were improved markedly in the fermented rice compared with unfermented rice (Table 1). The amounts of Ca^{++}, Mg^{++}, Fe^{+++}, Zn^{++}, Mn^{++} were 5.45±0.25, 4.86±0.28, 0.19±0.04, 0.64±0.04 and 0.21±0.02 ppm, respectively (Table 1).

The aroma of the food was dependent on volatile organic compounds (VOCs). Different alcohol-based volatile compounds experimented in both foods (fermented and unfermented rice) and observed that only methanol, butan-1-ol, propan-2-ol, and fatty alcohol were present in very little quantity in fermented rice (Table 1). Volatile compounds in unfermented rice were not detected.

Amino acid enrichment

A pool of essential (leucine, histidine, lysine, methionine, phenylalanine, and valine) and non-essential (arginine, serine, aspartic acid, glutamic acid, glycine, alanine, tyrosine, and proline) amino acids were present in the fermented rice. During fermentation of rice with the herbal starter, their quantity surprisingly improved compared with the unfermented rice (Table 2). There was a significant difference (P <0.05) between the two rice samples.

Estimation of oligosaccharide fragments

Cumulation of malto-oligosaccharide was evaluated in the fermented rice after completion of fermentation, but no such oligomers were found in the unfermented rice (Fig. 2). HPLC also confirmed the fragment of malto-oligomer (G3/maltotriose), and it remained in a concentration of 2.2 mg/g in the fermented sample.

Table 2 — Amino acid profile of unfermented\fermented rice sample

Amino acids	Concentration (mg/g)	
	Unfermented rice (C)	Fermented rice (T)
Arginine	0.45±0.04[b]	1.32±0.28[a]
Serine	0.15±0.02[b]	0.55±0.06[a]
Aspartic acid	1.12±0.22[b]	4.67±0.58[a]
Glutamic acid	0.56±0.18[b]	1.62±0.25[a]
Glycine	0.25±0.04[b]	0.82±0.04[a]
Alanine	-	0.15±0.02
Tyrosine	1.04±0.12[b]	4.95±0.55[a]
Proline	0.060±0.002[b]	0.52±0.04[a]
Methionine	-	0.23±0.02
Valine	0.095±0.004[b]	0.61±0.04[a]
Phenyle alanine	-	0.28±0.02[a]
Leucine	0.078±0.004[b]	0.45±0.04[a]
Histidine	0.35±0.02[b]	1.24±0.45[a]
Lysine	0.028±0.001[b]	0.36±0.02[a]

[Data are presented as mean±SD. Values in same row with different superscript (alphabets) indicate significant difference (P <0.05)]

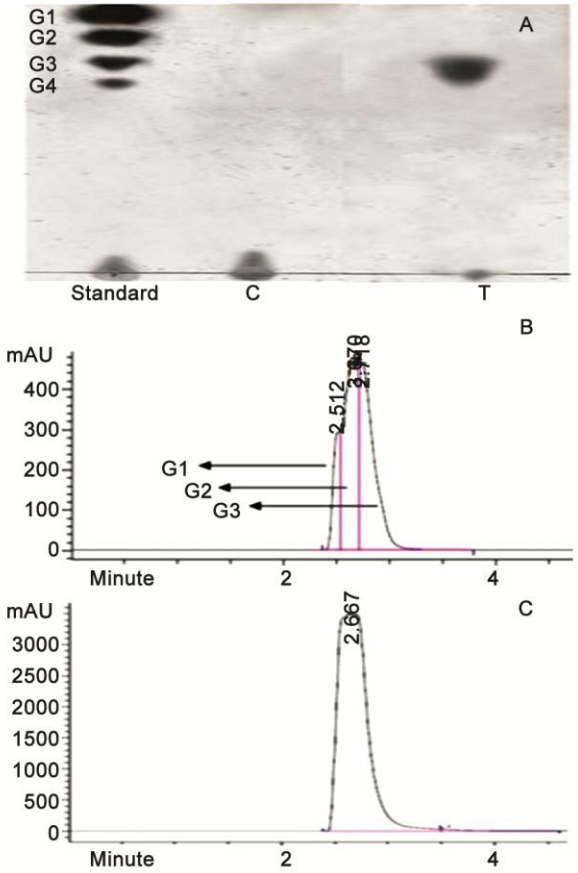

Fig. 2 — (A) Analysis of malto-oligosaccharides (G1, glucose; G2, maltose; G2, malto-triose; and G3, maltotetraose) using TLC method, where sample wells were marked as standard, 'C' and test 'T'; (B) HPLC chromatogram of standard malto-oligomers; and (C) The concentration (2.2 mg/g extracts) of malto-oligosaccharide in the 'T' sample analyzed from of area of HPLC chromatogram using the corresponding standards.

Fatty acid composition

The quantity of different fatty acid components in the fermented and unfermented rice has been given in Table 3. It was enumerated that the concentration of all types of fatty acids was significantly (P <0.05) higher in fermented rice than in the unfermented sample. Dodecanoate, pentadecanoate, and eicosenoate were absent in the unfermented rice, but their concentration increased in the fermented product and reached 62.48±1.12, 72.25±1.67 and 75.62±1.82µg/mL, respectively. Interestingly, the concentration of health beneficial unsaturated fatty acids such asoleate (ω9), linoleate (ω6), linolenate (ω3) and palmitoleate (ω7) was improved more than 2, 5, 7 and 10 folds, respectively, due to fermentation.

Phytochemical analysis

Total phenolic content, phenolic profile, and total flavonoid content of two samples (C and T) were evaluated for phytochemical analysis and are shown in Table 4. It was enumerated that the phenolic content of fermented rice was 2.17 mg/g, which is a much higher value than the unfermented rice (0.38 mg/g). Moreover, a notably more elevated amount of phenolic acids such asprotocatechuic acid, *p*-hydroxybenzoic acid, *p*-coumaric acid, ferulic acid, and sinapic acid were also identified in the fermented rice compared with unfermented extracts. Similarly, flavonoid content in the fermented rice was also increased more than 7 folds to differentiate with unfermented rice. Both the phenolics and flavonoids content was significantly (P <0.05) increased during fermentation of rice.

Table 3 — Fatty acid components of unfermented\fermented rice sample (µg/mL)

Fatty acids	Unfermented rice (C)	Fermented rice (T)
Methyl dodecanoate	-	62.48±1.12
Methyl myristate	65.82±1.45^b	345.65±3.45^a
Methyl pentadecanoate	-	72.25±1.67
Methyl palmitate	92.65±1.34^b	937.56±8.45^a
Methyl palmitoleate	12.67±0.38^b	92.55±1.12^a
Methyl heptadecanoate	17.96±0.85^b	116.59±2.67^a
Methyl stearate	155.52±2.23^b	412.45±2.35^a
Methyl octadecenoate	13.28^b±0.98	46.39±1.75^a
Methyl oleate	132.92±1.32^b	365.46±9.75^a
Methyl linoleate	976.45±3.66^b	3537.19±28.72^a
Methyl eicosenoate	-	75.62±1.82
Methyl linolenate	42.28±1.56^b	315.45^a±6.51^a

[Data are presented as mean±SD. Values in same row with different superscript (alphabets) indicate significant difference (P <0.05)]

Table 4 — Phytochemical components of unfermented and fermented rice sample

Parameters	Unfermented rice (C)	Fermented rice (T)
Total phenolic content (mg/g)	0.38±0.02^b	2.17±0.32^a
Protocatechuic acid (µg/g)	2.95±0.15^b	27.75±2.11^a
P-hydroxy-benzoic acid (µg/g)	1.53±0.12^b	10.37±0.64^a
P-Coumaric acid(µg/g)	0.045±0.002^b	0.58±0.06^a
Ferulic acid (µg/g)	0.76±0.03^b	1.28±0.12^a
Sinapic acid (µg/g)	1.24±0.09^b	9.25±0.34^a
Total flavonoids content (mg/g)	0.27±0.02^b	1.96±0.22^a

[Data are presented as mean±SD. Values in same row with different superscript (alphabets) indicate significant difference (P <0.05)]

Assay of *in vitro* antioxidant activity

In this study, DPPH, ABTS, hydroxyl (OH) and ferric reducing/antioxidant power (FRAP) radical scavenging methods were employed and calculate the IC_{50} values to estimate the antioxidant potentialities of the aqueous extract of unfermented and fermented samples (Fig. 3). It was observed that, the IC_{50} values against mentioned radical scavenging activities (DPPH, ABTS, OH^- and FRAP) of the fermented rice extract were lower more than 5, 6, 5 and 7 folds respectively compared to the unfermented one (P <0.05). But the IC_{50} values of fermented extract were comparable or very close to standard antioxidant.

Discussion

Fermented foods were prepared by interaction of microbes, either by naturally fermented or by adding starter culture method, which could modify the substrates biochemically into edible products and also made it more nutritious. The addition of plant based starter culture in rice fermentation in the Indian subcontinent is very popular. The endophytic microorganisms in the plant help to convert the substrate into value added food products.

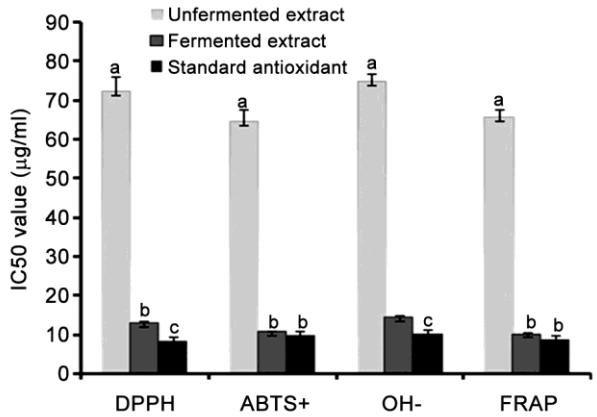

Fig. 3 — Scavenging effect in IC_{50} value of different standard antioxidants, fermented and unfermented extract on different free radicals (DPPH•, ABTS•+, OH• and Fe3+ to Fe2+ reducing). [The superscripted small letters (a, b and c) are significantly different at P <0.05].

In the present study, a fermented rice product was prepared using the root dust (rhizome) of *Elephantopus scaber* L. directly as a starter material. Its functional potentialities include nutrient profiling, phytochemical analysis, *in vitro* antioxidant activity were evaluated. It was noted that the newly formulated rice fermented food was enriched with a group of endophytic microbes such as aerobic and anaerobic bacteria, LAB, Bifidobacteria, mold, and yeast (Fig. 1). Rice fermentation by an herbal starter is a polyphonic type of fermentation; at the beginning, aerobic bacterium started the fermentation process and influenced the microorganisms. Aerobic microbes and its hydrolytic activity set the environment for the growth of yeast and mold. LAB and Bifidobacteria were dominant at the end of the fermentation process, revealing that the microorganisms might be outsourced from the herbal medicinal residues used for the fermentation[5,23]. The affluence of LAB, *Bifidobacterium* sp., and yeast, typically considered health-beneficial microbes, makes the product a probiotic food[8]. The progression of microbes from herbal plant to the foods was first established by our group[4]. Dietary supplementation of fermented rice containing sufficient numbers of microbes may assist and upgrade health through gut microbiota modulation.

Titratable acidity and pH are two interconnected theories in food analysis that allocate with acidity. Fermented rice was more acidic as it accumulated a higher level of lactic acid and acetic acid than the unfermented rice (Table 1). During fermentation, the growth of anaerobic microbes was increased with anaerobiosis and lowering of pH, and that help in forming of a group of enzymes for decomposing the food matrix dietary fibers[24]. Many evidences proposed that the presence of lactic acid and other metabolites in the fermented food could inhibit the intestinal pathogen, balance the texture of food, improve the number of micronutrients and consider as an available energy source for any living organisms, which results in cholesterol-lowering, anti-ulcer, antitumor, and also have anti-allergic activities[13].

The data about the proximate composition of any food are essentials for health professionals and dieticians to promote the particular food**.** The comparatively higher protein content in the fermented rice was likely due to generation of yeast biomass, which is served as the source of single-cell protein, while enhancement of fat content was occurred by hydrolysis of starchlipids complex and synthesis of cell membrane phospholipids by fungus[3]. In our previous report, enhancement of protein (10.25 g %) and fat (1.3 g %) content was also identified, where boiled rice was fermented with the root of *Asparagus racemosus* as herbal starter than unfermented milled rice[4]. In contrast, due to the enzymatic degradation process, the content of carbohydrates was lower in fermented rice than in the unfermented one. Improving lactic acid production during the fermentation process may also help break down the rapidly digestible starch (RDS) and slow digestibility starch (SDS) complex, which improves the glycaemic index[25,26]. The amount of crude fibre was significant, but the energy content was low in the fermented product because of carbohydrate breakdown. Hence, the microbial interaction during fermentation made the fermented rice more nutritious and healthier than unfermented rice.

The newly formulated rice fermented products contained a sufficient amount of vitamins (vitamin B_{12}, folic acid, riboflavin, thiamine and vitamin C), minerals (calcium, magnesium, iron, zinc and manganese), and volatile compounds (methanol, butan-1-ol, propan-2-ol and fatty alcohol) (Table 1). The quantities of the micronutrients are more accurate than the recommended daily allowance (RDA) level for Indian people. The content of vitamin B_{12}, folic acid and vitamin C was markedly improved in the fermented rice. Folic acid (vitamin B_9) helps in systematic DNA replication and repair. The proliferation of leucocytes, erythrocytes and enterocytes requires a considerable amount of folic acid[27]. The women of developing countries are primarily deficient in folic acid and its RDA is 75-150 μg, and the supplementation of fermented rice can easily manage this deficiency. Microbial consortia, especially LAB and yeast, might produce the vitamins during fermentation as suggested elsewhere[13]. Moreover, it is well known that LAB could produce phytase enzyme, which is responsible for the degradation of anti-nutrientphytic acid, liberates the free minerals, and thus bioenrich the substrate[28]. In connection to this, the fermented material was found enriched with the minerals, and thus herbal starter-based newly formulated fermented rice could be used as calcium-magnesium-rich food. Our findings were comparable with the previous study[4]. Volatile organic compounds (VOCs) are

related to a food's aroma. Different alcohol-based volatile compounds were estimated in both foods and observed that only methanol, butan-1-ol, propan-2-ol, and fatty alcohol were present in very little quantity in fermented rice. These volatile compounds were also reported by Banik *et al*.[29]. The quantity of amino acids was improved amazingly at the end of fermentation compared to the control rice sample as like the study of Hor *et al*.[4]. Actually, the complex microbial structure augmented in the fermented rice liberated different hydrolytic enzymes, which might help in the release of the nutrients. They may also secret the vitamins and volatile compounds. Hence, the herbal-based starter significantly enriched the rice with vitamins, minerals and volatile compounds, exhibiting different health benefits to the consumers.

The quantity of essential and non-essential free amino acids increased unexpectedly during the curse of fermentation of boiled rice with herbal starter. Amino acids are the main compositional unit of protein. In the present scenario, supplementations of quality protein are at a risk for rural population of least developed and developing countries. Herbal starter based fermentation in cereal-containing foods provides a natural way to concentrate and enhance free amino acid synthesis. Presents of lysine in the fermented rice considered as a measure of quality protein. Equally, yeast contained fermented rice also showed it efficacy with a large number of free amino acid production and develops a positive nitrogen balance[30]. Thus, the newly formulated rice fermented product was compatible with other traditional fermented beverages like chang, apong and judima.

The microbes ferment the starchy material by releasing a group of carbohydrate-active enzymes (CAZymes) that hydrolyzed rice's starch and produced simple sugars such as mono-, di-, oligo-saccharides. After completion of fermentation, accumulation of only malto-oligosaccharide was detected in the fermented rice. Maltooligosaccharides (G3 and G4) were also found in the end products of rice-fermented beverages (*haria*)[10]. These maltooligosaccharides have multifunctional health benefits such as increased the growth of indigenous gut flora whose metabolic end product is a short-chain fatty acid (SCFA), induced mucin production and stimulated gut-associated lymphoid tissues (GALTs)[31].

Fatty acids are important dietary fuel sources for animals and essential structural components for cells. The fermented rice was also enriched by a group of fatty acids, such as linolenate (ω3), linoleate (ω6), palmitoleate (ω7) and eicosenoate (ω9). The concentration of mentioned unsaturated fatty acids were also improved more than 3.2, 2.1, 2.3, and 25 folds in the herbal starter (*Asparagus racemosus*) used another fermented rice compared with unfermented one[4]. These fatty acids (FAs) may play important nutraceuticals related to functional potentialities of the brain and nervous system[17]. Moreover, monounsaturated fatty acid (MUFA) and polyunsaturated fatty acid (PUFA) based diets help improve glycemic index and lipid profile in serum. They are preferable for treating and preventing obesity, diabetes and insulin resistance syndromes[31]. It was suggested that mould and yeast synthesize fatty acids encumbered by slow growth and a requirement for strictly anaerobic culture conditions[32,33]. The bio-enrichment of rice might be due to the participation of active microbes from plants.

During fermentation, microbes originating hydrolytic enzymes (cellulases, esterases, glycosidase, polyphenol hydrolase, etc.) may lead to cleavage and denaturation of the cellulosic backbone as well as polyphenolic structures; therefore, flavonoid and phenolic components are separated from the harboring molecule and become free[34]. Recently, the interest in dietary consumption of flavonoids and phenolic acids has been attracted much because they have a variety of beneficial biological properties and play an essential role in the prevention and protection of many human diseases[35]. The phenolic and flavonoid contents were significantly higher in fermented rice than the unfermented ones. Different types of phenolic compounds were accumulated in fermented rice, and their concentration varies during fermentation. Comparatively higher amounts of phenolic acids, such as protocatechuic acid, *p*-hydroxybenzoic acid, *p*-coumaric acid, ferulic acid, and sinapic acid were also present in the fermented rice. Emerging evidence highlighted that these phenolic compounds are bioactive and exert many health-beneficial activities (antioxidant, antibacterial, anticancer, anti-ulcer, anti-diabetic, anti-aging, etc.)[36]. Globally, interest in phenolics and flavonoids attracts much more, as they scavenge free radicals in the system and neutralize them before they damage body cell[13]. It was estimated that the fermented rice's IC_{50} values of DPPH, ABTS, FRAP, and Hydroxyl (OH) radical scavenging activities were significantly

improved due to fermentation by a consortium of microbes and comparable or very close to standard antioxidants. The "antioxidant power" of the fermented rice was incredibly related to its high phenolic and flavonoid contents because these molecules have the innate ability to donate an electron or hydrogen[37]. The herbal-based starter could significantly enrich the rice substrate with phenolics and flavonoids, exhibiting antioxidant activities.

Conclusion

This study demonstrated that rice fermentation with these selective herbal starters (rhizome of *Elephantopus scaber*) enriched rice with health-promoting microbes and nutraceuticals such as water-soluble vitamins and minerals, oligosaccharides, essential fatty acids, and a pool of amino acids. The fermented rice was also enriched with phytochemicals (phenolics and flavonoids) which exert significant antioxidant effects against different free radicals. However, this experimental evidence supported the traditional use of herbal starters to prepare a variety of functional foods. It may create a new opportunity to develop rice-based fermented food.

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Conflict of Interest

Authors declare no competing interests.

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